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The therapeutic mechanism of Shenyuan Gan in lipopolysaccharide-induced neuroinflammation in BV2 microglial cells

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ABSTRACT

Objective To study the therapeutic effects of Shenyuan Gan (参远苷, SYG) on the inflammatory response in BV2 microglial cells induced by lipopolysaccharide (LPS).

Methods The cytotoxicity of SYG to BV2 microglial cells was evaluated using a Cell Counting Kit-8 (CCK-8) assay, and the effect of SYG concentrations on LPS-induced BV2 microglial cells was studied. The morphological changes were observed using an optical microscope. The nitric oxide (NO) concentration in cell culture supernatant was determined using Griess reagent. The expression of cytokines and inflammatory mediators were also measured by an enzyme-linked immunosorbent assay (ELISA). Western blot analysis was used to determine the levels of inducible NO synthase (iNOS), nuclear factor-kappa B (NF-κB) p65, alpha inhibitor of NF-κB (IκB-α), phosphorylation-IκB-α (p-IκB-α), NOD-like receptor 3 (NLRP3), and caspase-1 expression. Moreover, the expression of iNOS, NLRP3, and ionized calcium binding adapter molecule 1 (Iba1) was also observed using immunofluorescent staining.

Results SYG had a low cytotoxic effect on BV2 microglial cells and could significantly decrease LPS-induced morphological changes of BV2 microglial cells ($P < 0.05$). ELISA results showed that SYG significantly inhibited the LPS-induced increase in interleukin (IL)-1β and IL-6 in BV2 microglia cells ($P < 0.05$), and Western blot analysis showed that the phosphorylation levels of iNOS, NF-κB p65, and IκB-α as well as NLRP3 and caspase-1 expression were also significantly decreased, and IκB-α expression was increased after SYG treatment ($P < 0.05$, compared with the LPS-treated group). The immunofluorescence results were consistent with the Western blot results, and Iba1 staining indicated that the cell morphology tended to be resting. These results indicate that SYG has a certain inhibitory effect on LPS-induced inflammation in BV2 microglial cells.

Conclusion SYG can inhibit LPS-induced release of inflammatory factors in BV2 microglial cells by affecting the phosphorylation levels of NF-κB p65 and IκB-α. SYG is a valuable candidate for treating neuroinflammation-related diseases.

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1 Introduction

Neuroinflammation is an immune response that involves local immune cells (e.g., microglia) and inflammation-related pathways within the central nervous system (CNS), and is a complex response in the CNS against internal and external infections [1]. Neuroinflammation is an important factor in various CNS diseases, such as depression and Alzheimer's disease [2-4]. Microglia are considered to be immune-competent cells in the CNS [5]. Microglia activate a stimulating inflammatory reaction by releasing pro-inflammatory cytokines upon any change in the brain microenvironment. Hence, resolving microglia inflammation bears promise as a novel treatment strategy for neuroinflammation-mediated CNS disease.

Chinese medicine formulas (CMFs), combinations of multiple decoctions under the guidance of traditional Chinese medicine (TCM) theory, have been used for thousands of years. Nowadays, with the modernization and standardization of TCM, the contents of CMFs have expanded to include many effective compounds [6, 7]. Renshen (Ginseng Radix et Rhizoma) and Yuanzhi (Polygalae Radix) are the most common herbal materials in traditional oriental medicines, including TCM [8]. These two herbs are critical components of a series of CMFs derived from Kanxin San (开心散), which is originally recorded in *Valuable Prescriptions for Emergency* (*Bei Ji Qian Jin Yao Fang*, 《备急千金要方》) and prescribed for brain disorders [9]. Total saponins of Renshen (Ginseng Radix et Rhizoma) (TSG) and total oligosaccharide esters of Yuanzhi (Polygalae Radix) (TOP) are the major effective compounds of Renshen (Ginseng Radix et Rhizoma) and Yuanzhi (Polygalae Radix) and the main constituents of our novel compound-based CMF, Shenyuan Gan (参远苷, SYG). SYG is a Chinese herbal mixture composed of TSG and TOP (2∶1). In our previous studies [10, 11], SYG has been demonstrated to be effective in ameliorating depressive-like behavior and enhancing cognitive ability in an *in vivo* chronic mild stress model. Considering that chronic stress-induced mental disorders are closely associated with neuroinflammation [12], we hypothesized that SYG could exert beneficial effects on the CNS via modulating neuroinflammation. Therefore, we investigated the effects of SYG on lipopolysaccharide (LPS)-induced BV2 microglia cell inflammation and aimed to investigate the underlying mechanism of its anti-inflammatory effect.

