

RESEARCH ARTICLE

Rauwolfia polysaccharide can inhibit the progress of ulcerative colitis through NOS2-mediated JAK2/STAT3 pathway

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Citation: Wu H, Jiang F, Yuan W, Zhao Y, Liu N, Miao X (2024) Rauwolfia polysaccharide can inhibit the progress of ulcerative colitis through NOS2-mediated JAK2/STAT3 pathway. PLoS ONE 19(4): e0301660. <https://doi.org/10.1371/journal.pone.0301660>

Editor: Qi Chen, University of Kansas Medical Center, UNITED STATES

Received: October 31, 2023

Accepted: March 20, 2024

Published: April 16, 2024

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Data Availability Statement: All relevant data are within the manuscript and its [Supporting Information](#) files.

Funding: The study was supported by National Natural Science Foundation of China (82160104), Key Research and Development Project of Hainan Province (ZDYF2022SHFZ099), Hainan Provincial Natural Science Foundation of China (822QN448 and 823RC564) and Innovative research project of graduate students in Hainan Province (Qhys2023-471).

Abstract

Background

Ulcerative colitis (UC) is an inflammatory disease of the digestive tract. Rauwolfia polysaccharide (Rau) has therapeutic effects on colitis in mice, but its mechanism of action needs to be further clarified. In the study, we explored the effect of Rau on the UC cell model induced by Lipopolysaccharide (LPS).

Methods

We constructed a UC cell model by stimulating HT-29 cells with LPS. Dextran sodium sulfate (DSS) was used to induce mice to construct an animal model of UC. Subsequently, we performed Rau administration on the UC cell model. Then, the therapeutic effect of Rau on UC cell model and was validated through methods such as Cell Counting Kit-8 (CCK8), Muse, Quantitative real-time polymerase chain reaction (RT-qPCR), Western blotting, and Enzyme-linked immunosorbent assay (ELISA).

Results

The results showed that Rau can promote the proliferation and inhibit the apoptosis of the HT-29 cells-induced by LPS. Moreover, we observed that Rau can inhibit the expression of NOS2/JAK2/STAT3 in LPS-induced HT-29 cells. To further explore the role of NOS2 in UC progression, we used siRNA technology to knock down NOS2 and search for its mechanism in UC. The results illustrated that NOS2 knockdown can promote proliferation and inhibit the apoptosis of LPS-induced HT-29 cells by JAK2/STAT3 pathway. In addition, in vitro and in vivo experiments, we observed that the activation of the JAK2/STAT3 pathway can inhibit the effect of Rau on DSS-induced UC model.

Competing interests: The authors have declared that no competing interests exist.

Conclusion

In short, Rauwolfia polysaccharide can inhibit the progress of ulcerative colitis through NOS2-mediated JAK2/STAT3 pathway. This study provides a theoretical clue for the treatment of UC by Rau.

1. Introduction

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD) that begins in the rectum and extends in a continuous fashion, eventually involving part or the entire colon [1]. The lesions of the disease mainly invade the rectal mucosa and submucosa, forming erosions and ulcers. UC has two peak incidence patterns. The first peak occurs between the ages of 20 and 30, and the second peak occurs between the ages of 50 and 70 [2, 3]. In addition, the incidence of UC is reported to be higher in developed countries than in developing countries. In North America and Northern Europe, the incidence rate and prevalence rate of UC are the highest, with an incidence of 9–20 cases per 100,000 person-years and a prevalence of 156–291 cases per 100,000 people [4, 5]. The incidence of UC in Asian countries, including China, is also increasing year by year. In Hong Kong, the incidence of IBD tripled from 1.0 to 3.1 per 100,000 people [6]. Among them, Crohn's disease, another inflammatory form of IBD, is more common than UC. In recent years, in the research progress of UC therapeutic drugs, traditional drugs such as 5-aminosalicylic acid, glucocorticoids and immunosuppressants have formed standardized treatment in the treatment plan [7–9]. Although there is a certain effect, the treatment effect is not ideal from the perspective of the tolerance of UC patients to the drug and the physiological health status after surgery [10].

Traditional Chinese medicine (TCM) is a conservative treatment for UC and is effective in relieving the symptoms of UC [11], primarily by regulating inflammatory cytokines and the immune system to protect the intestinal mucosa. Hainan Rauwolfia is a unique traditional Chinese medicine in Hainan and has a history of about 2000 years and is used to treat various human diseases [12]. It has the functions of clearing heat and detoxifying, anti-inflammatory and analgesic [13]. Moreover, Rauwolfia extract has been found to have an apparent inhibitory effect on prostate cancer [14], pancreatic cancer [15], ovarian cancer [16] and so on. The extract of Rauwolfia also has a therapeutic effect on colitis mice induced by DSS [17–19], but the mechanism needs to be further clarified.

In this study, we constructed a UC cell model by stimulating HT-29 cells with LPS and an animal model induced by DSS, and treated the model with Rau to explore the molecular mechanism of Rau in treating the UC model. This study provides a theoretical clue for the treatment of UC by Rau.

2. Methods

2.1. Chemicals and reagents

Rau (Customization, 5g) was obtained from Nanjing Daosifu Biotechnology Co., Ltd. LPS (L9143, 10mg) and DSS (60316ES25, 25g) was purchased from Sigma-Aldrich and Yeasen, respectively. Antibodies used in this study were as follows: iNOS (proteintech, 22226-1-AP), JAK2 (Affinity, AF6022), p-JAK2 (Abcam, ab195055), STAT3 (Zen-Bio, R22785), p-STAT3 (Cell signaling, 9145S), GAPDH (proteintech, 60004-1-Ig) and IgG (Zen-Bio, 511103). The IgG used for HE staining was also purchased from Zen-Bio (511103).

2.2 Cell culture

Human Colon Cancer Cell line HT-29 was obtained from iCell Bioscience Inc, Shanghai (iCell-h078). The cell line was cultured in McCoy's 5A medium mixed with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were cultured in an incubator at 37°C, 5% CO₂ and 95% air.

2.3 Establishment of rat model of ulcerative colitis

We purchased BALB/C mice from the First Affiliated College of Xi'an Jiaotong University. The mice were adaptively reared at 22–24°C and 20% humidity for one week and randomly divided into 4 groups (control group, DSS group, DSS+Rau group, DSS+Rau+colivelin group) with 9 mice in each group. The UC group mice were given 3% (w/v) DSS solution for free consumption for seven days, while the control group mice were fed with purified water for seven days. Rau(200mg/kg, 500μL) by gavage and colivelin(1mg/kg, 100μL) by intraperitoneal injection were administered daily for 7 days from the 8th day of modeling. The control and UC groups were given the same amount of distilled water. At 0d, 4d, 8d, 12d and 15d, the hair color, stool characteristics, stool occult blood and body weight of mice were observed and recorded. At the end of administration, the colon of the mice was dissected immediately after death, and the length of the colon was measured after laying it flat.

2.4 Cell proliferation

We completed cell proliferation by Cell Counting Kit-8 (MCE, HY-K0301). Firstly, HT-29 cells were inoculated into 96-well plates at 2×10^3 cells/well and dealt with LPS at different concentration gradients (0μg/mL, 40μg/mL, 60μg/mL, 100μg/mL, 200μg/mL) to construct UC cell model. Different concentration gradients of Rau (50μg/mL, 100μg/mL, 200μg/mL, 400μg/mL) were used to treat the UC cell model. At 450nm wavelength, we detected the absorbance value of the above groups.

