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Original Article

Grape Seed Extract Attenuates Demyelination in Experimental Autoimmune Encephalomyelitis Mice by Inhibiting Inflammatory Response of Immune Cells*

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ABSTRACT **Objective**: To examine the anti-inflammatory effect of grape seed extract (GSE) in animal and cellular models and explore its mechanism of action. **Methods**: This study determined the inhibitory effect of GSE on macrophage inflammation and Th1 and Th17 polarization *in vitro*. Based on the *in vitro* results, the effects and mechanisms of GSE on multiple sclerosis (MS)-experimental autoimmune encephalomyelitis (EAE) mice model were further explored. The C57BL/6 mice were intragastrically administered with 50 mg/kg of GSE once a day from the 3rd day to the 27th day after immunization. The activation of microglia, the polarization of Th1 and Th17 and the inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-12, IL-17 and interferon-γ (IFN-γ) secreted by them were detected *in vitro* and *in vivo* by flow cytometry, enzyme linked immunosorbent assay (ELISA), immunofluorescence staining and Western blot, respectively. **Results**: GSE reduced the secretion of TNF-α, IL-1β and IL-6 in bone marrow-derived macrophages stimulated by lipopolysaccharide ($P<0.01$), inhibited the secretion of TNF-α, IL-1β, IL-6, IL-12, IL-17 and IFN-γ in spleen cells of EAE mice immunized for 9 days ($P<0.05$ or $P<0.01$), and reduced the differentiation of Th1 and Th17 mediated by CD3 and CD28 factors ($P<0.01$). GSE significantly improved the clinical symptoms of EAE mice, and inhibited spinal cord demyelination and inflammatory cell infiltration. Peripherally, GSE downregulated the expression of toll-like-receptor 4 (TLR4) and Rho-associated kinase (ROCKⅡ, $P<0.05$ or $P<0.01$), and inhibited the secretion of inflammatory factors ($P<0.01$ or $P<0.05$). In the central nervous system, GSE inhibited the infiltration of $CD45^+CD11b^+$ and $CD45^+CD4^+$ cells, and weakened the differentiation of Th1 and Th17 ($P<0.05$). Moreover, it reduced the secretion of inflammatory factors ($P<0.01$), and prevented the activation of microglia ($P<0.05$). **Conclusion**: GSE had a beneficial effect on the pathogenesis and progression of EAE by inhibiting inflammatory response as a potential drug and strategy for the treatment of MS.

KEYWORDS grape seed extract, experimental autoimmune encephalomyelitis, demyelination, inflammatory cell, inflammatory factor

Multiple sclerosis (MS) is an immune-mediated chronic inflammatory demyelinating disease in the central nervous system (CNS), characterized by an increasing inflammatory response and extensive primary demyelination. The inflammation of MS is triggered by peripheral autoreactive $CD4^+$ T cells, which can pass through the blood-brain barrier (BBB) to induce an inflammatory reaction associating with leukocytes, reactive astrocytes and microglia and myelin-laden macrophages, and various combinations of myelin macrophages.[1] These inflammatory

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reactions can attack neurons and oligodendrocytes (OLs) through intercellular interaction and autocrine or paracrine pro-inflammatory cytokines, chemokines and adhesion molecules. It eventually leads to diffuse CNS inflammation and the destruction of OLs and neurons, and then leads to the damage of myelin sheath and axons. Therefore, inhibiting inflammatory responses may possibly have a better effect on the healing of MS.[2,3] And many reports also showed that anti-inflammatory treatments can prevent or delay the progression of the disease. For example, some natural small molecular substances such as nordihydroguaiaretic acid and small molecular peptides can alleviate the disease process of experimental autoimmune encephalomyelitis (EAE) by inhibiting inflammation.[4,5] EAE is characterized by inflammatory demyelination and neurodegeneration in the CNS, which leads to an approximation to the key pathological of MS patients.[6,7]

Grape seed from *Vitis vinifera* L. is the waste part of grape fresh food, juice and wine industry. Grape seed extract (GSE) containing important polyphenols (20%–55%) and potent antioxidants, has been widely studied and developed as health products and drugs for a variety of diseases.[8-10] Flavonoids are important components of phenolic compounds in GSE, which can be divided into several categories. There are C6-C3-C6 polyphenols with 2 hydroxyl benzene rings; A and B are connected by 3-carbon chain that is part of the heterocyclic ring C. According to the oxidation state of ring C, these compounds are classified into flavonols, flavan-3-ols and anthocyanins.[11]

Due to their antioxidant properties, phenolic compounds have been proved to have anti-inflammatory potential. For example, GSE can decrease the levels of proinflammatory cytokines interleukin-1β (IL-1β) and recombinant neutrophil to lymphocyte ratio (NLR) family, pyrin 3 (NLRP3) to reduce the damage of diabetic pancreas.[12] GSE also reduced the inflammatory capacity of lipopolysaccharide (LPS)-treated human primary monocytes, and reduced the expression and secretion of tumor necrosis factor-α (TNF-α), IL-1β and IL-6 genes.[13] In view of these findings, we set out to study a possible effect of GSE on mice with EAE.

METHODS

Animals

Female C57BL/6 mice (8–10 weeks old, weighing 18–20 g, clean degree, certificate No. SCXK (Jing) 2021-0006) were purchased from Vital River Laboratory Animal Technology Co. Ltd., China. Before the experiment, mice were fed without pathogens and maintained for a week in a 12/12 h reverse light/dark cycle in a temperature control room (25±2 ℃, humidity of 50%–60%). All experiments were conducted in accordance with the guidelines of the International Council for Laboratory Animal Science. The detailed experimental design of the study is shown in Appendix 1. The study was approved by the Ethics Committee of Shanxi University of Chinese Medicine, China.