2 Materials and methods

2.1 Cell culture

The BV2 microglia cell line was purchased from Wuhan University. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Procell, China) with 10% fetal bovine serum (FBS, Procell, China). The cells were incubated at 37 °C with 5% CO_2.

2.2 Drugs and treatments

TSG and TOP were purchased from the China Academy of Chinese Medical Sciences. SYG consisted of TSG and TOP at a ratio of 2∶1, which was dissolved in DMEM (100 μg/mL). LPS (Sigma, USA) was dissolved in phosphate-buffered saline (1 mg/mL) (Procell, China). In all subsequent experiments, cells were pretreated with SYG (30 – 120 μg/mL) for 2 h and stimulated with LPS (400 ng/mL) for 24 h.

2.3 Cell viability assay

Cell viability was assessed with a Cell Counting Kit-8 (CCK-8) assay (Biosharpe, China). Cells were inoculated in 96-well plates at a density of 8 ×10^3 per well. After the cells reached 80% confluence, LPS (400 ng/mL) and different concentrations of SYG (0, 1, 10, 20, 30, 60, and 120 μg/mL) were added as described in section 2.2, or SYG was added alone. After 24 h of incubation, 10% CCK-8 medium was added and the mixture was incubated for 2 h at 37 °C. Then, absorbance was measured at 450 nm in the microplate reader (Biotek, Elx800).

2.4 Nitric oxide (NO) assay

The NO concentration in cell culture supernatant was assessed with the Griess assay. Cells were inoculated in 96-well plates at a density of 8 ×10^3 per well. After the cells reached 80% confluence, they were treated as described in section 2.2. After 24 h of incubation, the supernatant was collected and NO determination was performed according to the manufacturer's protocol (Beyotime, China).

2.5 Measurement of inflammatory factor levels

Cell inflammatory factors interleukin (IL)-1β and IL-6 were assessed with an enzyme-linked immunosorbent assay (ELISA) kit (MULTI SCIENCES, China). Cells were inoculated in 96-well plates at a density of 8 ×10^3 per well. After the cells reached 80% confluence, they were treated as described in section 2.2. After 24 h of incubation, the cell culture supernatant was collected and centrifuged (500 ×g, 6 min) to collect the supernatant, then this supernatant was added to a 96-well plate in the ELISA kit, with 100 μL per well. The concentration of IL-6 and IL-1β was measured using the ELISA manufacturer's instructions.

2.6 Western blot analysis

Cells were seeded into 6-well plates at a density of 1×10^5 cells per well. After the cells reached 80% confluence, various concentrations of SYG were added to cells 2 h before incubation with or without LPS (400 ng/mL) for 24 h. After treatment with LPS and SYG, the supernatant was removed. Cells were lysed on ice for 30 min with RIPA buffer (MULTI SCIENCES, China), then centrifuged at 13 000 rpm for 15 min at 4 °C. The supernatant was collected, and the concentration was determined using a bicinchoninic acid protein measurement kit (MULTI SCIENCES, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used for 8% and 10% electrophoresis and the cells were then transferred to polyvinylidene fluoride membranes (Immobilon, USA). After blocking with 5% non-fat milk, the primary antibody was incubated at 4 °C overnight. The antibodies were as follows: inducible NO Synthase (iNOS, Cell Signaling Technology, CST, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Proteintech, China), nuclear factor-kappa B (NF-κB) p65 (Abcam, UK), phosphorylation-NF-κB p65 (Abcam, UK), alpha inhibitor of NF-κB (IκB-α, CST, USA), phosphorylation-IκB-α (p-IκB-α, CST, USA), NOD-like receptor 3 (NLRP3, Abcam, UK), and caspase-1 (Abcam, UK). Then, the membrane was washed with Tris-buffered saline-Tween buffer and incubated for 1 h at 37 °C with HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) (Proteintech, China) and HRP-conjugated Affinipure Goat Anti-Mouse IgG (H +L) (Proteintech, China). The protein bands were visualized by chemiluminescence (Millipore, USA). The GAPDH protein level was used as the protein loading control. According to the protein quantification data, the total amount of protein loaded into each line of gel was about 15 – 20 μg.