2.5 Cell apoptosis

Cell apoptosis was performed by cell apoptosis kit (Muse, MCH100105) based on the flow cytometer instrument. After cells were collected, we added Muse Annexin V & Dead Cell to the above groups. Then it was incubated at room temperature and shielded from light for 20min and tested on the machine. Finally, the cell apoptosis of each group was determined by calculating the sum of early and late apoptosis in each group.

2.6 RT-qPCR

Total RNA was extracted with Trizol from the collected cells. Nanodrop 2000 was used to detect RNA concentration and purity. RNA integrity was detected by 0.8% denaturing gel electrophoresis. Then, reverse transcribe 500ng RNA into cDNA with a TAKARA reverse transcription kit. The primers for the amplification of NOS2/JAK2/STAT3 cDNA were listed in [Table 1](#). The RT-qPCR system included the primers, cDNA and SYBR High-Sensitivity qPCR SuperMix. Based on the fluorescence quantitative instrument (ABI 7500), we completed the PCR process and the PCR conditions were as follows: 1) 1min at 95°C; 2) 45 cycles of 20s at 95°C, 20s at 60°C and 30s at 72°C; 3) 4°C ∞.

2.7 Western blotting assay

After cells were collected, we extracted total protein by cell lysate containing protease inhibitors. Soon afterwards, we transferred them to sodium dodecyl sulfate-polyacrylamide gel

Table 1. Primer sequence of NOS2/JAK2/STAT3.

| Name | Forward primer (5'-3') | Reverse primer (5'-3') |
|-------|-------------------------|-------------------------|
| NOS2 | GCTCTACACCTCCAATGTGACC | CTGCCGAGATTTGAGCCTCATG |
| JAK2 | CCAGATGGAAACTGTTCGCTCAG | GAGGTTGGTACATCAGAAACACC |
| STAT3 | CTTTGAGACCGAGGTGTATCACC | GGTCAGCATGTTGTACCACAGG |
| GAPDH | GGAGCGAGATCCCTCCAAAAT | GGCTGTTGTCATACTTCTCATGG |

<https://doi.org/10.1371/journal.pone.0301660.t001>

(SDS-PAGE) for separation and then transferred them to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked with the blocking solution containing 5% non-fat milk and were incubated with primary antibodies at 4°C overnight. After cleaning the PVDF membrane with TBST, the membrane was incubated with the secondary antibody for 1h at room temperature. Finally, electrochemiluminescence (ECL) solutions were prepared in a dark chamber to visualize the target protein bands. The exposure time was ascertained in light of the fluorescence intensity of the target protein and the light intensity of the target protein was then analyzed using ImageJ.

2.8 ELISA

After 24h of incubation, the expression of IL-6 (JL20268), IL-1 β (JL18442) and TNF- α (JL10484) in serum of colitis mice was detected by the enzyme-linked immunosorbent assay (ELISA) kit provided by Jianglai biology. Continuously measure the optical density (OD) of each well at 450nm using a multifunctional microplate reader (BioTek, Elx-800).

2.9 Disease activity index (DAI)

At 0d, 4d, 8d, 12d and 15d, the hair color, stool characteristics, fecal occult blood and body weight of the mice were observed and recorded. The DAI score was calculated according to the described method [20] to evaluate the severity of the disease.

2.10 HE staining

After the mice were killed at the end of the experiment, the whole colon was separated and rinsed with cold PBS, and then a part of the colon was fixed with 10% formalin and embedded in paraffin to make tissue sections. Subsequently, tissue sections were stained with hematoxylin and eosin according to the standard of practice [21] to assess the severity of colitis.

2.11 Statistical analysis

Data was expressed as mean \pm standard deviation (SD). SPSS software 20.0 and GraphPad Prism 8.3.1 software were used to complete data collation and analysis. One-way analysis of variance was used for multiple group comparisons. $p < 0.05$ indicates a statistically significant difference.

3. Results

3.1 Ulcerative colitis cell model was constructed by LPS-induced HT-29 cells

We constructed a cell model of UC by stimulating HT-29 cells with a series of concentrations of LPS (0 μ g/mL, 40 μ g/mL, 60 μ g/mL, 100 μ g/mL, 200 μ g/mL). Firstly, the cell viability of each group was measured with the CCK8 kit. In Fig 1A, the cell viability of HT-29 cells decreased after treatment with various concentrations of LPS (all $p < 0.001$). LPS 60 μ g/mL was