Isolation, Stimulation and Treatment of Bone Marrow-Derived Macrophages

The C57BL/6 mice were decapitated and sacrificed after intraperitoneal injection of chloral hydrate anesthesia, and were perfused intracardially with normal saline (NS), then the bone marrow was isolated. A 25G needle was used in a 5 mL syringe, moved up and down repeatedly to disperse cells aggregates. The cell suspension was passed through a cell strainer (70 μm) to get a single cell suspension, then centrifuged at 400×*g* for 10 min. The cells were resuspended in the medium with 50 ng/mL macrophage colony stimulating factor (M-CSF), inoculated in 10 cm sterile bacterial plastic petri dish, and cultured in cell culture box with 5% CO_2 at 37 ℃. On day 7, bone marrow-derived macrophages (BMDMs) induced by M-CSF were inoculated into 24-well plates and stimulated with LPS (1 μg/mL), and 50 μg/mL GSE was added at the same time. After 24 h, the supernatants were collected for enzyme linked immunosorbent assay (ELISA) to detect related cytokines, including TNF-α, IL-1β and IL-6.[14]

Purification and Differentiation of $CD4^+$ T Cells

Initial $CD4^+$ T cells were purified from spleen of C57BL/6 mice by negative selection using $CD4^+$ T cell isolation kit Ⅱ (lot No. YS-SO-0001221, Miltenyi Biotec, USA). Cells in the normal group were suspended in high-glucose dulbecco's modification of eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). In vehicle group and GSE group, cells were suspended in high-glucose DMEM (10% FBS) containing 1 μg/mL hamster anti-CD28 (37.51; lot No. 553294, eBioscience, USA), 2 ng/mL transforming growth factor-β (TGF-β, lot No. 100-21, Peprotech, USA), 20 ng/mL IL-23 (lot No. 200-23, Peprotech), 20 ng/mL IL-6 (lot No. 200-06, Peprotech), 5 μg/mL anti-IL-4 (lot No. 504101,

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Biolegend, USA) and 5 μg/mL anti-interferon-γ (IFN-γ, lot No. 513207, Biolegend), and cultured in 24-well plates pre-coated with 0.25 μg/mL hamster anti-CD3 for the differentiation of Th17 cells. In addition to the anti-CD3CD28 antibody, anti-IL-4 and anti-IFN-γ antibodies were used in the same method as Th17 differentiation, an additional 20 ng/mL IL-12 (lot No. 210-12, Peprotech) was added to induce Th1 differentiation. The cells of GSE group were also cultured with 50 μg/mL GSE for 7 days.[15]

Preparation of Spleen Monocyte Suspension

Spleens of vehicle group and GSE group were removed under aseptic conditions and then prepared by grinding the organ through a 40 μm nylin mesh in medium. Erythrocytes in the suspensions were osmotically lysed. Splenocytes were washed 3 times and re-suspended in high-glucose DMEM containing 10% FBS. These cells would be used to analyze the inflammatory response by flow cytometry, ELISA and Western blot. In addition, splenocytes from untreated EAE mice immunized for 9 days can also be used to detect the inhibitory effect of GSE (50 μg/mL) on inflammation *in vitro*.

Induction and Clinical Evaluation of EAE Mice

Mouse myelin oligodendrocyte glycoprotein peptide 35-55 (MOG35-55, MEVGWYRSPFSRVVHLYRNGK) was produced in an automatic synthesizer (CL. Bio-Scientific. Company, China). The purity of the peptide was 95% by high performance liquid chromatography (HPLC). Chronic EAE was induced by subcutaneous immunization with 300 μg complete Freund's adjuvant (344291, Sigma, USA), 3 mg/mL MOG35-55 and *Mycobacterium tuberculosis* H37Ra (231141, BD Difco, USA, 400 μg/mouse). At the same time of immunization and 48 h later, 200 ng pertussis toxin (Enzo Life Sciences, USA) was injected into mice by intraperitoneal injection.[16]

Animals were weighed and evaluated for clinical score every other day in a blinded fashion by at least 2 investigators. Clinical score of EAE was graded according to the following criteria: 0: healthy; 1: limp tail; 2: ataxia and/or paresis of hindlimbs; 3: paralysis of hindlimbs and/or paresis of forelimbs; 4: tetraparalysis; and 5: moribund or death.[1] After immunization of mice, the model was successful if the clinical symptoms were tail paralysis in mice, and the clinical score reached 1 point. When the clinical score reached 3 points, the mice would be taken special care. The food was softened by water in the plate, added nutrients such as eggs, and the plate was placed at the bottom of the cage so as to obtain food, water and nutrition. The severity of clinical symptoms in EAE mice was partially compared with clinical scores.

Grouping and Intervention

The animals were randomly divided into 3 groups according to body weights, including normal group, vehicle group, and GSE group (*n*=10 per group). From day 3 post immunization (p.i.) to day 27 p.i., mice in the GSE group were intragastrically administered with GSE (batch No. 002-2008055-24, Tianjin Jianfeng Natural Product R&D Co., Ltd., China) dissolved in NS at a dose of 50 mg/kg (equivalent to twice the clinical dosage). Mice in the vehicle and normal groups were administered with NS in a similar manner. All mice were sacrificed on day 28 p.i. for the further laboratory evaluation.

Tissue Preparation

The mice were decapitated and sacrificed after intraperitoneal injection of chloral hydrate anesthesia, and were perfused intracardially with NS. The enlarged area of cervical and lumbar spinal cord was fixed with 4% paraformaldehyde for 6 h. Spinal cord was then covered with 30% sucrose solution and kept at 4 ℃ for 24 h. The prepared spinal cord was embedded, cut into slices (10 μm) with a cryostat microtome (Leica CM1950, USA) and stored at 4 ℃ for pathological staining. Mononuclear cells (MNCs) were isolated from the remaining spinal cord and brain by percoll density gradient method. Then the expressions of inflammatory chemokines, cytokines, activated T cells and macrophages in the CNS were detected by real-time polymerase chain reaction (PCR) and flow cytometry, respectively.

