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ORIGINAL RESEARCH

Cannabidiol Alleviates Intestinal Fibrosis in Mice with Ulcerative Colitis by Regulating Transforming Growth Factor Signaling Pathway

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Objective: The aim of this study is to investigate the protective effect of Cannabidiol (CBD) on DSS-induced colitis in C57BL/6 mice and its related pathways.

Methods: A mouse model of ulcerative colitis (US) was induced by DSS. Enzyme-linked immunosorbent assay (ELISA), quantitative reverse transcription polymerase-chain reaction (qRT-PCR), Western blot (WB) and immunofluorescence (IF) were used to identify the key factors involved in inflammatory response, oxidative stress and intestinal fibrosis. In addition, we transfected si-RNA into CCD-18Co cells.

Results: The research suggests that CBD significantly improves intestinal inflammation by up-regulating the nuclear factor erythroid 2-related factor 2 (Nrf2) expression, inhibiting the classical Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κb) pathway, and inhibiting the release of IL-6 (Interleukin), IL-1β, Tumor Necrosis Factor-α (TNF-α) and other factors. At the same time, CBD plays an antioxidant role by regulating Nrf2/ HO-1 (Heme Oxygenase-1) pathway and activating HO-1 activity. On the other hand, CBD may regulate Transforming growth factor beta (TGF-β)/SMADs signaling pathway by inhibiting the expression of TGF-β1, thereby inhibiting the expression of α-SMA, Collagen1, TIMP1 and other factors, thus playing an anti-fibrotic role. Notably, when Nrf2 is inhibited or lacking, CBD loses the above protective effect against DSS-induced colon injury.

Conclusion: CBD affects the classical NF-κb pathway, Nrf2/ Heme Oxygenase-1 (HO-1) pathway, and Transforming growth factor beta (TGF-β)/SMAD pathway by regulating Nrf2, thereby reducing colonic inflammation and oxidative stress and improving the progression of colonic fibrosis.

Keywords: Ulcerative colitis, cannabidiol, Nrf2, intestinal fibrosis, oxidative stress

Introduction

Ulcerative colitis (UC) is a kind of intermittent diarrhea, abdominal distension, abdominal pain as the typical symptoms of chronic nonspecific intestinal inflammatory diseases. Lesions were diffuse distribution of prevalence rate of more than 400/100,000.[1] At present, the pathogenesis of UC has not been fully studied, and its etiology is complex.[2] In addition to common factors such as inflammation, oxidative stress, immunity, and environmental factors, intestinal fibrosis is a common outcome in the natural course of UC, and it is also the basis of most complications.[3,4] In patients with UC, submucosal fibrosis was detected in almost 100% of UC colectomy specimens.[5] Because UC has the characteristics of low cure rate and high recurrence rate, it is difficult to achieve the ideal effect after conventional drug treatment in clinical practice.[6] Therefore, it is particularly necessary to seek a new drug with high safety and good therapeutic effect.

Cannabidiol (CBD) is considered to be a non-psychoactive compound derived from the cannabis plant. CBD has a wide range of biological effects, including antioxidant and anti-inflammatory effects. Hao Chen et al[7] found that CBD inhibited the phosphorylation and expression of p65 NF-κB, and then inhibited the activation of Toll-like receptor 4

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(TLR4)/Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κb) pathway, thus playing an anti-inflammatory role. Wang et al[8] showed that CBD could significantly improve the oxidative stress response of hepatocytes by regulating the expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and antioxidant enzymes. In addition, it is believed that in the case of anti-inflammatory qualities of CBD, its effect on the intestinal system is negligible.[9] In conclusion, CBD can regulate the transcriptional level of Nrf2 and NF-κB redox-sensitive activity, inhibit the classical pathway of inflammatory response, and play a multi-directional antioxidant and anti-inflammatory effect.[10]

Fibrosis is usually caused by chronic inflammation, which manifests as an immune response lasting several months, in which inflammation, tissue remodeling, and repair processes occur simultaneously.[11] Despite diverse etiology and clinical presentation, what most chronic fibrotic diseases have in common is sustained growth factor, proteolytic enzyme, angiogenic factor, and fibrotic cytokine production. These secretions stimulate the deposition of connective tissue components, gradually remodeling and destroying normal tissue architecture.[12] The anti-fibrosis effect of CBD is drawing attention. Recent studies have shown that CBD can regulate key factors such as Transforming growth factor beta (TGF-β) or inhibit Reactive Oxygen Species (ROS) to improve cellular oxidative stress to antagonizing chronic inflammation and fibrosis formation in various tissues.[13,14]

At present, there is no study on the treatment of intestinal fibrosis in UC patients, and there is also a lack of relevant animal experiments. A large number of previous studies have shown that CBD has anti-inflammatory and anti-fibrosis therapeutic effects with high biological safety.[15,16] This makes plant-derived CBD a great advantage over other chemically synthesized small-molecule compounds for clinical application. At present, there is a lack of effective treatment for intestinal fibrosis in UC patients. The above discussions indicate that CBD may have potential in the treatment of intestinal fibrosis in UC patients. In this study, animal models and in vitro cytological experiments were established to study the effect of CBD on colitis and intestinal fibrosis, and to explore the therapeutic mechanism.

