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Catalpol alleviates the lipopolysaccharide-induced inflammatory response of BV2 cells

Sijie Liu[1], Jiayi Zhu[1], Hao Huang[1], Jiaxiang Xu[1], Biao Zhang[2], and Yi Luo[1]

[1]Department of Neurology, Suzhou Hospital of Integrated Traditional Chinese and Western Medicine, Suzhou, China; [2]Department of Central Laboratory, Suzhou Hospital of Integrated Traditional Chinese and Western Medicine, Suzhou, China.

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Abstract

This study aimed to investigate the effect of catalpol on the inflammatory response of murine microglial cell line (BV2 cells) induced by lipopolysaccharide. Cell proliferation activity was detected by CCK-8 assay. The morphology of BV2 was observed by an optical microscope. The inflammatory factors interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) were detected by enzyme-linked immunosorbent assay (ELISA). Reactive oxygen species (ROS) were detected by flow cytometry. Induced nitric oxide synthase (iNOS) level was detected by immunofluorescence. The results showed that 5 μg/mL catalpol did not effect the proliferation of BV2 cells, while 10 μg/mL catalpol significantly decreased the viability of BV2 cells. Then the experiment was carried out with 5 μg/mL catalpol. Catalpol can improve the morphology of lipopolysaccharide-induced BV2 cells, decrease the level of inflammatory factors, and reduce the production of iNOS and ROS. Therefore, catalpol can inhibit the lipopolysaccharide-induced activation of BV2 cells and has anti-inflammatory effects.

Introduction

Neuroinflammation is an innate and adaptive immune response initiated by various immune cells to injuries by releasing inflammatory mediators (Ni Chasaide et al., 2020). Neuroinflammation leads to the production of pro-inflammatory cytokines, such as interleukin, tumor necrosis factor (TNF), and some small molecular messengers of nitric oxide (NO) and reactive oxygen species (ROS), leading to nerve damage (Leng et al., 2021). Numerous studies have demonstrated the important role neuroinflammation plays in the pathophysiology of ischemic stroke (Alsbrook et al., 2023) and other neurodegenerative disorders (Thakur et al., 2023) as well as how the condition develops. Recent years have seen a strong increase in the interest in neuroinflammation as a target for therapeutic interventions and diagnostic testing due to its important involvement in various neurological illnesses.

The resident immune cells of the central nervous system are called microglia, which are key regulators of the inflammatory response of the central nervous system (Bhusal et al., 2023). Activated microglia experience structural alterations during neuroinflammation, accompanied by a markedly increased production of ROS and inflammatory cytokines, accelerating the development of the disease (Estes et al., 2014). As a result, numerous research have used therapeutic approaches that target microglia in treating neurodegenerative disorders (Gao et al., 2023) and ischemic stroke (Jurcau et al., 2021). Through the microglia regulating

nerve inflammation is one of the most widely used treatment strategies.

Rehmannia glutinosa is a common traditional herbal medicine in China, often used to treat diseases related to aging and metabolism. Catalpol, an iridoid glucoside, is the main active ingredient from this plant root. To date, catalpol has shown significant neuroprotective effects in experimental models of Alzheimer's disease (Wang et al., 2009), Parkinson's disease (Xu et al., 2010), and ischemic stroke (Zhu et al., 2010), which may be related to its pharmacological effects such as antioxidation, anti-inflammation, and free radical scavenging (Bhattamisra et al., 2019). It is unclear, nevertheless, if microglia contribute to the neuroprotective benefits of catalpol.

As the immortalized murine microglial cell line (BV2 cell) has been extensively employed in research about diseases of the central nervous system (Stansley et al., 2012), this study examined the neuroprotective effect of catalpol on lipopolysaccharide-stimulated BV2 cells by subjecting BV2 cells to lipopolysaccharide treatment, which replicates the inflammatory milieu in the brain.

Materials and Methods

Cell culture

BV2 cells were obtained from iCell (China). The BV2 cells cultured in DMEM (Bdbio, L100-500) supplemented with 10%fetal bovine serum and penicillin-streptomycin. Cells were routinely cultured at 37°C in a 5%CO_2 incubator. When the cells reached 80% abundance, they were digested with 0.25% trypsin and subcultured for further passage.