Histopathological and Immunofluorescence Staining

To further judge the severity of clinical symptoms in mice, the range of demyelinating area and slides of spinal cord stained with Luxol fast blue (LFB) were measured. The demyelinating level was displayed by color difference, and its color was lighter than that of normal tissue. Hematoxylin and eosin (HE) staining was used to determine the number of CNS-infiltrating cells. In addition, spinal cord slice was blocked with 1% body surface area (BSA) at room temperature (RT) for 60 min and stained with anti-myelin basic protein (MBP,

1:1000, lot No. ab209328, Abcam, UK), anti-allograft inflammatory factor 1 (anti-Iba-1) antibody (1:1000, lot No. ab178846, Abcam), ani-CD4 antibody and anti-CD11b antibody (1:1000, lot No. ab184308, Abcam) at 4 ℃ for overnight. After washed 3 times with phosphate buffer saline (PBS), it was incubated with the relevant Cy5- and AF488-fluorescent antibodies at RT for 2 h. The images of staining were captured by Leica upright fluorescent microscopy (MAB326, Germany). Quantification analysis was performed by Image-Pro Plus v8.0 software (Media Cybernetics, USA) on 3 sections in each group of mice.

Isolation of CNS Infiltrating Cells

The brain and spinal cord of each mouse were placed in a 50 mL centrifuge tube with ice-cold PBS. The organ was pressed through the cell strainer by the back of sterile 5 mL syringe plunger (100 μm). The suspension was collected into a 50 mL tube on ice, then centrifuged for 8–10 min at 390×*g*. The cell precipitate was resuspended in 20 mL 30% percoll and overlaid onto 10 mL 70% percoll. After centrifugation at 600×*g* at RT for 20 min, the myelin component on the top of tube was removed. The cells were collected from the interface and washed twice with PBS.(17)

Cytokine ELISA Assay

The homogenate supernatant of whole spinal cord was collected and detected for TNF-α (lot No. DY410-05), IL-1β (lot No. DY401-05), IL-6 (lot No. DY406-05), IL-12 (lot No. DY419-05), IL-15 (lot No. DY447-05), IFN-γ (lot No. DY485-05) and IL-17 (lot No. DY421-05) by a sandwich ELISA kit purchased from the R&D Company (USA). Splenocytes (6×10^5/200 μL/well) were inoculated in 96 wells and incubated for 48 h in the presence of MOG35-55 (10 μg/mL). Supernatants were collected and measured for cytokines by sandwich ELISA kits. In addition, the cytokines secreted by activated initial T cells and macrophages were also detected by ELISA. The determination was performed in duplicate in 3 independent experiments.

Flow Cytometry Analysis

For cell surface staining, after blocking Fc receptor with anti-mouse CD16/32 antibody (lot No. 101301, eBioscience), the CNS-infiltrating MNCs were stained for 20 min at RT in BSA/PBS buffer with the following panel of antibodies: PE-cy7-CD45 (lot No. MHCD12), PE-cy5-CD4 (lot No. MCD0418), and APC-cy7-CD11b (lot No. A15389), all from eBioscience. The cells were washed twice and permeated with Fix & Perm reagent A (Fixation Medium, lot No. GAS003, Thermo, USA) for 20 min at RT. Then they were centrifuged and resuspended in 500 μL of Fix & Perm reagent B (permeabilization medium) with PE-IL-17 (lot No. 12-9171-82, eBioscience) and BV-711-IFN-γ (lot No. 553294, BD Biosciences, USA). Cells were gated using forward and sideward scatter characteristics for lymphocytes and monocytes and at least 100,000 gated events were collected using flow cytometer (BD Biosciences). The same dye was also used for plenocytes and activated primary T cells to stain CD4, IL-17 and IFN-γ.(15) All these data were analyzed by Flow Jo 10.8.1 software (BD, USA).

Western Blot Analysis

Proteins were obtained from splenocytes isolated from each mouse by using protein extraction kit (MCL1, Sigma). A cocktail of protease inhibitors (lot No. 87785, Thermo) was added to the cleavage system to maintain the integrity of the protein. The mixture was centrifuged at 13,000×*g* for 20 min at 4 ℃ and the supernatants were collected. Protein concentrations were determined by a bicinchoninic acid (BCA) protein assay. Equal amounts of protein (30 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Millipore, Immobilon-P, USA). After non-specific binding was blocked with 5% non-fat dry milk, membranes were treated with anti-toll-like-receptor 4 (anti-TLR4) antibody (1:1000, lot No. 14358, CST, USA), anti-Rho-associated kinase (anti-ROCKⅡ) antibody (1:1000, lot No. 47012, CST) and anti-β-actin antibody (1:1000, lot No. BS6499, Bioworld, USA) overnight at 4 ℃. Bands were visualized with an enhanced chemiluminescence (ECL) system (32209, Thermo).

Real-Time PCR

Spinal cords were repeatedly snap-frozen and ground with liquid nitrogen. RNA was extracted from spinal cord by using mammalian total RNA miniprep kit (lot No. RNT70, Sigma). The extracted RNA was converted to cDNA by using the first strand cDNA synthesis kit (lot No. K1622, Thermo) according to the protocol of manufacturer. Next, the chemokine macrophage inflammatory protein-1alpha (MIP-1α) and monocyte chemotactic protein-1 (MCP-1) mRNA expression was detected by quantitative real-time PCR. The primers were designed by Shanghai BioTNT as follows: MIP-1α: forward primer 5'-CAATTCATCGTTGACTATT-3' and reverse primer 5'-CAGTGATGTATTCTTGGA-3'; MCP-1: forward primer

5'-GAGTAGGCTGGAGAGCTACAAGAG-3' and reverse primer 5'-AGGTAGTGGATGCATTAGCTTCAG-3'. The $2^{-\triangle\triangle Ct}$ method was used to calculate mRNA expression of MIP-1α and MCP-1.