2.7 Immunofluorescence staining of iNOS, NLRP3, and ionized calcium binding adapter molecule (Iba1)

BV2 microglial cells were seeded into 24-well plates at a density of 1×10^4 cells per well, and were treated as described above. After incubation for 24 h, the supernatant was removed. Cells were fixed with 4% paraformaldehyde for 20 min and 0.1% Triton X-100 was used for 20 min for permeabilization. The cells were blocked with 3% BSA for 1 h and incubated overnight at 4 °C with primary antibodies. The antibodies were as follows: iNOS, NLRP3 (Boster, China), and Iba1 (Abcam, UK). The following day, cells were incubated with the following secondary antibodies: fluorescein-conjugated Affinipure Goat Anti-Rabbit IgG (H +L) (Abcam, UK), Alexa Flour® 594-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) (Abcam, UK), and Donkey Anti-Goat IgG (H +L) (Alexa Fluor® 555) (Abcam, UK) in a 1% Bovine Serum Albumin (BSA) dilution for 1 h at 37 °C. The cells were treated with DAPI (Sigma, USA) for 10 min. All images were captured with a fluorescence microscope (Olympus, lx83-CBH).

2.8 Statistical analysis

The data were statistically analyzed by GraphPad Prism 8.0 and SPSS 21.0 software. The measurement data were expressed as mean ± standard deviation (SD). The difference between the two groups of sample means was statistically analyzed by *t* test or with a one-way analysis of variance. Post hoc least significant difference comparisons were performed when appropriate [13], and $P < 0.05$ indicated that the difference was statistically significant [14].

3 Results

3.1 Effects of SYG and LPS on cell viability and morphology

The CCK-8 assay was performed after treatment with various concentrations of SYG to determine the effect on the viability of BV2 microglial cells. Figure 1A shows that none of the concentrations of SYG affected the viability of BV2 microglial cells in the range of 0 – 120 μg/mL. Thus, the concentration range of 30 – 120 μg/mL was used for the subsequent experiments. In the range of 0 – 120 μg/mL of SYG, SYG at different concentrations showed no significant toxic effects on BV2 microglial cells ($P > 0.05$); interestingly, SYG also showed a growth-promoting effect on BV2 microglial cells ($P < 0.05$). In addition, in order to determine the combined toxicity of LPS and SYG on BV2 microglial cells, we added LPS (400 ng/mL) to determine the cell viability on the basis of the previous (Figure 1B). No significant effect on cell viability was found ($P > 0.05$). To determine whether SYG alters the LPS-induced morphology of BV2 microglial cells, the cells were pretreated with SYG (120 μg/mL) for 2 h, followed by treatment with LPS for 24 h, and were then observed under a microscope. As indicated in Figure 1C and 1D, after stimulation with LPS, the cell morphology changed from the resting to the activated state and showed decreased pseudopodia, and the cell body became larger and rounder. There was also a significant increase in the proportion of activated cells ($P < 0.05$). However, SYG effectively decreased these changes and the proportion of activated cells. These results showed that an optimal concentration of SYG can effectively decrease the activation of microglia induced by LPS.