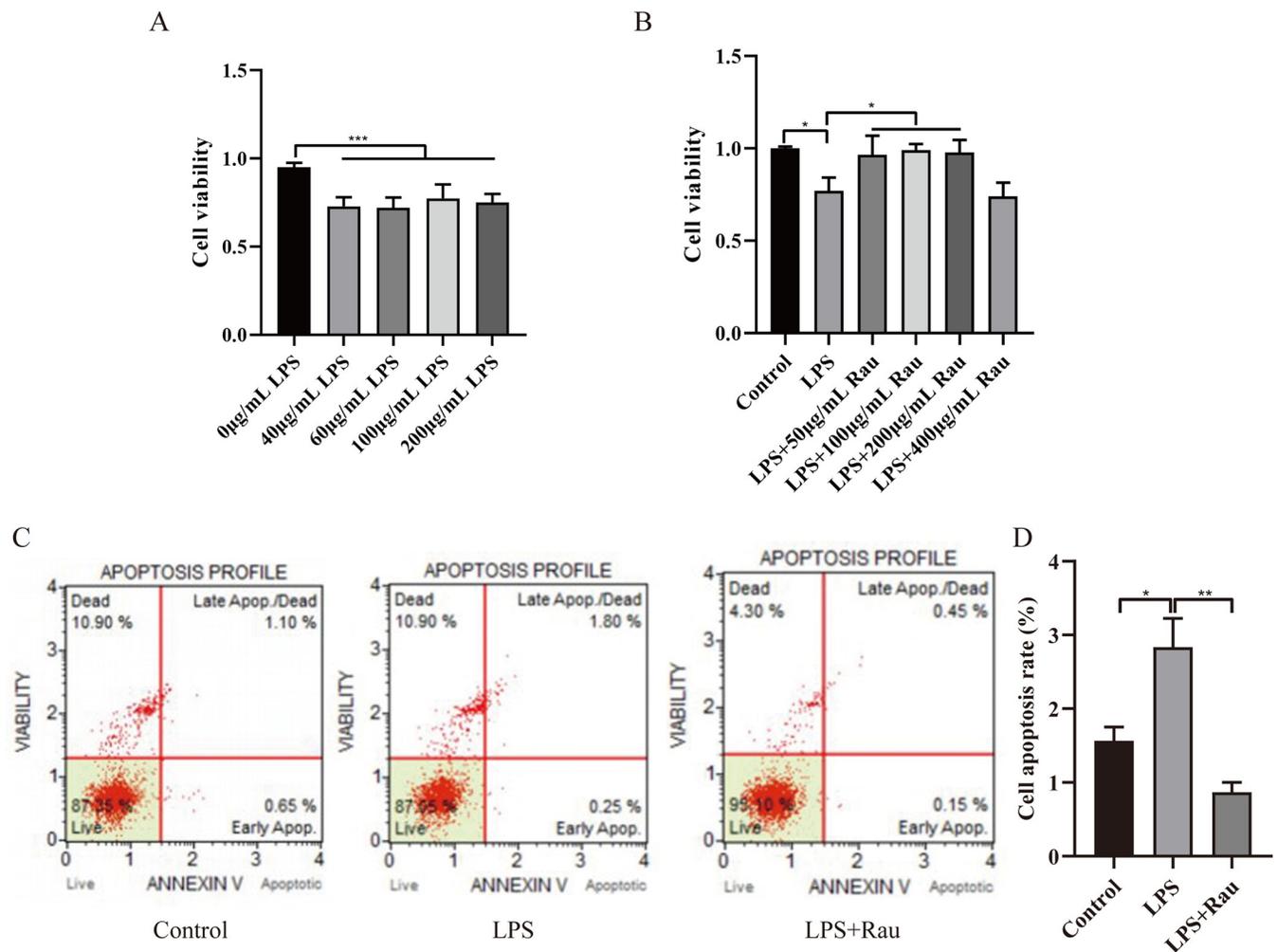


Fig 1. Rau can promote the proliferation and inhibit the apoptosis of the UC cell model induced by LPS. A: The cell viability of HT-29 cells treated with a series of concentrations of LPS (0µg/mL, 40µg/mL, 60µg/mL, 100µg/mL, 200µg/mL) was detected by CCK8. B: The cell viability of the UC cell model induced by LPS and treated with different concentrations of Rau (50µg/mL, 100µg/mL, 200µg/mL, 400µg/mL) was detected by CCK8. C: We observed cell apoptosis in the control group, the LPS group and the LPS+Rau group using Muse. D: The proportion of cell apoptosis in the control group, the LPS group and the LPS+Rau group was analyzed. The sum of the percentage of early apoptosis and the percentage of late apoptosis was the apoptosis ratio. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

<https://doi.org/10.1371/journal.pone.0301660.g001>

considered to be the optimal concentration, so this concentration was selected for the next experiment.

3.2 Rau can promote the proliferation and inhibit the apoptosis of LPS-induced HT-29 cells by NOS2/JAK2/STAT3 pathway

Studies have shown that the polysaccharides from Rau have anti-inflammatory effects, so we chose Rau to treat the UC cell model for molecular mechanism research. In Fig 1B, the toxicity of different concentrations of Rau (50µg/mL, 100µg/mL, 200µg/mL, 400µg/mL) on the model was detected by CCK8 kit. Compared with the group of LPS, low concentration of Rau (50µg/mL, 100µg/mL, 200µg/mL) can restore cell activity (all $p < 0.05$), while a high concentration of Rau (400µg/mL) can reduce cell viability. When the concentration of Rau was 100µg/mL, the cell viability of this group was comparable to that of the control group. Therefore, we chose Rau 100µg/mL to explore the following experiments.

In Fig 1C, we observed cell apoptosis of the control group, the LPS group and the LPS+Rau group, and the apoptosis ratio in each group was 1.75%, 2.05% and 0.60%, respectively. Soon afterwards, we conducted a statistical analysis of apoptosis in each group (Fig 1D). Compared with the control group, apoptosis was significantly increased in the LPS group ($p = 0.033$), and the addition of Rau can significantly reduce cell apoptosis ($p = 0.004$). The evidence indicated that Rau can promote the proliferation and inhibit the apoptosis of the UC cell model induced by LPS.

Moreover, we found that the mRNA expression of *NOS2*, *JAK2* and *STAT3* was notably increased by LPS stimulation ($p = 0.003$, $p = 0.030$, $p < 0.001$), while *NOS2*, *JAK2* and *STAT3* mRNA expression was significantly decreased after Rau was added ($p = 0.003$, $p = 0.030$, $p = 0.003$, Fig 2A–2C). After LPS stimulation, the expression of *NOS2*, *JAK2*, p-*JAK2*, *STAT3* and p-*STAT3* was also significantly increased ($p < 0.001$, $p = 0.001$, $p < 0.001$, $p = 0.003$, $p < 0.001$). However, the protein expression of *NOS2*, *JAK2*, p-*JAK2*, *STAT3* and p-*STAT3* was reversed by the addition of Rau ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p = 0.002$, $p < 0.001$), as shown in Fig 2D–2I. The results displayed that Rau can inhibit the expression of *NOS2*/*JAK2*/*STAT3* in the UC cell model induced by LPS. What's more, the expression of IL-6, IL-1 β and TNF- α was also checked in the control group, the LPS group and the LPS+Rau group (Fig 2J–2L). The expression of IL-6, IL-1 β and TNF- α in the LPS group was significantly higher than those in the control group (all $p < 0.001$). Nevertheless, the addition of Rau can inhibit the expression of IL-6, IL-1 β and TNF- α induced by LPS ($p < 0.001$, $p < 0.001$, $p = 0.005$). The above results displayed that Rau can promote the proliferation and inhibit the apoptosis of LPS-induced HT-29 cells by *NOS2*/*JAK2*/*STAT3* pathway.

3.3 *NOS2* knockdown can promote proliferation and inhibit the apoptosis of LPS-induced HT-29 cells by *JAK2*/*STAT3* pathway

To further explore the role of *NOS2* in LPS-induced ulcerative colitis, we designed and synthesized siNOS2-1, siNOS2-2 and siNOS2-3. In Fig 3A–3C, we tested the expression of *NOS2* mRNA and *NOS2* protein by RT-qPCR and western blot, respectively. The siRNA with the best knockdown effect was siNOS2-2 and siNOS2-3 (all $p < 0.001$). Next, we examined the effect of siNOS2-2 and siNOS2-3 on the proliferation and apoptosis of LPS-induced UC cell model (Fig 4A–4C). In Fig 4A, after the transfection of siNOS2, the proliferation ability of the LPS-induced UC cell model increased (all $p < 0.001$). Conversely, by contrast with the LPS group (apoptosis ratio: 2.93%), the apoptosis of cells transfected with siNOS2-2 and siNOS2-3 decreased (apoptosis ratio: 1.25% and 1.35%, respectively). The results showed that *NOS2* knockdown can promote proliferation and inhibit the apoptosis of the UC cell model induced by LPS (Fig 4B and 4C).

Besides, we noticed that siNOS2-2 and siNOS2-3 reversed the expression of IL-6 ($p = 0.011$, $p = 0.027$), IL-1 β ($p < 0.001$, $p = 0.001$) and TNF- α (all $p < 0.001$) in UC cell model induced by LPS (Fig 5A–5C). Meanwhile, we observed that siNOS2-2 and siNOS2-3 can inhibit the mRNA expression of *JAK2* ($p = 0.036$, $p = 0.032$) and *STAT3* ($p = 0.006$, $p = 0.004$) when compared with the LPS group (Fig 5D–5F). In comparison to the LPS group, siNOS2-2 and siNOS2-3 can significantly restrain the expression of *JAK2* ($p = 0.000$, $p = 0.022$), *STAT3* ($p < 0.001$, $p < 0.001$), p-*JAK2* ($p < 0.001$, $p < 0.001$), p-*STAT3* ($p = 0.001$, $p < 0.001$), as shown in Fig 5G–5L. From the above data, we speculated that Rau may promote proliferation and inhibit apoptosis of UC cell model through *NOS2*-mediated *JAK2*/*STAT3* pathway.