Drug intervention

Catalpol (C110215, Aladdin, China) was prepared into a solution (5 mM) with PBS as a solvent, ultrasound-promoted, and preserved at -20°C. Subsequently, the medium is sufficiently diluted to the desired concentration. Lipopolysaccharide (GC205009, Servicebio, China) was prepared into a solution (1 mg/mL) with PBS as solvent, ultrasound-assisted, and stored at -20°C. The medium was then completely diluted to 1 μg/mL.

Cell proliferation assay

The impact of catalpol on the viability of BV2 cells was assessed through the CCK-8 assay. The cell proliferation experiment was conducted with CCK-8 assay kit (Medicalbio, CCK-08). BV2 cells in 5,000 cells per hole density inoculated into 96-well plates for the night, add different concentrations of catalpol（1, 2, 5, 10, 20, and 40 μg/mL), incubation for 24 hours. CCK-8 reagent (10 μL) was added to each well after the end cell treatment. After incubation at 37°C for 1 hour, the absorbance of BV2 cells at a wavelength of 450 nm was measured using a microplate reader（Bao et al., 2019）.

Morphological observation

The morphology of BV2 cells under different drug interventions was observed by an optical microscope (Olympus, IX73). BV2 cells were inoculated into 6-well plates at a rate of 4 × 10^5 cells/well and incubated overnight. They were divided into a control group, lipopolysaccharide group, and lipopolysaccharide + catalpol group with 3 multiple pores in each group. After cell adhesion, the lipopolysaccharide + catalpol group was pretreated with 5 μg/mL catalpol for 2 hours and then added with 1 μg/mL lipopolysaccharide, while the lipopolysaccharide group was only added with 1 μg/mL lipopolysaccharide. After drug intervention, the cell morphology was observed under a light microscope after incubation for 24 hours.

ELISA

Inflammatory factors IL-1β, IL-6, and TNF-α were detected by ELISA kit (Multisciences, China, EK 201, EK 206, EK282). BV2 cells were inoculated into 6-well

Box 1: Immunofluorescence detection

Principle

The detection is based on antigen-antibody binding using fluorescent detection.

Requirements

Paraformaldehyde (Servicebio, G1101), PBS (Servicebio, G4202), Triton X-100 (Servicebio, GC204003), BSA (Servicebio, GC305010), iNOS (Zen-Bioscience, 340668), Cy3 conjugated goat anti-mouse IgG (H+L) (Servicebio, GB21301), DAPI (Servicebio, G1012).

Procedure

Step 1: Slides coated with polyl-lysine were placed in 24-well plates.

Step 2: BV2 cells were inoculated in 24-well plates at a density of 1 × 10^4 cells/well and administered in groups for 24 hours.

Step 3: After the culture-medium was removed, the cells were washed 3 times with PBS, fixed with 4% paraformaldehyde at room temperature for 10 min, and then infiltrated with 0.3% Triton X-100 for 15 min.

Step 4: The cells were closed with 5% BSA solution for 30 min, anti-rabbit iNOS (1:100) was added, and incubated at 4°C overnight.

Step 5: The cells were washed 3 times with PBS, incubated with second antibody (Cy3 conjugated goat anti-mouse IgG (H+L), 1:500) for 1 hour, and treated with DAPI (5 μg/mL) at 37°C for 10 min.

Step 6: All images were taken using a fluorescence microscope.

References

Zhao et al.,2021

plates at 4 × 10^5 cells/well, and incubated overnight. The supernatant was collected 24 hours after the cells were administered according to the group and method mentioned above. The levels of inflammatory factors in the supernatant of each group were detected according to the instructions of each ELISA kit (Li et al., 2023).

Flow cytometry

ROS levels were detected by flow cytometry. According to the above density, BV2 cells were inoculated in 6-well plates and cultured overnight. After 24 hours of intervention, the culture medium was discarded, and the cells were washed with PBS 3 times. The reactive oxygen species assay kit (Yeasen, 50101ES01) was diluted in serum-free medium at a ratio of 1:1000. The cells were re-suspended in diluted solution and cultured in a cell incubator at 37°C for 20 min. After washing with PBS again, the cells were collected. After being re-suspended with 500 µL PBS, the ROS content was detected by flow cytometry (Beckman, DxFlex) (Zhang et al., 2022).

Statistical analysis

For each group of at least three separate experiments, all data are expressed as mean ± standard deviation (SD). GraphPad Prism 8.0 (GraphPad Software, USA) experimental data were used for analysis. One-way analysis of variance (ANOVA) followed by the Tukey test was used for comparison among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect on BV2 cells viability

To determine the potential effect of catalpol on the cytotoxicity of BV2 cells, the effects of different concentrations of catalpol on the viability of BV2 cells was measured by the CCK-8 method. As shown in Figure 1, 10 µg/mL catalpol significantly reduced the viability of BV2 cells. Meanwhile, the effect of 5 µg/mL catalpol on the proliferation of BV2 cells was limited. Therefore, 5 µg/mL catalpol was used in the following experiments.