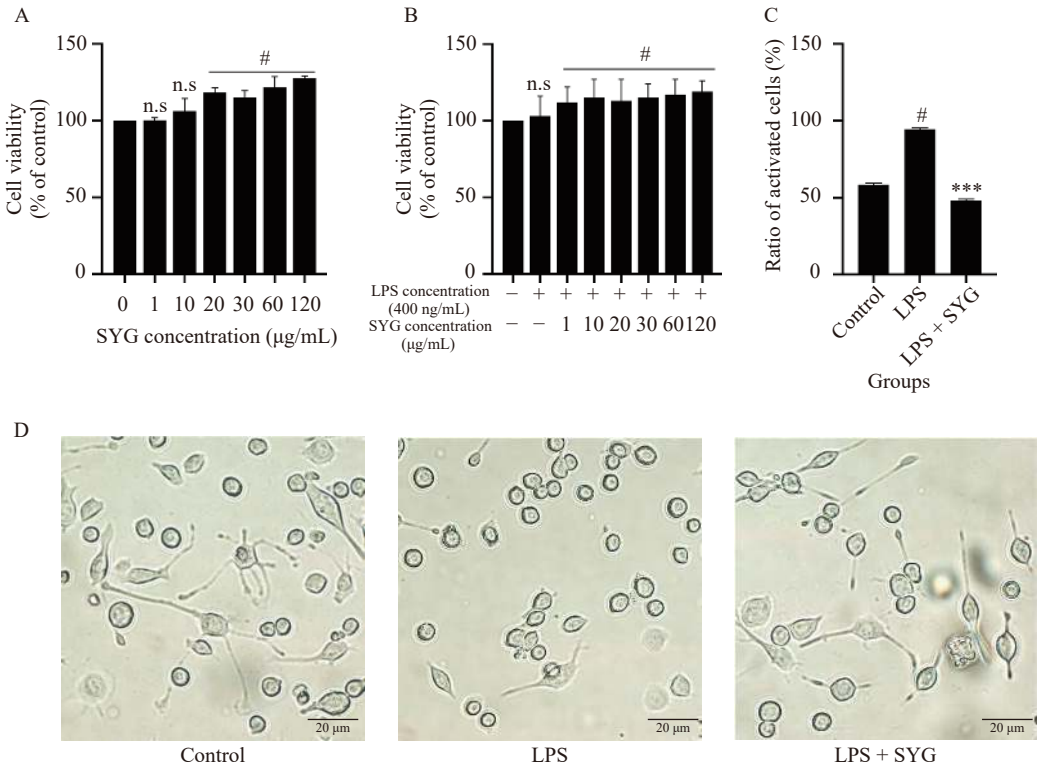

Figure 1 Effects of SYG on the viability and activation state of BV2 microglia cells

A, the effects of different concentrations of SYG on cell viability. B, the effects of different concentrations of SYG and LPS (400 ng/mL) on cell viability. C, the ratio of activated cells stimulated with LPS. D, the cell morphology change under different treatments. The data of three independent repeated experiments were analyzed, and the results are denoted by mean ± SD. n.s represents no significant difference. $^{\#}P < 0.05$, compared with the control group; $^{***}P < 0.001$, compared with the LPS-treated group.

3.2 Effects of SYG treatment on inflammatory cytokine levels

To investigate the potential regulatory effects of SYG on proinflammatory responses, BV2 microglial cells were treated as described above. The NO level significantly increased after LPS treatment ($P < 0.001$), whereas pretreatment with SYG significantly decreased LPS-induced NO production in a dose-dependent manner ($P < 0.001$). The highest concentration (120 μg/mL) resulted in a decrease to about one-half of that of the LPS group (Figure 2A). ELISA and Western blot were used to investigate whether SYG regulated the expression of inflammatory cytokines. The IL-6 and IL-1β levels increased in the cell culture media in the LPS treatment group, and pre-treatment with different concentrations of SYG resulted in a dose-dependent decrease in cytokine production (Figure 2B and 2C). Consistent with the findings above, immunofluorescence and Western blot showed that SYG significantly reduced LPS-induced iNOS expression ($P < 0.05$, Figure 2D and 2E).