Materials and Methods

Materials

Smooth muscle actin Polyclonal antibody (14395-1-AP) was purchased from Proteintech Group Wuhan Sanying Biological Company (1:2000); TIMP2 Antibody (#DF6454) was purchased from Affinity Biosciences (1:1000); NRF2, NFE2L2 Polyclonal antibody (16396-1-AP) was purchased from Proteintech Group Wuhan Sanying Biological Company (1:5000). Other reagents are attached in supplementary information (Table S1).

DSS-Induced Colitis Model and Treatment Methods

Eight-week-old female C57BL/6 mice weighing about 22–24 g were used. CBD power (purity 99%) was purchased from Zhongsheng Biotechnology Co., Ltd., (Heilongjiang, China). All mice were randomly divided into 6 groups, namely: Control group (Control), CBD group (60mg/kg), model group chronic enteritis group (DSS), DSS+CBD-L (20 mg/kg), DSS+CBD-M (40 mg/kg), DSS+CBD-H (60 mg/kg). In the study of CBD increasing Nrf2 expression to alleviate intestinal fibrosis, the mice were divided into Control group (Control), DSS model group, DSS+CBD treatment group, and DSS+ Nrf2 inhibition group (DSS+ ML385+CBD), 30 mg/kg ML385. There were 10 mice in each group. In the study on the effect of CBD on the fibrosis of CCD-18Co cells stimulated by TGF-β1, there were six groups: normal group, 20μM CBD, TGF-β1, TGF-β1+CBD 5μM, TGF-β1+CBD10μM, TGF-β1+CBD20μM. Different concentrations of CBD (5μM, 10μM, 20μM) were used to stimulate the incubation with 5.0 μg/L TGF-β1 for 24 hours. In the study of CBD inhibiting the TGF-β/SMADs signaling pathway in CCD-18co, there were six groups: normal group, 20μM CBD, TGF-β1, TGF-β1 +CBD 5μM, TGF-β1 +CBD 10μM, TGF-β1 +CBD 20μM. Different concentrations of CBD (5μM, 10μM, 20μM) were used to stimulate the incubation with 5.0 μg/L TGF-β1 for 12 hours. ML385 (30 mg/kg) pretreatment was intraperitoneally given an hour prior to CBD administration. The main reagents used in this study and related information from sources are shown in the supplementary materials.

Modeling and administration: Experimental chronic colitis was induced in mice by three cycles of DSS as shown in Figure I. Each cycle consisted of 7 days of 2% DSS followed by 14 days of sterile drinking water. The first and last days of the experiment were designated as day 1 and day 50, respectively. First, the experimental mice were anesthetized to

take blood from the posterior orbital vein, and then killed. The weight of the mice was recorded daily and the spleen weight was measured at the end. This study was approved by Biological and Medical Ethics Committee, School of Basic Medicine, Jiamusi University (No. JDJCYXY20240044).

Colonic Histopathological Score

The following criteria were used for evaluation, shown as Table 1.

CCK-8 Detects the Optimal Stimulation Concentration and Time

The CCD-18Co obtained from the American Type Culture Collection (ATCC, Georgetown, DC, USA) was seeded into 96 well plates with a cell density of 4×10^3 cells per well, and the 200 μL culture medium was added into per well for 12 h incubating at 37 °C. Then, the original solution was replaced by a culture medium containing different concentrations TGF-β1 (1.25, 2.5, 5, 10 μg/L) or CBD (5 μM, 10 μM, 20 μM, 40 Mm) with incubating at 37 °C for 24 h, respectively. Subsequently, the 100 μL CCK-8 working solution was used under dark conditions. After 2 h incubation, the absorbance value was detected at 450 nm to evaluate cell viability.

Cell Culture and Model Establishment

CCD-18Co cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS and 1% penicillin/streptomycin. Co-incubation with CBD and TGF-β1 demonstrated that CBD affected TGF-β1 signaling by increasing Nrf2 expression. CCD-18Co cells were stimulated with 5.0 μg/L TGF-β1 for 24 hours. Co-incubation with TGF-β1 was performed with 20μM CBD for 24 hours.

Cell transfection technique was used to transfect si-RNA into CCD-18Co cells and inhibit the expression of Nrf2. Taking the six-well plate as an example, 100 pmol siRNA and 5 μL Lipofectamine 2000 transfection reagent were dissolved in two tubes of 250 μL Opti-MEM serum-free culture solution, mixed evenly, left at room temperature for 5 min, then the two tubes of solution were mixed, gently shaken, and left at room temperature for 20 min. After standing for 20 min, the mixed solution was added to each hole of the six-well plate by drops, and replaced with the complete culture solution 4–6 h later. The expression of Nrf2 in CCD-18Co cells was detected by Western blot (WB) to prove the successful transfection of siRNA.