Effect on morphological changes of BV2 cells induced by lipopolysaccharide

The BV2 cells in the control group had spherical or fusiform cell bodies, bright cytoplasm, and distinct borders, all indicating that they were quiescent. Activation of BV2 microglia was observed in the lipopolysaccharide group; these microglia had larger cell bodies and fuzzy borders. At the same time, partially activated BV2 cells showed amoeba-like changes. The morphology of BV2 cells in the lipopolysaccharide + catalpol group showed a considerable improvement with gradually cleaner margins, translucent cell bodies, and fewer processes when compared to the lipopolysaccharide group (Figure 2).

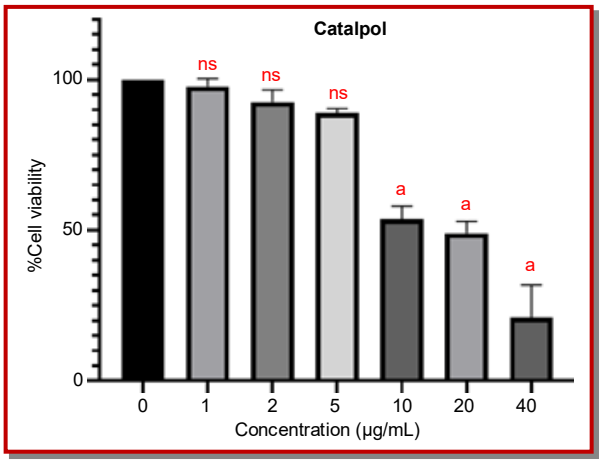

Figure 1: The viability of BV2 cells after treatment with varying concentrations of catalpol was evaluated using the CCK-8 assay. Compared with the control group (catalpol concentration 0 µg/mL). [a]p<0.01

Effect on lipopolysaccharide-induced production of inflammatory factors in BV2 microglia

The levels of inflammatory cytokines in BV2 cells were detected by ELISA. Compared with the normal control group, the contents of IL-1β, IL-6, and TNF-α in the cell supernatant of the lipopolysaccharide group were significantly increased (p<0.01). Compared with the lipopolysaccharide group, administration of catalpol

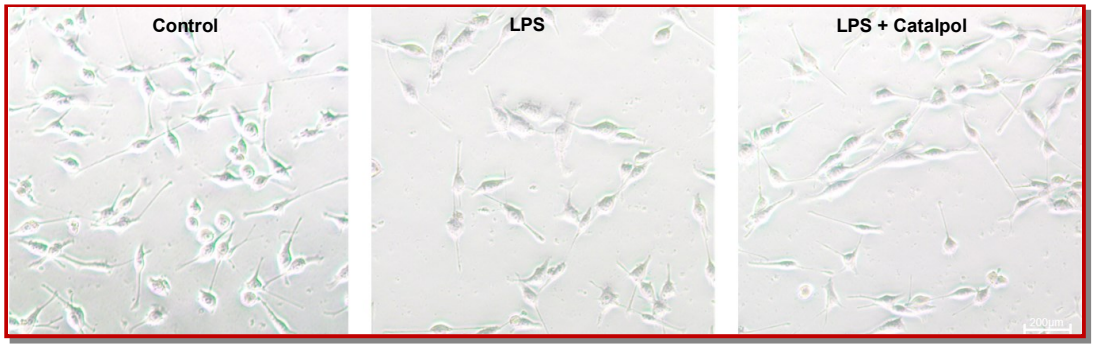

Figure 2: Effects of LPS (1 µg/mL) and catalpol (5 µg/mL) on the morphology of BV2 cells