3.3 The regulatory effects of SYG on NF-κB activity

The NF-κB pathway has long been considered a prototypical proinflammatory signaling pathway. Thus, we hypothesized that SYG may inhibit the NF-κB signaling pathway to regulate the neuroinflammation response. NF-κB and IκB-α expression was detected by Western blot analysis. As shown in Figure 3, after LPS stimulation, there was a marked decrease in the IκB-α protein level but an increase in the phosphorylation levels of NF-κB p65 and IκB-α compared with the control group ($P < 0.05$). However, the decrease in LPS-induced neuroinflammation by SYG could be attributed to its inhibition of IκB phosphorylation.

3.4 SYG suppressed the NLRP3-mediated inflammatory response

The activation of the NLRP3 inflammasome is considered a key contributor to the development of neuroinflammation. To investigate whether SYG affects its activation, BV2 microglia cells were treated as mentioned above, and the NLRP3 and caspase-1 expression levels were examined by Western blot analysis. As shown in Figure 4A and 4B, the addition of SYG effectively reduced the increase in NLRP3 and caspase-1 in BV2 microglia cells caused by LPS stimulation ($P < 0.05$). In the immunofluorescence image of Iba1 and NLRP3 (Figure 4C), NLRP3 expression also showed enhancement after LPS stimulation. After SYG treatment, the expression of NLRP3 decreased, and the cell morphology also changed to the resting state.

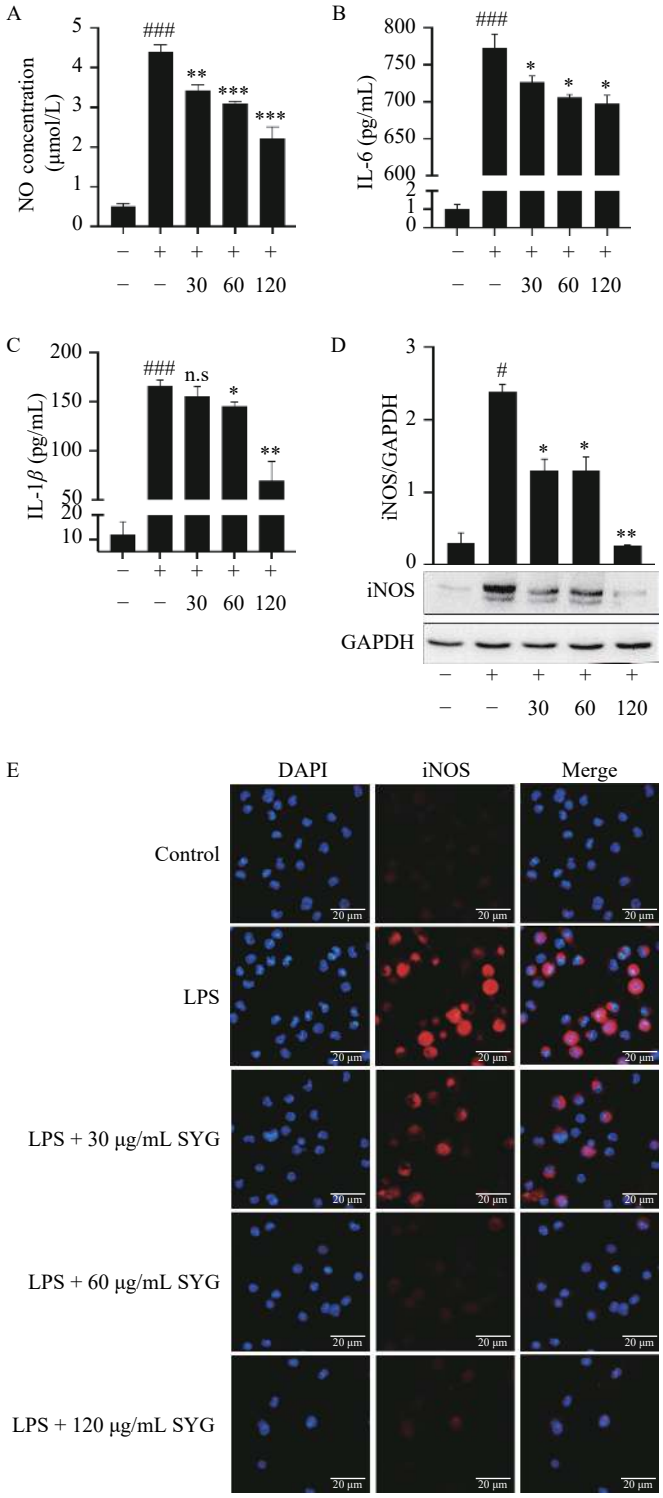

Figure 2 Effects of SYG on the inflammatory cytokines in LPS-induced BV2 microglial cells