Statistical Analysis

All the experiments were repeated thrice and GraphPad Prism 8.0 software (USA) was used for statistical analysis. Data were presented as mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variance (ANOVA) was used for multiple comparisons and followed by Tukey's *post-hoc*. Single comparison was evaluated by unpaired *t* test. A statistically significant difference was assumed at P<0.05.

RESULTS

GSE Inhibited Inflammatory Responses *in vitro*

GSE significantly inhibited the secretion of TNF-α, IL-1β and IL-6 in BMDMs (Figure 1A, P<0.01). At the same time, GSE inhibited the differentiation of Th17 and Th1 induced by anti-CD3CD28 antibody and the secretion of their effectors, IL-17 and IFN-γ *in vitro* (Figures 1B and 1C, P<0.01 or P<0.05). The effect of GSE on splenocytes of EAE mice immunized for 9 days was detected. GSE inhibited the production of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12, IL-17 and IFN-γ (P<0.05 or P<0.01), which were induced by MOG35-55 peptide (Figure 1D).

GSE Ameliorated Clinical Severity, Delayed Onset and Attenuated Demyelination and Inflammatory Infiltration of EAE Mice

GSE treatment significantly decreased the clinical scores and reduction in weight loss of EAE mice compared with the vehicle group (P<0.05, Figure 2A). EAE clinical symptoms were firstly detectable around day 12 p.i. The symptoms reached a peak at day 22 p.i., and the disease course was maintained roughly for a month. Most of GSE-treated mice substantially delayed disease onset and extended time to disease peak compared with the vehicle group (P<0.05, Figure 2B). Compared with the vehicle group, the white matter demyelination and inflammatory cell infiltration in GSE-treated mice were significantly reduced (P<0.05, Figures 2C and 2D). As shown in Figure 2E, immunofluorescence (IF) staining of spinal cord showed that MBP expression decreased significantly and the amount of fragmented MBP increased in EAE mice (P<0.01), which was reversed by GSE treatment (P<0.05). IF staining showed that the infiltration of CD11b$^+$ and CD4$^+$ cells in the white matter of spinal cord in GSE-treated mice was greatly reduced (P<0.01, Figure 2F).

GSE Inhibited Inflammatory Reaction in Periphery

Compared with the vehicle group, GSE treatment significantly lowered the levels of TNF-α, IL-1β, IL-6 and IL-12 (P<0.05 or P<0.01), while had no effect on IL-15 (P>0.05, Figure 3A). In addition, the differentiation of Th17 and Th1 T cells was inhibited, thereby reducing the secretion of IL-17 and IFN-γ (P<0.05 or P<0.01, Figures 3B and 3C). To explore the molecular mechanism of GSE, we found that GSE significantly inhibited the expression of TLR4 and ROCKⅡ (P<0.05 or P<0.01, Figure 3D).

GSE Altered Immune Cell Response and Cytokines in CNS

Compared with the vehicle group, GSE significantly

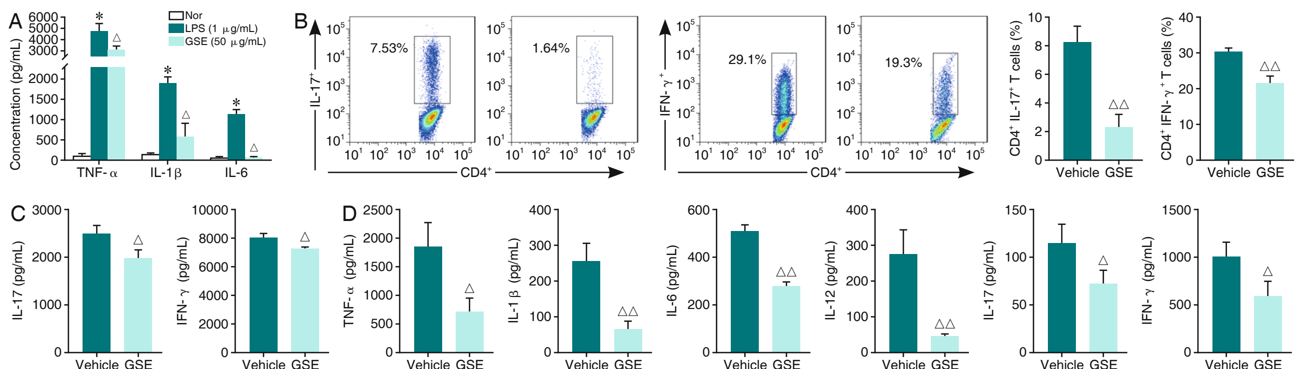


Figure 1. GSE Inhibited Inflammatory Responses *in vitro* ($\bar{x} \pm s$, n=10)

Notes: Cytokines were measured by ELISA and Th cells were analyzed by flow cytometry. (A) Secretion of TNF-α, IL-1β, and IL-6 from initial macrophages; $^*P$<0.01 *vs*. normal group; $^{\triangle}P$<0.01 *vs*. LPS group. (B) Triple differentiation of CD4$^+$IFN-γ$^+$ and CD4$^+$IL-17$^+$ T cells; (C) levels of IFN-γ and IL-17; (D) levels of TNF-α, IL-1β, IL-6, IL-12, IL-17 and IFN-γ. $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. vehicle group. GSE: grape seed extract; Nor: normal; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; IFN-γ: interferon-γ; and the same below