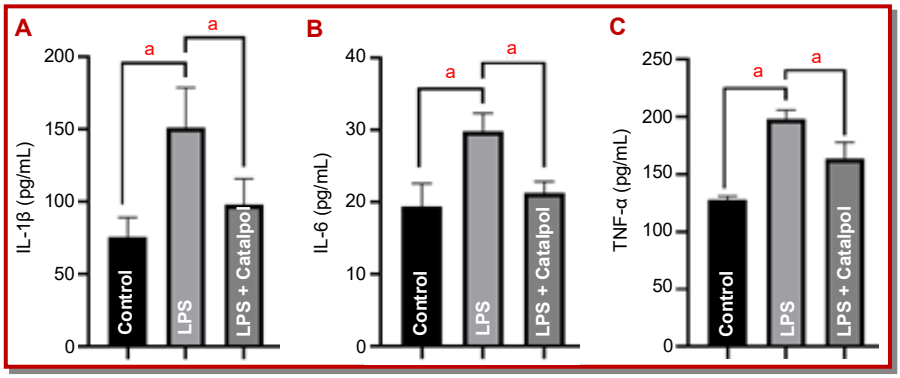

Figure 3: Effects of catalpol on lipopolysaccharide-induced production of inflammatory cytokines in BV2 cell. Levels of the inflammatory cytokines IL-1β (A), IL-6 (B), and TNF-α (C) were measured in the supernatants of BV2 cells using ELISA. [a]p<0.05

decreased the contents of IL-1β (p<0.01), IL-6(p<0.01) and TNF-α (p<0.05) produced by lipopolysaccharide-induced BV2. It has been proved that catalpol can inhibit the inflammatory factors produced by BV2 cells induced by lipopolysaccharide (Figure 3).

Effect on ROS level of BV2 cells induced by lipopolysaccharide

Flow analysis was used to determine the effect of catalpol on ROS induced by lipopolysaccharide in BV2 cells. Compared with the control group, the ROS content of BV2 cells induced by lipopolysaccharide was significantly increased (p<0.01), while catalpol could reduce the ROS content of BV2 cells (p<0.01). It was demonstrated that catalpol can reduce ROS produced by lipopolysaccharide-induced BV2 cells (Figure 4).

Effect on iNOS produced by lipopolysaccharide-induced BV2 microglia

Induced nitric oxide synthase (iNOS) content in BV2 cells was detected by immunofluorescence. Compared with the control group, the iNOS content of lipopolysaccharide-induced BV2 cells was significantly increased (p<0.01), and catalpol significantly decreased the iNOS level of LPS-induced BV2 cells (p<0.01). It was proved that catalpol could reduce iNOS produced by lipopolysaccharide-induced BV2 cells and had anti-inflammatory effect (Figure 5).