A, NO concentration of different treatment groups of cells. B and C, levels of IL-6 and IL-1β of the BV2 microglial cells, respectively. D, iNOS expression of the BV2 microglial cells. E, representative fluorescence microscopy images of the expression of iNOS (red) in LPS-activated BV2 microglial cells. The nuclei were stained with DAPI (blue). The data of three independent repeated experiments were analyzed, and the results are denoted by mean ± SD. n.s represents no significant difference. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the LPS-treated group.

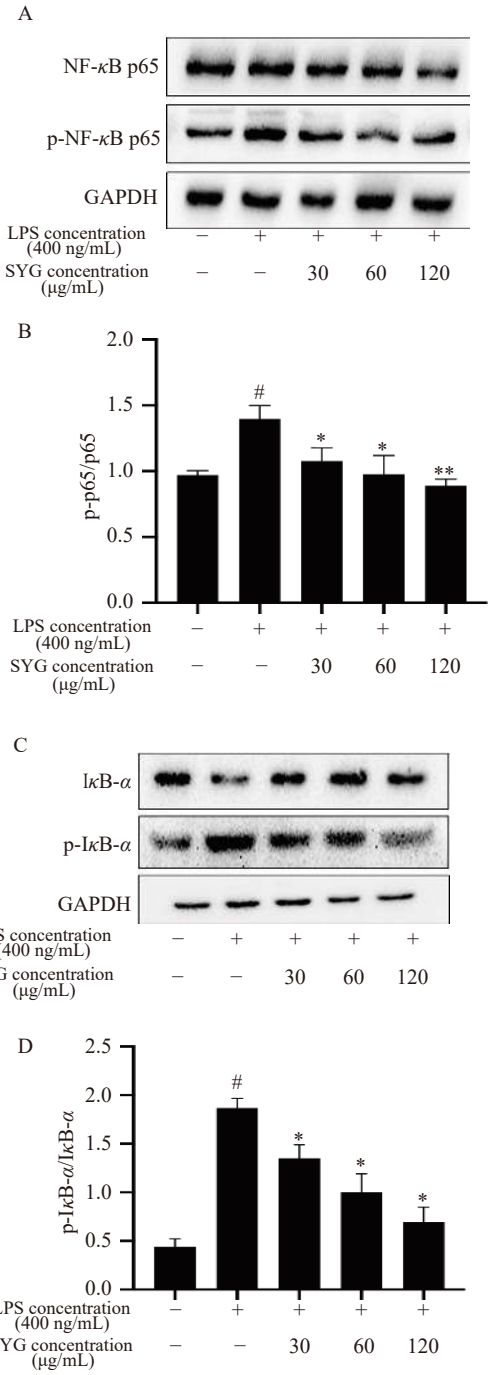

Figure 3 Effects of SYG on NF-κB activation by LPS-induced BV2 microglial cells

A, Western blot analysis of NF-κB p65 and phospho-NF-κB p65. B, quantitation of Western blot analysis of NF-κB p65 and phospho-NF-κB p65. C, Western blot of IκB-α and phospho-IκB-α. D, quantitation of Western blot analysis of IκB-α and phospho-IκB-α. The data of three independent repeated experiments were analyzed, and the results are denoted by mean ± SD. $^{\#}P < 0.05$, compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the LPS-treated group.