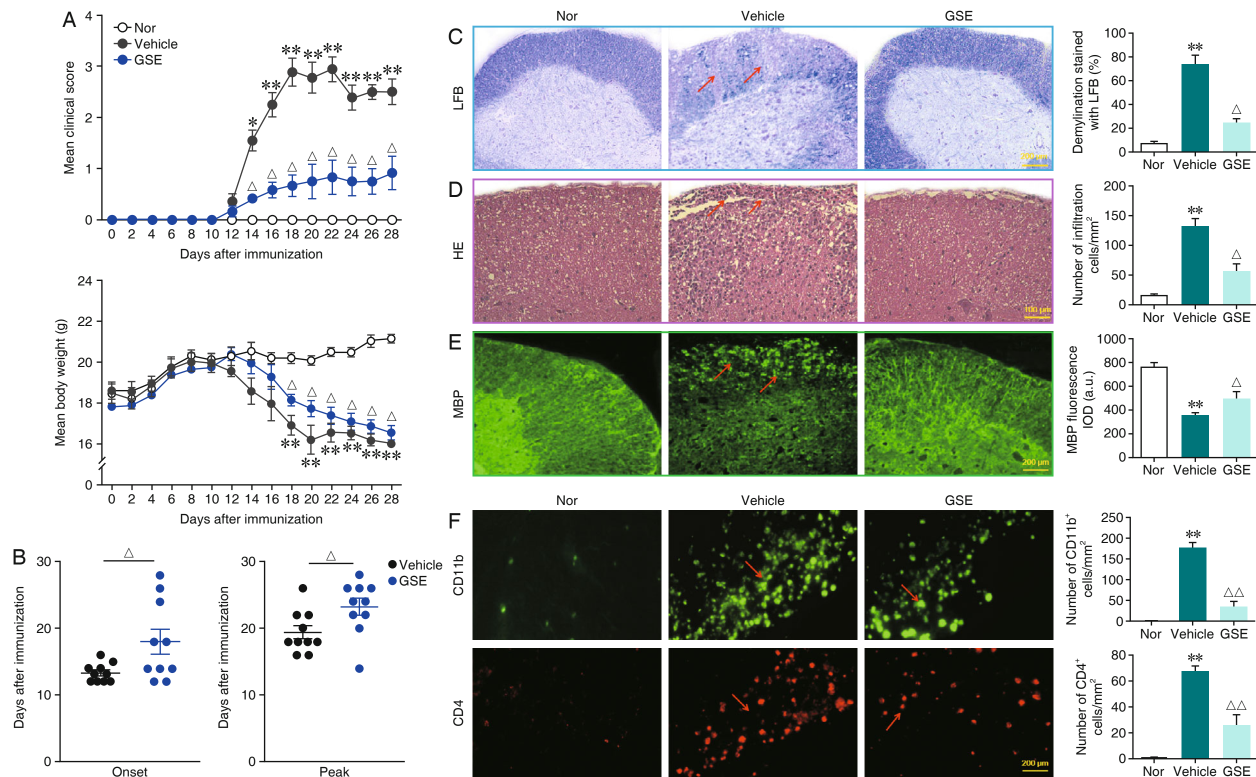


Figure 2. GSE Ameliorated Severity of EAE, Delayed Onset and Attenuated Demyelination and Inflammatory Infiltration ($\bar{x} \pm s$, n=10)

Notes: (A) Clinical scores and body weights of vehicle- and GSE-treated EAE mice. (B) Histogram of average onset and peak score of disease (onset defined at 1 score) for all groups. (C) Demyelination stained with LFB (×20). Total white matter in LFB was manually outlined, and pixel area (%) of demyelination in total white matter was calculated, scale bar=200 μm. The arrows indicate areas of demyelination. (D) Color photos in HE staining were automatically converted to black and white (×20), and number of inflammatory foci (>20 mononuclear cells/focus) in total white matter were calculated, scale bar=100 μm. The arrows indicate the inflammatory foci. (E) IF staining for MBP in total white matter (×20), and the MBP fluorescence IOD were calculated, scale bar=200 μm. The arrows indicate areas of the position of lower MBP fluorescence. (F) IF staining for CD11b and CD4 in total white matter (×20), and the number of $CD11b^+$ (green) and $CD4^+$ (red) cell were calculated. Scale bar=200 μm. The arrows indicate areas where CD4 and CD11b cells are dense. $^*P$<0.05, $^{**}P$<0.01 *vs*. normal group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. vehicle group. EAE: experimental autoimmune encephalomyelitis; LFB: Luxol fast blue; HE: hematoxylin and eosin; MBP: myelin basic protein; IF: immunofluorescence; IOD: integrated option density; and the same below

reduced the numbers of $CD45^+CD11b^+$macrophages and Iba-1^+ microglia in spinal cord (P<0.05 or P<0.01, Figures 4A and 4B). And the levels of TNF-α, IL-1β, IL-6 and IL-12 were all obviously decreased in GSE-treated mice (P<0.01), while IL-15 remained unchanged (P>0.05, Figure 4C). Consistent with the pathological results, the percentage and absolute number of $CD45^+CD4^+$ T cells in the CNS of EAE mice were significantly reduced after GSE treatment (P<0.05, Figure 5A). Accordingly, GSE also inhibited the differentiation of T cells to pathogenic Th17 and Th1 cells compared with vehicle group (P<0.05, Figure 5B), and accompanied by the decreases of IL-17 and IFN-γ (P<0.01, Figure 5C). These results were consistent with the results in the periphery. We detected the related chemokines, and found that GSE treatment reduced the expression of MIP-1α and MCP-1 mRNA (P<0.01, Figure 5D).

DISCUSSION

In this study, we explored whether GSE had a beneficial effect on EAE animal models. The results showed that GSE significantly alleviated the clinical symptoms to inhibit spinal cord demyelination, and weakened the activation of resident microglia in the spinal cord, the infiltration of peripheral macrophages and $CD4^+$ T cells in the CNS, the expression of inflammatory factors and the differentiation of Th1/Th17. In addition, GSE treatment also inhibited the inflammatory response of peripheral T cells and macrophages. In conclusion, our results suggested that GSE had therapeutic potential for EAE by inhibiting neuroinflammation.