3.4 Activation of *JAK2*/*STAT3* pathway can inhibit the effect of Rau on LPS-induced HT-29 cells

To determine whether the effect of Rau on LPS-induced HT-29 cells was related to the *JAK2*/*STAT3* pathway, we introduced the *JAK2*/*STAT3* pathway activator-colivelin. First of all, we

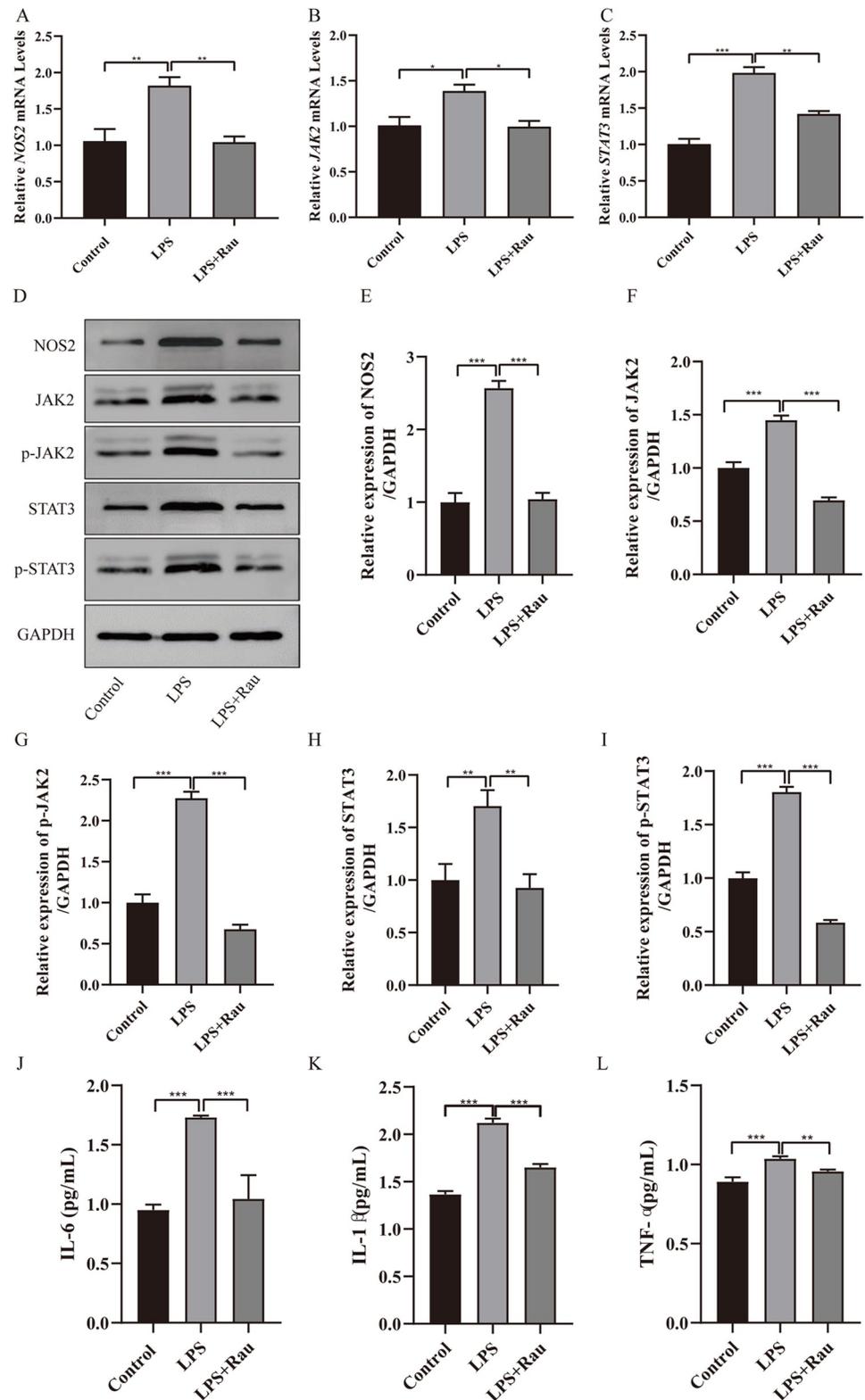


Fig 2. Rau can inhibit the expression of NOS2/JAK2/STAT3 and IL-6/IL-1 β /TNF- α in UC cell model induced by LPS. A-C: The mRNA expression of JAK2 and STAT3 in the control group, the LPS group and the LPS+Rau group were detected by RT-qPCR. D: The protein expression of NOS2, JAK2, STAT3, p-JAK2 and p-STAT3 in the control group, the LPS group and the LPS+Rau group were detected by Western blotting assay. E-I: Perform grayscale analysis

using Image J software. J-L: The protein expression of IL-6, IL-1 β and TNF- α in the control group, the LPS group and the LPS+Rau group were detected by ELISA. Three independent samples were used for statistics. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

<https://doi.org/10.1371/journal.pone.0301660.g002>

examined the effect of different concentrations (0 μ M, 0.5 μ M, 1 μ M, 2 μ M, 4 μ M) of colivelin on HT-29 cell viability, and the results showed that different concentrations of colivelin did not affect cell viability (Fig 6A). Also, as the concentration of colivelin increased, STAT3 and p-STAT3 expression increased significantly (Fig 6B–6D). According to the related articles [22, 23], we finally selected 1 μ M colivelin for follow-up experiments.

We tested the role of colivelin in LPS-induced HT-29 cells treated with Rau (Fig 6E–6G). In Fig 6E, the cell viability of the group of LPS+Rau+colivelin was significantly reduced in contrast to the group of LPS+Rau ($p < 0.001$). However, the apoptosis ratio in group of LPS+Rau and LPS+Rau+colivelin was 1.97% and 5.45%, respectively, illustrating that the cell apoptosis of LPS+Rau+colivelin group was significantly increased in contrast to LPS+Rau group ($p < 0.001$). These results suggested that colivelin can play a negative role in LPS-induced HT-29 cells treated with Rau.

Meanwhile, we estimated JAK2 and STAT3 expression level in the control, LPS, LPS+Rau and LPS+Rau+colivelin group (Fig 6H–6N). In Fig 6H, 6I, we observed that LPS+Rau stimulation can reduce the mRNA expression levels of JAK2 and STAT3, while the addition of colivelin can increase the levels ($p = 0.038$, $p < 0.001$). In Fig 6J–6N, in the LPS+Rau and LPS+Rau+colivelin group, we observed the same upward trend. Compared to the LPS+Rau group, JAK2 and STAT3 protein expression level were rising in the LPS+Rau+colivelin group ($p = 0.036$, $p < 0.001$). The phosphorylation level of JAK2 and STAT3 protein were also increased ($p = 0.002$, $p < 0.001$). These data confirm that JAK2/STAT3 pathway was involved in LPS-induced HT-29 cells treated with Rau. And activation of JAK2/STAT3 pathway can have a negative effect on LPS-induced HT-29 cells treated with Rau. In Fig 6O–6Q, the expression of IL-6, IL-1 β and TNF- α was significantly increased (the LPS+Rau group vs. the LPS+Rau+colivelin group, $p < 0.001$, $p = 0.033$, $p = 0.026$). This result confirmed the negative effect of JAK2/STAT3 pathway activation in LPS-induced HT-29 cells treated with Rau.