4 Discussion

Compound-based CMF is a bridge between traditional and modern Chinese medicine. In addition to the synergistic effects of TCM, compound-based CMF has the

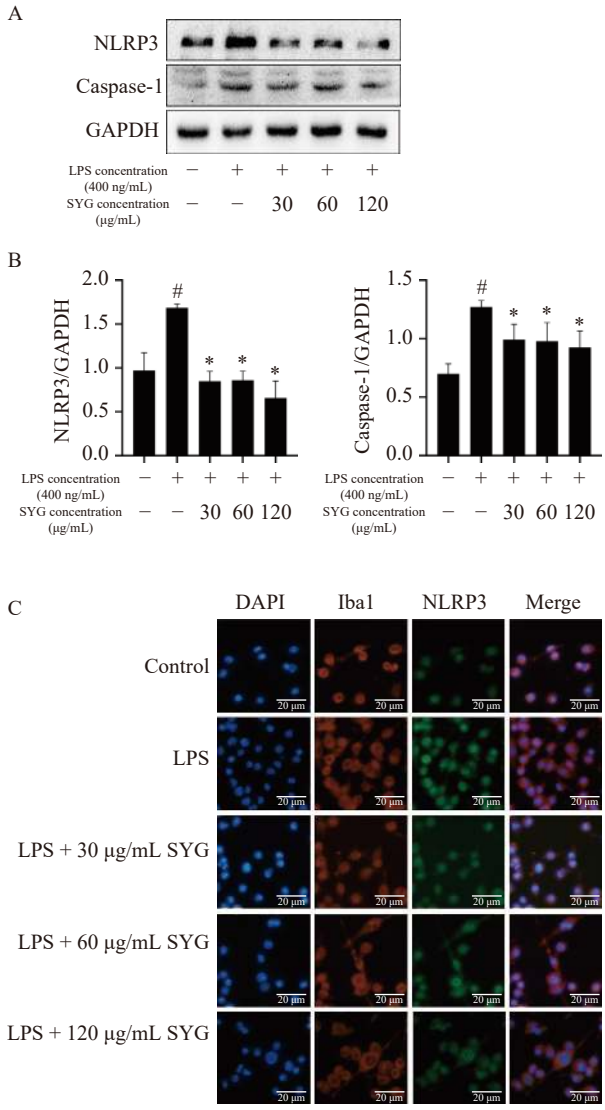

Figure 4 Effects of SYG on NLRP3 activation by LPS-induced BV2 microglial cells

A, Western blot analysis of NLRP3 and caspase-1. B, quantitation of Western blot analysis of NLRP3 and caspase-1. C, representative fluorescence microscopy images of the expression of NLRP3 (green) and Iba1 (orange) in LPS-activated BV2 cells. The nuclei were stained with DAPI (blue). The data of three independent repeated experiments were analyzed, and the results are denoted by mean ± SD. $^{\#}P < 0.05$, compared with the control group; $^{*}P < 0.05$, compared with the LPS-treated group.

advantages of clear chemical composition and controllable quality. It is helpful to better comprehend the underlying mechanisms of classical prescription and interpret the rules of herbal combination to some extent [15]. Due to neuroprotective, anti-oxidative stress, anti-apoptotic,anti-inflammatory,andneurotrophiceffects,bothRenshen (Ginseng Radix et Rhizoma) and Yuanzhi (Polygalae Radix) have been traditionally used to treat cognitive deficits and other brain diseases in TCM [16-19]. The combination of TSG and TGP would exert a better curative effect and lower toxic potential [20].

This study found that SYG was effective in inhibiting BV2 microglial cell inflammation. In the range of 0 – 120 μg/mL, SYG showed no significant toxicity towards BV2 microglial cells and could significantly decrease the activated morphological changes and proinflammatory cytokine levels. In normal states, microglia do not produce any pro-inflammatory factors, and they are considered to be homeostasis maintainers in the brain. In response to any stimuli, such as injury, harmful toxins, infection, or inflammation, microglial cells produce pro-inflammatory mediators, such as IL-6, IL-1β, and chemokines. LPS is the most widely used inflammatory mediator, and it can trigger a generalized pro-inflammatory response in microglia [21] to further induce many inflammation-related signal pathways, such as the NF-κB pathwaty. The transcription of chemokines, cytokines, and pro-inflammatory enzymes is under the control of NF-κB [22, 23]. Hence, NF-κB is the central transcription factor of inflammation. In addition, we noticed that NF-κB was activated by LPS stimulation and increased the level of IL-6, IL-1β, iNOS, and NO. In resting cells, inactivated NF-κB is located in the cytoplasm that integrates with the inhibitor IκB. The presence of LPS stimuli results in the phosphorylation of IκB protein and the proteasomal degradation of IκB [24]. This change causes NF-κB p65 to phosphorylate at the Ser536 site and release NF-κB from Iκ-B. The released NF-κB translocates into the nucleus and initiates a series of transcriptions, activates downstream inflammatory response factors, and causes a series of inflammatory reactions [25]. Therefore, the NF-κB signaling pathway plays a key role in neuroinflammation-related disease control [26].