Oxidative damage is now considered to be the main cause of axonal degeneration in the MS

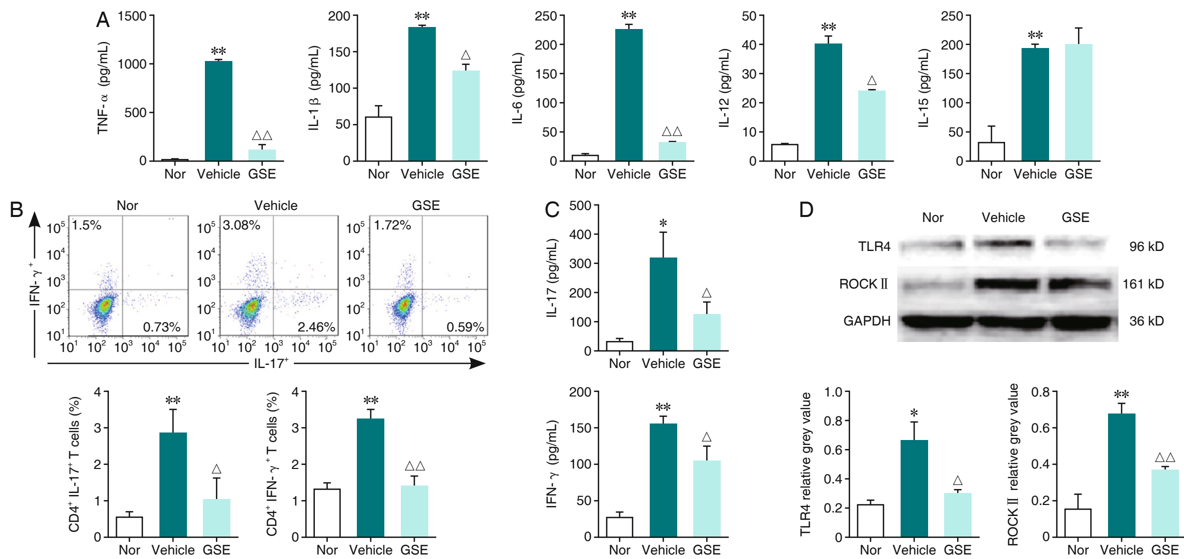


Figure 3. GSE Inhibited Peripheral Immune Response in EAE Mice ($\bar{x} \pm s$, n=10)

Notes: MNCs were isolated from the spleen of vehicle- and GSE-treated EAE mice on day 28 p.i. (A) The levels of macrophage/microglia-derived cytokines TNF-α, IL-1β, IL-6, IL-12 and IL-15 were analyzed by ELISA. (B) $CD4^+$ T cells producing pathogenic IL-17 and IFN-γ were analyzed by flow cytometry, and the percentages in the CNS were shown. (C) The levels of IL-17 and IFN-γ derived from T cells were measured by ELISA kits. (D) TLR4 and ROCK Ⅱ expression were detected by Western blot, and densitometry of the bands were quantified by using Image J software. $^*P$<0.05, $^{**}P$<0.01 *vs*. normal group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. vehicle group. MNCs: mononuclear cells; CNS: central nervous system; TLR4: toll-like-receptor 4; ROCK Ⅱ: Rho-associated kinase; EAE: experimental autoimmune encephalomyelitis; ELISA: enzyme linked immunosorbent assay; and the same below

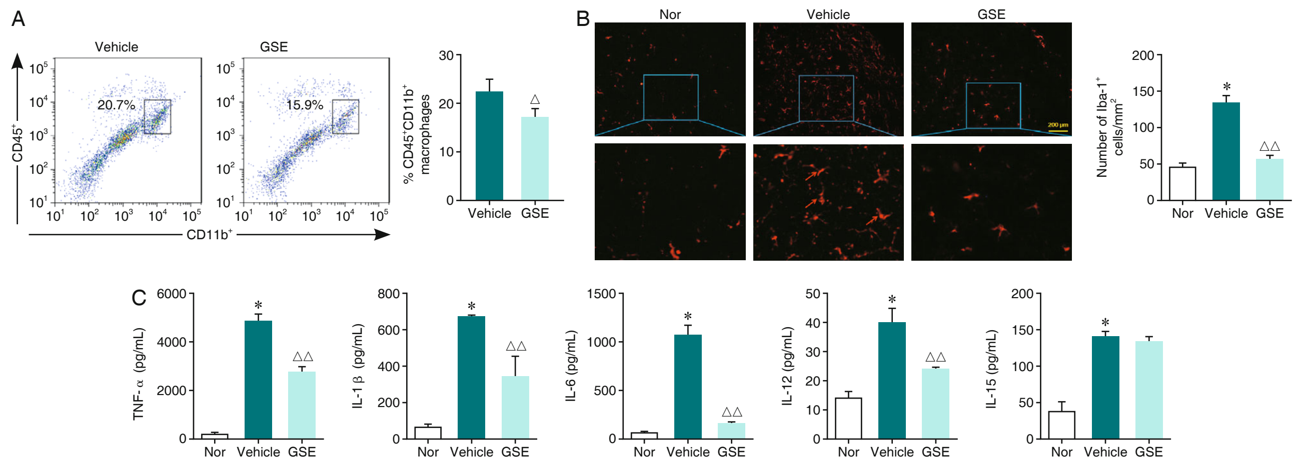
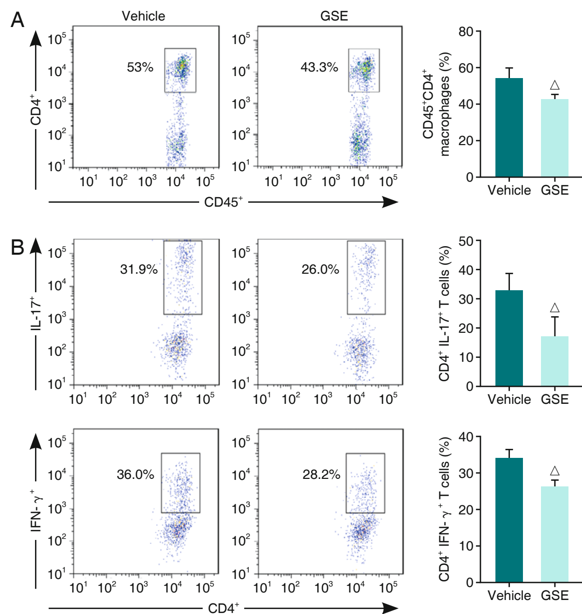