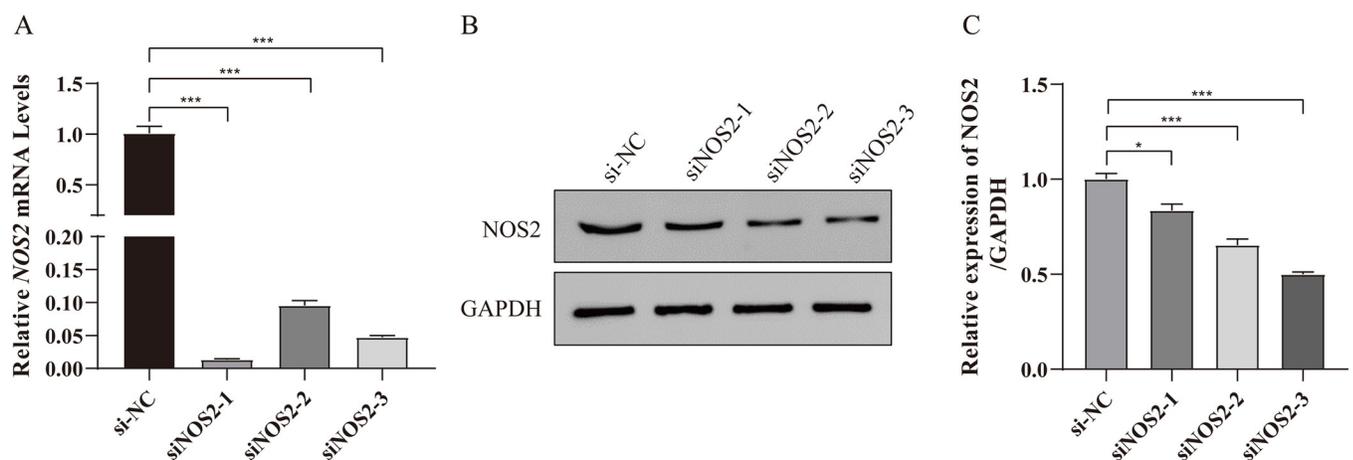


Fig 3. Screening NOS2 siRNA. A-B: After siNOS2-1, siNOS2-2 and siNOS2-3 transfected into the LPS-induced UC cell model, we tested the expression of NOS2 mRNA expression and NOS2 protein expression by RT-qPCR and Western blotting assay, respectively. C: Perform grayscale analysis using Image J software. Three independent samples were used for statistics. * $p < 0.05$, *** $p < 0.001$.

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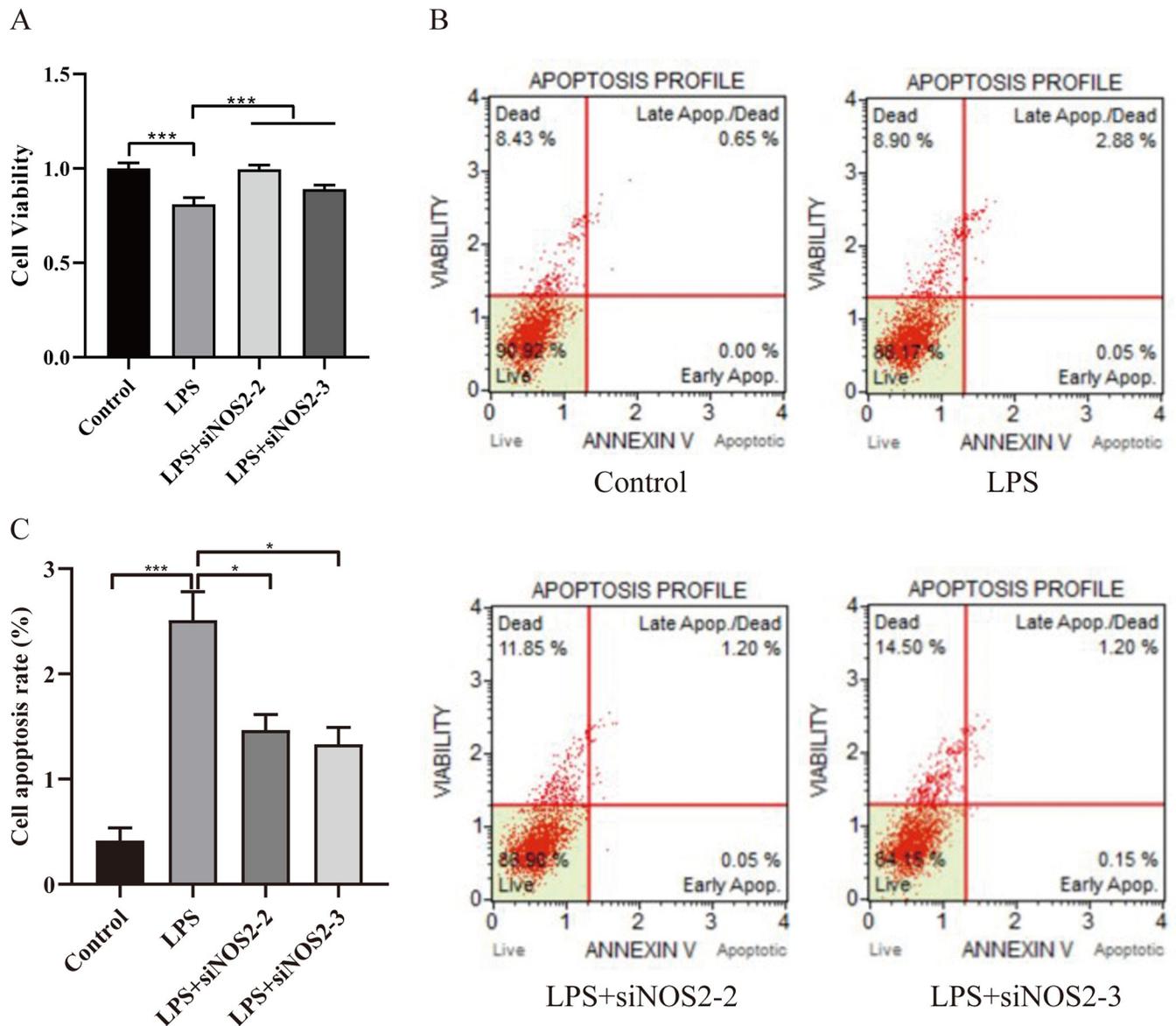


Fig 4. NOS2 knockdown can promote proliferation and inhibit the apoptosis of the UC cell model induced by LPS. A: We examined the effect of siNOS2-2 and siNOS2-3 on cell proliferation of LPS-induced UC cell model by CCK8. B: We examined the effect of siNOS2-2 and siNOS2-3 on cell apoptosis of LPS-induced UC cell model by Muse. C: The proportion of apoptosis in the control group, the LPS group, the siNOS2-2 group and the siNOS2-3 group was analyzed. The sum of the percentage of early apoptosis and the percentage of late apoptosis was the apoptosis ratio.