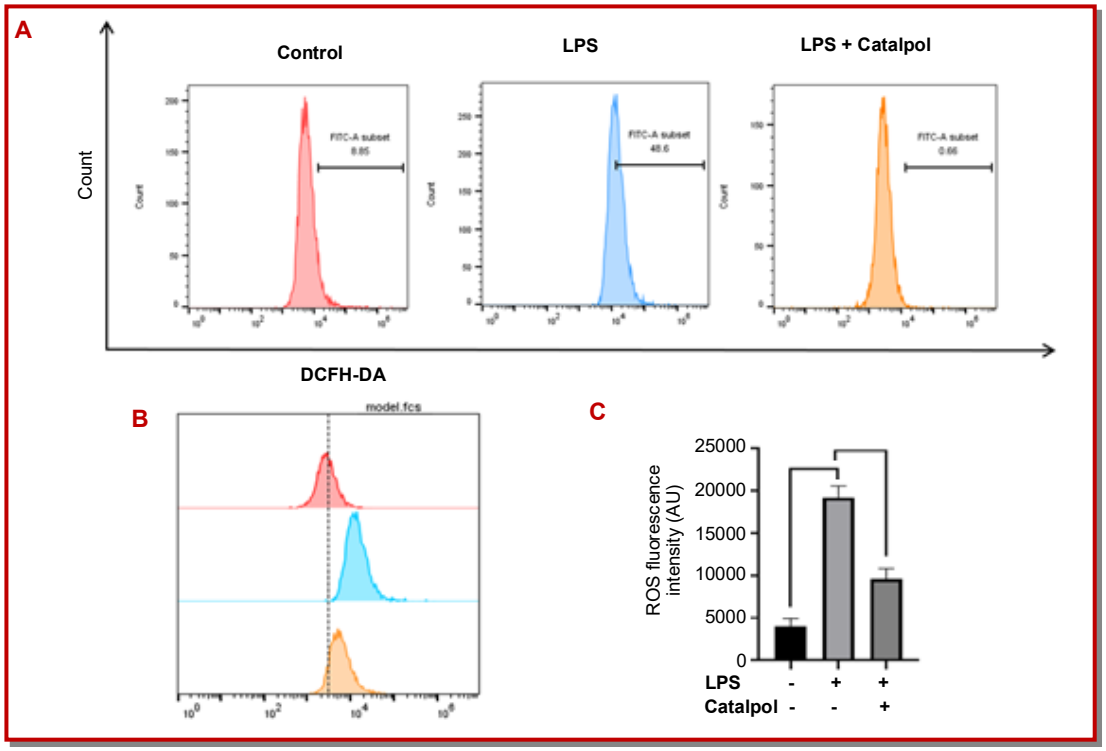

Figure 4: Effect of catalpol (5 μg/mL) on lipopolysaccharide (1 μg/mL)-induced ROS levels in BV2 microglia. Comparison of ROS levels produced by BV2 in different groups by flow cytometry (A, B). Statistical results of ROS levels in different groups of BV2 cells (C). [a]p<0.05

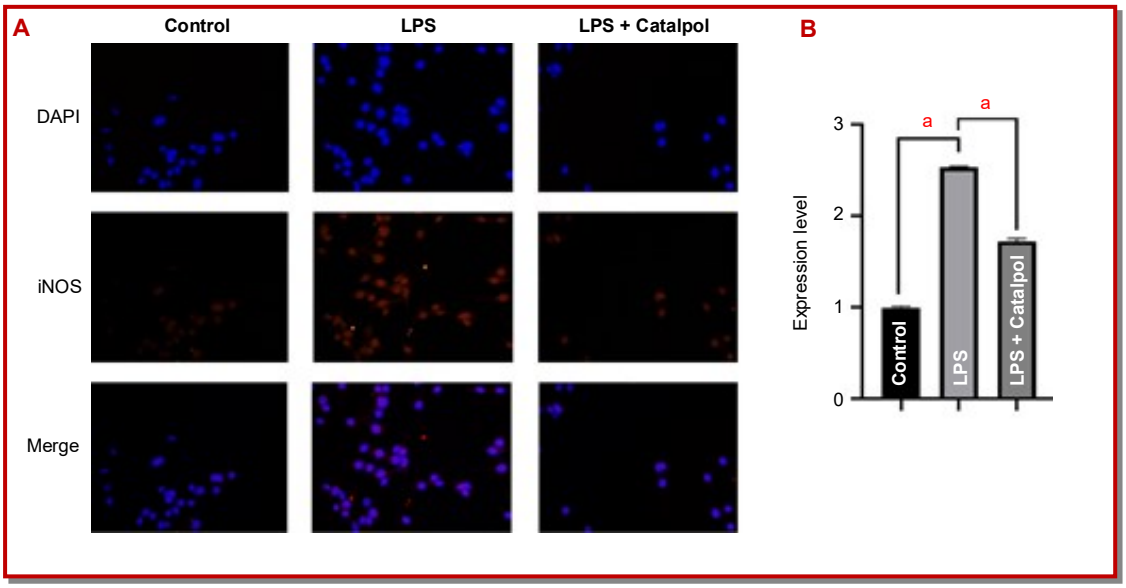

Figure 5: Effect of catalpol (5 μg/mL) on the iNOS level of BV2 cells induced by lipopolysaccharide (1 μg/mL). iNOS produced by different groups of BV2 cells were detected by immunofluorescence (A). Statistical results of iNOS fluorescence in different groups of BV2 cells (B). $^{a}p<0.05$

Discussion

The purpose of this work was to look into how catalpol protects BV2 cells against lipopolysaccharide-induced inflammatory responses. A series of experiments demonstrated the effects of catalpol on the expression of inflammatory cytokines, ROS level, iNOS expression and morphological changes in lipopolysaccharide-induced BV2 cells. The experimental results showed that 5 μg/mL catalpol had minimal impact on BV2 cell proliferation, while 10 μg/mL catalpol significantly inhibited BV2 cells viability. Therefore, catalpol at a concentration of 5 μg/mL was selected for subsequent experiments. The results showed that catalpol could reduce the expression of inflammatory factors IL-1β, IL-6, and TNF-α-induced by lipopolysaccharide-induced BV2 cells, reduce the production of ROS and iNOS, and improve the morphological changes of BV2 cells. These findings provide evidence for catalpol's potential as a therapeutic agent for neuro-inflammation.