Figure 4. GSE Inhibited Infiltration of Peripheral Macrophages in CNS of EAE Mice, Activated Microglia and Inhibited Production of Inflammatory Cytokines ($\bar{x} \pm s$, n=10)

Notes: MNCs were isolated from the spinal cord of vehicle- and GSE-treated EAE mice on day 28 p.i. (A) Cells were analyzed for expression of $CD45^{high}CD11b^+$ by flow cytometry. The percentages of $CD45^{high}CD11b^+$ cells in the CNS were shown. (B) IF staining for Iba-1 (red) showed the absolute number of Iba-1^+ cells in CNS (×40). Scale bar=200 μm. (C) Levels of TNF-α, IL-1β, IL-6, IL-12 and IL-15 were analyzed by ELISA. $^*P$<0.01 *vs*. normal group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. vehicle group

brain. In fact, some markers of oxidative stress, such as malondialdehyde, reduced form of nicotinamide adenine dinucleotide phosphate oxidase complex, nitric oxide (NO) and reactive oxygen species, are highly expressed in MS patients and EAE models.[18] These oxidative stress factors can cause irreversible oxidative damage by acting on different pathological processes, including activation of immune cells (microglia, T cells and B cells), destruction of BBB and promotion of leukocyte migration. Therefore, the use of antioxidants is attractive for the treatment of MS,[18,19] and has excellent research prospects.[19] The clinical research progress of dimethyl fumarate-activated nuclear-related factor 2 (Nrf2) antioxidant signal cascade reaction showed that inhibiting oxidative stress is essential for clinical treatment.[20] In this study, we found that GSE inhibited the peripheral inflammatory response of EAE mice on day 9 p.i. After intervening EAE mice on



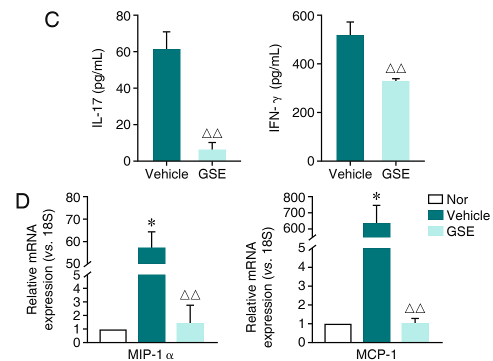


Figure 5. GSE Reduced Encephalitogenic T Cell Infiltration into CNS in EAE Mice ($\bar{x}\pm s$, n=10)

Notes: MNCs were isolated from CNS of vehicle- and GSE-treated EAE mice on day 28 p.i. (A) Cells were analyzed for expression of $CD4^+$ in the lymphocyte gate by flow cytometry. The percentages of $CD4^+$ cells in the CNS were shown. (B) The pathogenic IL-17- and IFN-γ-producing $CD4^+$ T cells were analyzed by flow cytometry, and the percentages in the CNS were shown. (C) The contents of IL-17 and IFN-γ were detected by ELISA. (D) mRNA expression of MIP-1α and MCP-1 by real-time PCR. $^*P$<0.01 *vs*. normal group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. vehicle group

day 3 p.i. with GSE, it was found that GSE significantly delayed clinical disease onset and extend time to disease peak, indicating that GSE plays a critical role in modulating the immune-inflammatory response driving the onset and early pathological events of EAE.

Myeloid cells in the CNS such as resident microglial cells and infiltrating macrophages play an important role in the development of the disease.[21-23] As a source of pro-inflammatory cytokines and chemokines, they can further amplify the recruitment of immune cells.[24] Here, we firstly confirmed the inhibitory action of GSE on the infiltration of peripheral macrophages and the activation of microglia, and found that GSE reduced the secretion of some cytokines from macrophage/microglia, such as TNF-α, IL-1β, IL-6 and IL-12. These factors aggravate the pathological process of EAE.[25,26] TNF-α can act on T cells, and promote the activation and proliferation of initial and effector T cells, further determining the size of the pathogenic conventional T cell pool.[27] TNF-α also directly induce the surface expression of Ca^{2+} permeable-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and N-methyl-D-aspartate receptors, and decrease inhibitory gamma-aminobutyric acid A receptors on neurons.[28-30] IL-1β and IL-6 are important factors that induce Th17 cell differentiation. Notably, neutralizing IL-1β and/or IL-6 significantly decreased IL-17 expression and Th17 cell population.[31] IL-12-modulated Th1 cells readily produce IFN-γ and granulocyte-macrophage colony stimulating factor (GM-CSF) in the CNS of mice and induce a severe form of EAE.[31] IL-15 is highly expressed in serum and cerebrospinal fluid of MS patients and EAE mice, which means that IL-15 is essential to the pathogenesis of MS. However, our experimental results showed that GSE did not inhibit IL-15 secretion. High level of IL-15 expressed in the serum and cerebrospinal fluid of MS patients[32] and mice with EAE, which means IL-15 is critical for the pathogenesis of MS. IL-15 can induce $CD8^+$ T cell activation and memory T cells, transforming these T cells into pathogenic Th17 cells, resulting in tissue damage in EAE mice.[33,34] And exon-6-lacking IL-15△E6 mRNA isoform, preventing the infiltration of pathogenic T lymphocytes and macrophages into the CNS, can be induced by the activated immune cells.[35] Therefore, the effect of GSE on IL-15 has further research value and the specific mechanism will be our next research content.