<https://doi.org/10.1371/journal.pone.0301660.g004>

3.5 Activation of the JAK2/STAT3 pathway can inhibit the effect of Rau on DSS-induced UC mice model

In vivo, we used DSS to construct UC mice model to investigate the role of JAK2/STAT3 pathway activation in LPS-induced UC cell model treated with Rau. By observing the hair color, stool character, stool occult blood of DSS-induced UC mice model, and recording the change of body weight, the DAI score was calculated. In Fig 7A, DSS significantly increased the DAI score of mice ($p < 0.001$), while Rau inhibited DAI score of mice ($p = 0.015$). On the contrary, in Fig 7B and 7C, in the presence of 200 mg/kg/d Rau, the reduction in colon length induced by DSS in UC mice was greatly restored ($p < 0.001$). Unfortunately, the addition of colivelin

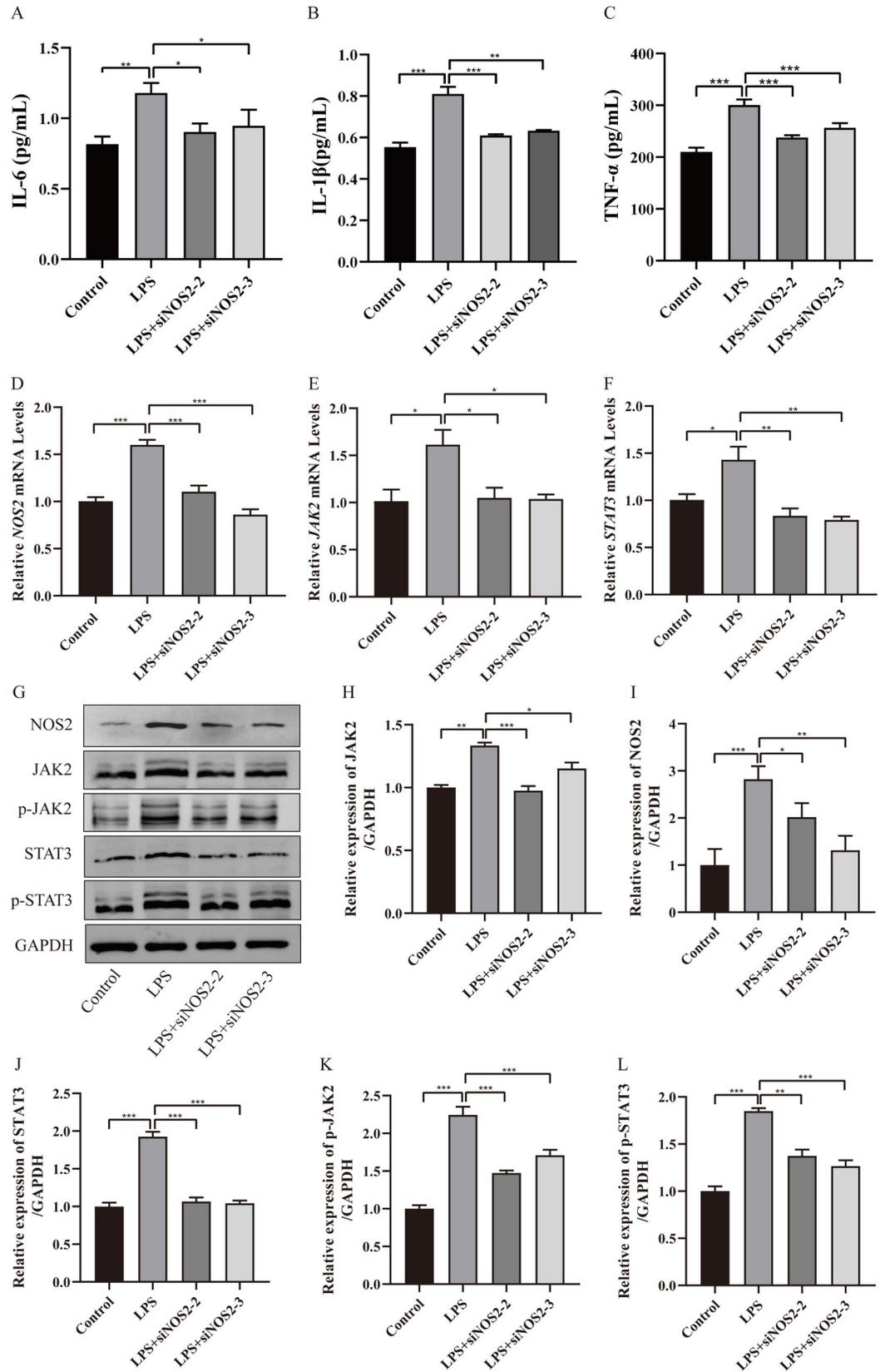


Fig 5. NOS2 knockdown can inhibit the expression of IL-6/IL-1 β /TNF- α and JAK2/STAT3 in UC cell model induced by LPS. A-C: The protein expression of IL-6/IL-1 β /TNF- α in the control group, the LPS group, the siNOS2-2 group and the siNOS2-3 group was detected by ELISA. D-F: The mRNA expression of JAK2 and STAT3 in the control group, the LPS group, the siNOS2-2 group and the siNOS2-3 group was detected by RT-qPCR. G: The protein expression of JAK2, STAT3, p-JAK2, p-STAT3 in the control group, the LPS group, the siNOS2-2 group and the siNOS2-3 group were detected by Western blotting assay. H-L: Perform grayscale analysis using Image J software. Three independent samples were used for statistics. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

<https://doi.org/10.1371/journal.pone.0301660.g005>

did not significantly change the DAI score and colon length of the UC mice ($p = 0.202$, $p = 0.744$).

Besides, we observed that Rau can significantly reduce the degree of colon mucosal tissue separation, intestinal epithelial tissue injury and inflammatory cell infiltration in DSS-induced UC mice (Fig 7D). And in Fig 7E–7G, Rau inhibited the protein expression of TNF- α /IL-1 β /IL-6 ($p < 0.001$, $p < 0.001$, $p = 0.002$) in DSS-induced UC mice. Similarly, Rau can inhibit the mRNA expression of JAK2/STAT3 ($p = 0.002$, $p = 0.001$) and the protein expression of JAK2, p-JAK2, STAT3, p-STAT3 ($p = 0.005$, $p < 0.001$, $p < 0.001$, $p < 0.001$) in DSS-induced UC mice (Fig 7H–7N). However, in the presence of colivelin, we observed that it can aggravate the degree of inflammatory damage-mild separation of colonic mucosa, injury of intestinal epithelium, infiltration of inflammatory cells in the mucosa (Fig 7D). It can also suppress the protein expression of TNF- α /IL-1 β /IL-6 in serum of colitis mice ($p < 0.001$, $p < 0.001$, $p = 0.003$), the mRNA expression of JAK2/STAT3 ($p = 0.028$, $p = 0.038$), the protein expression of JAK2/STAT3 ($p = 0.007$, $p < 0.001$) and the phosphorylation level of JAK2/STAT3 ($p < 0.001$, $p < 0.001$) in colonic tissue of DSS-induced UC mice treated by Rau (Fig 7H–7N). Generally, Rau significantly alleviated the symptom of UC mice, but the activation of the JAK2/STAT3 pathway can inhibit the effect of Rau on DSS-induced UC mice.