Previous studies have shown that catalpol exhibits significant neuroprotective effects in experimental models of Alzheimer's disease, Parkinson's disease (Zhang et al., 2023), and ischemic stroke (Liu et al., 2014), primarily due to its anti-inflammatory properties. Catalpol has been shown to reduce the production of inflammatory cytokines by inhibiting the NF-κB signaling pathway (Zhang et al., 2019). In addition, catalpol attenuates ROS generation by activating Nrf2-ARE signaling pathway and enhancing antioxidant defense (You et al., 2016). The findings of this study are generally consistent with the existing literature. Our study further validated the association between catalpol and its anti-inflammatory effects on microglia.

Microglia exhibit a variety of reactive phenotypes, and M1 microglia undergo morphological changes and are responsible for releasing a variety of pro-inflammatory cytokines (Savage et al., 2019). Specifically, abnormally activated M1-type microglia produce multiple inflammatory mediators (iNOS), inflammatory cytokines (IL-1β, IL-6, and TNF-α) (Nam et al., 2018), and reactive oxygen species (ROS) (Xia et al., 2021). Meanwhile, iNOS serves as a proinflammatory marker for M1 microglia (Guo et al., 2022). In the present study, catalpol effectively inhibited lipopolysaccharide-induced iNOS expression and reduced levels of inflammatory cytokines in microglia. This suggests that catalpol has the potential to inhibit the polarization of microglia towards an M1 phenotype, thereby reducing the secretion of inflammatory factors and ROS production by microglia, ultimately mitigating neuroinflammation (Simpson et al., 2020). The experimental results suggest that catalpol can improve the morphology of microglia, which further confirms this speculation. In addition, catalpol reduces NO by inhibiting the expression of iNOS, and reduces the level of ROS to relieve oxidative stress. The interplay between these mechanisms highlights the potential utility of catalpol in a wide range of neuroprotective applications.

Catalpol has been shown several earlier investigations to be reasonably safe to use in rodent models, with no discernible toxic side effects throughout the therapeutic dose range (Jiang et al., 2008). Additionally, a clinical investigation that evaluated catalpol on 345 patients with colon cancer showed that the treatment is safe, well-tolerated, and non-toxic (Fei et al., 2018). These results indicate that catalpol can be safely used in clinical practice. However, in this study, catalpol at a

high concentration (10 µg/mL) was found to inhibit the viability of BV2 cells. The discovery that catalpol may have two different effects-protective at low levels and possibly detrimental at large doses makes this finding valuable. This is crucial for developing safe and effective dosing strategies for therapeutic use. Several factors might explain these inconsistencies: First, differences in experimental conditions between in vitro and *in vivo* studies might lead to different outcomes. The *in vitro* environment is much simpler than the complex physiological environment *in vivo*, where catalpol might interact with other physiological factors. Second, the effects of different doses of catalpol may vary across models, suggesting a dose-dependent mechanism that warrants further investigation. Finally, as an immortalized microglial cell line, BV2 cells may respond differently to catalpol compared to primary microglial cells or *in vivo* cells, leading to discrepancies in the results (Henn et al., 2009).

The results of this study strongly support existing theoretical models related to neuroinflammation. This study found that catalpol exerts its antioxidant and anti-inflammatory effects by reducing the expression of inflammatory factors, ROS, and iNOS, which is consistent with the established role of microglia in neuroinflammation (Wu et al., 2022). Furthermore, results suggest that catalpol may inhibit microglial polarization to M1, thereby, alleviating neuroinflammation, and providing new insights into therapeutic strategies. Based on these findings, we propose an updated theoretical model in which catalpol exerts its neuroprotective effects by modulating the activation states of microglial cells.

Despite the meaningful findings of this study, there are some limitations to consider. Initially, the research was carried out exclusively *in vitro*, potentially restricting the applicability of the findings as it does not accurately capture the intricate physiological milieu *in vivo*. The absorbability of catalpol *in vivo*, its metabolites, and its effects are still unclear. Furthermore, the primary emphasis of this investigation was acute inflammatory reactions, the assessment of catalpol's role in chronic neuroinflammation was not conducted, which may have implications for our comprehension of the drug's long-term effects.

Conclusion

This study shows that catalpol reduces lipopolysaccharide-induced inflammation in BV2 microglial cells by inhibiting the production of inflammatory cytokines, ROS, and iNOS, underscoring its potential as a treatment for neuroinflammation.

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Ethical Issue

This study does not require and ethical approval.

Conflict of Interest

Authors declare no conflict of interest

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Author Info
Yi Luo, Biao Zhang (Principal contact)
e-mail: yiluomdyy@126.com; zhangbmed@126.com
First three authors contributed equally