Demyelination induced by autoreactive T cell response has long been considered a key pathological process in MS and EAE models. The detection results of T cells showed that GSE inhibited the infiltration of T cells and reduced the number of MS-related pro-inflammatory or pathogenic $CD4^+$ T cells—Th17 and Th1 cells. $CD4^+$ Th1 and $CD4^+$ Th17 mainly secrete pathogenic IL-17 and IFN-γ. In the CNS, IL-17 and IFN-γ induce the release of additional pro-inflammatory mediators and the increase of antigens to enhance immune response, or directly affect the activity of CNS resident cells to cause CNS injury.[36] For example, IL-17 can promote immune responses by acting on macrophages, microglia, astrocytes and oligodendrocytes through its receptors and by increasing the secretion of proinflammatory cytokines, chemokines and other effector proteins in synergy

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with other related signaling pathways.[37,38] IFN-γ respectively stimulates immune and structural cells, such as macrophages and epithelial cells, to release chemokines that are responsible for the recruitment and infiltration of inflammatory cells, and therefore amplifies the immune response.[39] And experimental results showed that targeting IL-17 and IFN-γ can effectively treat MS. It has been reported that dimethyl fumarate used clinically in the treatment of MS can also reduce Th17 response.[40,41] Therefore, GSE can improve the central inflammatory microenvironment and play a therapeutic role by inhibiting the differentiation of Th17 and Th1.

Chemotactic cytokines are classified into 4 groups (CXC, CX3C, CC and C) based on the position of the first 2 highly conserved cysteines in the amino acid sequence. Many studies have shown that chemokines are involved in the generation and development of EAE by inducing the extravasation of leukocytes and regulating the activation, migration and infiltration of monocytes, dendritic cells and T cells.[42,43] For example, MCP-1, regulated the expression and secretion of activated normal T cells (RANTES) and MIP-1α have remarkable synergistic effects during the relapses and remissions of EAE.[44] In this study, we detected 5 chemokines in spinal cords and found that GSE downregulated the expression of MCP-1 and MIP-1α mRNA. It is reported that the production of MCP-1 and MIP-1α is associated with acute symptoms in both rats and mice EAE models,[45] which is associated with chemotactic effect, but more importantly with chemotactic factor to influence the polarization of the Th cells and inflammatory cytokine secretion. Chemotactic effect can also affect the polarization of Th cells and the secretion of inflammatory cytokines. For example, on the one hand, MIP-1α can promote the differentiation of Th1 cells and the secretion of IFN-γ when stimulated by T cell receptor, indicating that MIP-1α not only directly affects lymphocyte recruitment, but also indirectly affects tissue damage and proinflammatory cytokine secretion.[10] On the other hand, increasing MCP-1 may inhibit IL-5$^+$/IL-13$^+$ Th2 differentiation and normal cell function, thereby inhibiting Th2-mediated anti-inflammatory effect. MCP-1 can also lead to pathogenesis to differing disease conditions characterized by dysregulated monocyte/macrophage tissue recruitment.[46] And it is reported that the upregulation of MCP-1 expression was detected in macrophage-rich lesions and treatment with an anti-MCP-1 antibody significantly reduced macrophage infiltration and inflammatory responses.[47] Therefore, inhibiting MCP-1 and MIP-1α may effectively alleviate the demyelination of EAE by inhibiting the infiltration of immune cells into the CNS.

Several investigations also proved the roles played by TLR4 and ROCK Ⅱ in MS pathogenesis. For example, TLR4 was demonstrated to be essential for the induction of LPS-induced oligodendrocyte death in the CNS of an EAE model. Inhibition of TLR4 signal transduction pathway showed a certain inhibitory effect on the occurrence of EAE.[48] Based on another study,[49] N-adamantyl-4-methylthiazol-2-amine, a potential therapy for neuroinflammatory disorders, such as MS, can decline TNF-α, IL-1β, inducible nitric oxide synthase (iNOS), and NO levels through inhibition of TLR4, and subsequent activation of nuclear factor kappa-B (NF-κB) signaling pathway induced by LPS in microglia culture. ROCK is highly expressed in the peripheral regions of MS patients, and ROCK upstream effector RhoA is upregulated in plaques,[49] which regulates the recruitment and activation of inflammatory cells in the injured and stressed sites, and aggravates the inflammatory response and demyelination.[50] Our study showed that GSE treatment inhibited the levels of TLR4 and ROCK Ⅱ, and was able to sustain the down-regulation of multiple inflammatory factors in EAE. From this we speculate that GSE may inhibit inflammatory cytokines by inhibiting TLR4 and ROCK Ⅱ.

In conclusion, GSE, as a powerful antioxidant, can alleviate neuroinflammation by inhibiting the abnormally activated macrophages/microglia, the differentiation of Th1 and Th17 and chemokines. It provides an experimental basis for the therapeutic potential of GSE in EAE. However, the inhibitory effect of GSE on TLR4 and ROCK Ⅱ and its possible mechanism are still worthy of further study.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

Wang Q and Yang ZC completed most of the work together and wrote the manuscript. Ma CG and Xiao BG helped coordinate and draft the manuscript. Miao Q, Chen YY and Dong YW mainly assisted in cell experiment. Li YQ and Wang J mainly assisted in animal experiments. Yuan HJ and Yu JZ were involved in the revisions. Ma CG and Xiao BG revised and finalized the

manuscript. All authors read and approved the final manuscript.

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