4. Discussion

UC is an inflammatory disease of the digestive tract, mainly located in the colon and rectum, with a chronic, lifelong, and easily recurrent course. Due to the lack of specific measures to treat UC and its serious complications, the World Health Organization has designated it as a difficult disease to treat in modern times [24]. Although researchers have been studying for many years, they have not yet discovered a clear cause of UC. In the study, we observed that NOS2 knockdown can promote proliferation and inhibit the apoptosis of LPS-induced HT-29 cells by JAK2/STAT3 pathway. In addition, based on the LPS-induced UC cell model and DSS-induced UC mice model, the activation of the JAK2/STAT3 pathway was observed to inhibit the effect of Rau on UC model. Therefore, we speculate that Rau can inhibit the progress of ulcerative colitis through NOS2-mediated JAK2/STAT3 pathway.

NOS2 encodes inducible nitric oxide synthase, which can catalyze the production of nitric oxide, thus contributing to antibacterial and anti-pathogen activity [25]. Some researchers reveal that the mRNA expression and protein expression of NOS2 in UC samples are significantly up-regulated than that in control subjects [26, 27]. These data indicated that NOS2 may take part in the development of UC. And, selective NOS2 may have therapeutic promise in the treatment of UC [28]. Wei et al found that NOS2 gene is a key gene in the treatment of UC by Gegen Qinlian Decoction through network pharmacology and molecular docking technology [29]. In addition, in an article reported by Zhang et al [30], compared with the DSS-induced colitis mouse model, NOS2 expression in the colon tissue of mice was significantly decreased after administration of protoberberine rhizocorrhizine. The results showed that NOS2 may be a key target for UC development as well as for TCM treatment of UC. In our research, we found that Rau can inhibit the expression of NOS2 in the UC cell model induced by LPS,

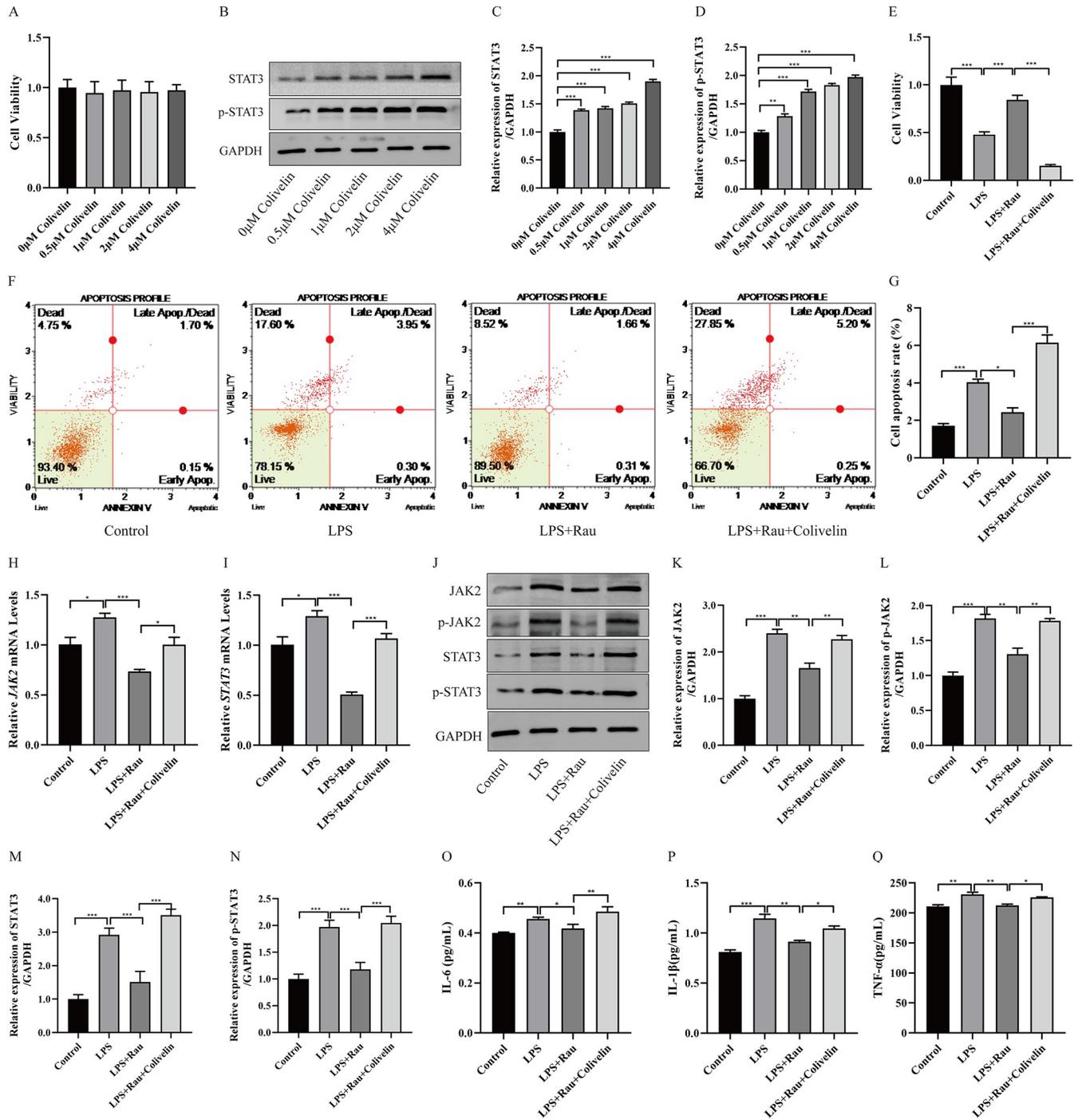


Fig 6. Activation of JAK2/STAT3 pathway can inhibit the effect of Rau on LPS-induced HT-29 cells. A: The UC cell model was treated with colvelin (0μM, 0.5μM, 1μM, 2μM, 4μM), the activator of JAK2/STAT3 pathway, and the cell activity was detected by CCK8. B: The UC cell model was treated with colvelin (0μM, 0.5μM, 1μM, 2μM, 4μM), and the protein expression level and phosphorylation level of STAT3 was detected by Western blotting assay. E: Cell proliferation in the control group, the LPS group, the LPS+Rau group and the LPS+Rau+colvelin group was detected by CCK8. F: Cell apoptosis in the control group, the LPS group, the LPS+Rau group and the LPS+Rau+colvelin group was detected by Muse. G-I: The mRNA expression of JAK2 and STAT3 in the control group, the LPS group, the LPS+Rau group and the LPS+Rau+colvelin group was detected by RT-qPCR. J: The protein expression of JAK2, STAT3, p-JAK2, p-STAT3 in the control group, the LPS group, the LPS+Rau group and the LPS+Rau+colvelin group was detected by Western blotting assay. C-D and K-N: Perform grayscale analysis using Image J software. O-Q: The protein expression of IL-6/IL-1β/TNF-α in the control group, the LPS group, the LPS+Rau group and the LPS+Rau+colvelin group was detected by ELISA. Three independent samples were used for statistics. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