Moreover, studies have shown that activated NF-κB can effectively activate the NLRP3 inflammasome signaling pathway, and IL-1β secretion is closely controlled by the activation of NLRP3 inflammasomes [27, 28]. Once overexpressed, NLRP3 activation promotes the cleavage of procaspase-1 [27]. The NLRP3 inflammasome and activated NF-κB work together to increase IL-1β transcription and thereby trigger inflammatory responses [29].

5 Conclusion

In summary, our results indicate that SYG can alleviate BV2 microglia-related neuroinflammation by affecting NF-κB activity and inflammasome assembly to treat related neurological diseases. On the other hand, it is necessary to conduct further *in vivo* experimental research on SYG to determine its component metabolism changes in the body to carry out follow-up treatment research.

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Competing interests

The authors declare no conflict of interest.

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参远苷抑制脂多糖诱导的 BV2 小胶质细胞炎症机制研究

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【摘要】目的 研究参远苷对脂多糖诱导的 BV2 小胶质细胞炎症反应的治疗作用。**方法** 采用细胞增殖-毒性检测（CCK-8）法评价参远苷对 BV2 小胶质细胞的细胞毒性，并研究不同浓度参远苷对脂多糖（LPS）诱导的 BV2 小胶质细胞活力的影响；光学显微镜观察细胞形态变化；使用 Griess 试剂测定细胞培养上清液一氧化氮（NO）浓度；通过酶联免疫吸附试验（ELISA）测量细胞因子和炎症介质的表达；蛋白质印迹分析（Western blot）用于确定诱导型一氧化氮合酶（iNOS）、核因子-κB p65（NF-κB p65）、磷酸化-核因子 κB p65（p-NF-κB p65）、核因子 κB-α 抑制蛋白（IκB-α）和磷酸化核因子-κB-α 抑制蛋白（p-IκB-α）、核苷酸结合寡聚化结构域样受体蛋白 3（NLRP3）以及 caspase-1 表达；免疫荧光染色观察 iNOS、NLRP3 和离子钙结合衔接分子 1（Iba1）的表达。**结果** 参远苷对 BV2 小胶质细胞具有较低的细胞毒性作用，并能显著降低 LPS 诱导的 BV2 小胶质细胞形态变化（$P<0.05$）。ELISA 结果表明参远苷能显著抑制 LPS 诱导的 BV2 小胶质细胞中白细胞介素-1β（IL-1β）和白细胞介素-6（IL-6）表达量的增加（$P<0.05$）。Western blot 结果显示，参远苷处理后的 iNOS、NF-κB p65 和 IκB-α 磷酸化水平以及 NLRP3 和 caspase-1 的表达明显降低，IκB-α 表达升高（$P<0.05$，与 LPS 处理组相比）。免疫荧光结果与 Western blot 结果一致，并且 Iba1 染色表明细胞形态趋于静息状态。这些结果表明，参远苷对 LPS 诱导的 BV2 小胶质细胞的炎症有一定的抑制作用。**结论** 参远苷可能通过影响 NF-κB p65 和 IκB-α 的磷酸化水平来抑制 LPS 诱导的 BV2 小胶质细胞炎症因子释放。参远苷是治疗神经炎症相关疾病的有价值的候选药物。

【关键词】参远苷；神经炎症；促炎因子；BV2 小胶质细胞；脂多糖