<https://doi.org/10.1371/journal.pone.0301660.g006>

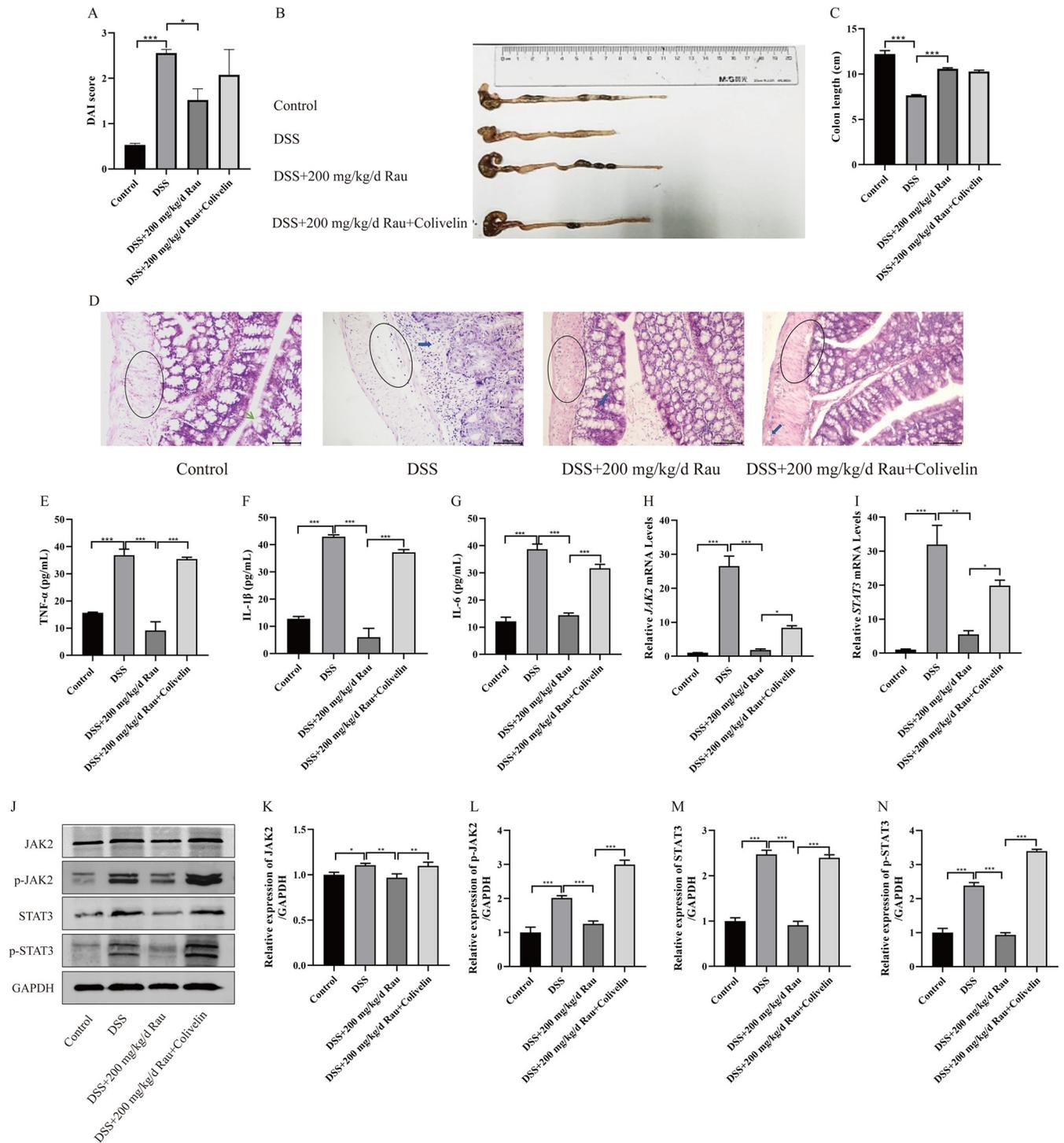


Fig 7. Activation of the JAK2/STAT3 pathway can inhibit the effect of Rau on DSS-induced UC mice model. A: DAI score of the control group, the DSS group, the DSS+200mg/kg/d Rau group and the DSS+200mg/kg/d Rau+colvelin group was calculated. B: Colon length of the control group, the DSS group, the DSS+200mg/kg/d Rau group and the DSS+200mg/kg/d Rau+colvelin group was calculated by ruler. C: The comparison results of colon length of the control group, the DSS group, the DSS+200mg/kg/d Rau group and the DSS+200mg/kg/d Rau+colvelin group were compared. D: Inflammatory reaction was detected in the control group, the DSS group, the DSS+200mg/kg/d Rau group and the DSS+200mg/kg/d Rau+colvelin group by HE. Blue circle: goblet cells; Red circle: goblet cells; Green scissors: Inflammatory cells. E-G: The protein expression of IL-6/IL-1 β /TNF- α in the control group, the DSS group, the DSS+200mg/kg/d Rau group and the DSS+200mg/kg/d Rau+colvelin group was detected by ELISA. H-I: The mRNA expression of JAK2 and STAT3 in the control group, the DSS group, the DSS+200mg/kg/d Rau group and the DSS+200mg/kg/d Rau+colvelin group was detected by RT-qPCR. J: The protein expression of JAK2,

STAT3, p-JAK2, p-STAT3 in the control group, the DSS group, the DSS+200mg/kg/d Rau group and the DSS+200mg/kg/d Rau+colvelin group was detected by Western blotting assay. K-N: Perform grayscale analysis using Image J software. Three independent samples were used for statistics. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

<https://doi.org/10.1371/journal.pone.0301660.g007>

illustrating that NOS2 was the target for Rau treatment of UC. To further explore the role of NOS2 in UC progression, we used siRNA technology to knock down NOS2 and search for its mechanism in UC. In the present study, siNOS2 can inhibit the mRNA expression of JAK2 and STAT3 and significantly restrain the expression of JAK2, STAT3, p-JAK2, and p-STAT3. The results displayed that NOS2 knockdown can inhibit the expression of JAK2/STAT3 in the UC cell model induced by LPS. Therefore, we suppose that we speculated that Rau may promote proliferation and inhibit apoptosis of UC cell model through NOS2-mediated JAK2/STAT3 pathway, yet, further studies are warranted.

JAK2/STAT3 signaling pathway is one of the main signaling pathways of inflammation and is responsible for the production of cytokines, as well as the recruitment and activation of cytokines [31, 32]. Numerous studies have shown that JAK2/STAT3 pathway is an important pathway inflammation influence colitis. Recently, blocking the JAK2/STAT3 pathway can regulate immune responses and alleviate chronic inflammation of the intestine [33]. Wu et al adopt this approach to confirm that butyric acid enhances the therapeutic effect of EHLJ7 on ulcerative colitis by inhibiting the JAK2/STAT3 signaling pathway [34]. Furthermore, researchers have revealed that the anti-inflammatory mechanism of traditional Chinese medicine is related to the blocking of JAK2/STAT3 signaling pathway [35]. To further test this idea, we used JAK2/STAT3 activator to inversely verify that the failure of Rau's anti-inflammatory mechanism in UC progress was associated with activation of the JAK2/STAT3 signaling pathway. We observed that the activation of the JAK2/STAT3 pathway can inhibit the effect of Rau on DSS-induced UC model.

In conclusion, the above data demonstrated that Rau can inhibit the progress of ulcerative colitis through NOS2-mediated JAK2/STAT3 pathway. In future, it is necessary for Rau to regulate UC models through NOS2/JAK2/STAT3 in further experiments.

Supporting information

S1 Fig. Flow diagram.
(JPG)

S1 Raw image.
(ZIP)

Acknowledgments

We thank all the participants and volunteers involved in this study.

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