Pathological Observation

After the treatment, the mice were sacrificed. Then, the samples were taken out and placed in 4% paraformaldehyde solution for 1 days, and were embedded with paraffin. The embedded samples are sliced by a paraffin slicing machine, and the slice thickness is set to 6 μm. Then, the slice was stained with masson's trichrome staining according to the manufacturer's instructions for histology analysis and immunohistochemical staining.

For immunohistochemical staining of α-SMA and collagen I, sections were treated with 0.25% pepsin (ab64201, Abcam) at 37°C for 20 min for antigen retrieval. After washing 5 times with PBS, the endogenous peroxidase was removed with 3% hydrogen peroxide methanol solution and the nonspecific antigen binding was blocked with 10% goat serum. Primary α-SMA antibody (1:100, Proteintech Group, 14395-1-AP) and collagen I antibody (1:100, Proteintech Group, 14695-1-AP) was applied overnight at 4°C. The next day, rabbit anti-mouse IgG (1:200, bs-0296R-HRP, Bioss)

Table 1 Colonic Histopathological Score

Item	Score
No evidence of inflammation	0
Low-grade inflammation with scattered mononuclear cells (1–2 lesions)	1
Moderate inflammation with multiple mononuclear lesions	2
High inflammation; increased vascular density; wall thickening	3
Severe inflammation with transmural leukocyte infiltration and goblet cell loss	4

was applied for 60 min. Subsequently, the nuclei were counterstained with hematoxylin. The sections were dehydrated and mounted with transparent neutral gum seals for microscope observation.

Western Blotting (WB) and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) in Colonic Tissues

Proteins were isolated with SDS lysis buffer from cultured cells and tissues at 4 °C. The lysates were heated at 95 °C for 10 min, quantified by NanoDrop 2000, loaded with 16x β-Blue (20% β-mercaptoethanol and 0.08% bromophenol blue) and stored at −80 °C. Fifty micrograms of proteins extracted from cultured cells or tissues were loaded in each well, separated via SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Antibodies including α-SMA Polyclonal antibody (Proteintech, 14395-1-AP), Collagen Type I Polyclonal antibody (Proteintech, 14695-1-AP), NRF2 Polyclonal antibody (Proteintech, 16396-1-AP), H0-1/HMOX1 Polyclonal antibody (Proteintech, 10701-1-AP), NF-KB p65 Recombinant antibody (Proteintech, 80979-1-RR), IL-6 Antibody (Affinity Biosciences, #DF6087), IL-1 beta Antibody (Affinity Biosciences, #AF5103) and TGF Beta 1 Polyclonal antibody (Proteintech, 21898-1-AP) were applied to analyze different proteins.

After colonic tissue was cleaned, total RNA of colonic tissue was extracted using an TRIzol Reagent, and reverse transcribed using RevertAid First-Strand cDNA Synthesis Kit. A real-time quantitative PCR was executed to quantify gene expression using an UltraSYBR mixture in a Roche light cycler 96 Real-Time System. The relative amount of each transcript was normalized to the amount of GAPDH in the same cDNA and data were analyzed according to the $2^{-\Delta\Delta Ct}$ method. The primer sequence was shown in the Table S2.

Immunofluorescence (IF)

Cells were cultured in 12-well plates with glass coverslips. At harvest, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with the above mentioned antibodies, followed by the corresponding Alexa Fluor 488- or 594-conjugated secondary IgG antibodies (Invitrogen, A-11008/A-48262/A11032). Cellular nuclei were stained with DAPI (Solarbio). Images were captured via an LSM 880 confocal microscope.

Statistics

All data were expressed as mean ± standard deviation, and SPSS 26.0 software was used for the analysis. Two-side $p < 0.05$ was considered significant difference. Significant differences were assessed by a one-way analysis of variance.

Results

CBD Reduces DSS-Induced Colonic Inflammatory Injury

The results, shown in Figure 1A, showed that with increasing CBD concentration, different concentrations of CBD ameliorated visible DSS-induced damage and shortening of the colon macroscopically. In addition, according to the results of HE and Masson staining (Figure 1B and C), CBD could reduce DSS-induced pathological tissue damage such as disordered mucosal gland structure, inflammatory infiltration, and intestinal fibrosis. Compared with the DSS group, the CBD treatment group had significant down-regulation of inflammatory markers NF-κb p65 and IL-1β, and fibrosis markers TGF-β1 and α-SMA, while the expression of antioxidant markers Nrf2 and HO-1 was significantly up-regulated (Figure 1D–F). In addition, as shown in Figure 1G and H, the expression of TGF-β1 and Nrf2 was the same as above, while the above conditions were reversed after adding CBD, and the improvement effect was more significant with the increase of CBD concentration.

CBD Reduces Colitis Disease Activity by Increasing Nrf2 Expression

Body weight loss was improved in the mice treated with CBD compared with the DSS group (Figure 2A). As shown in Figure 2B and C, the DSS group had the shortest colon length, and CBD treatment improved macroscopic DSS-induced visible damage and colon shortening, while colon length was not significantly improved in the Nrf2 inhibition group. In addition, CBD significantly attenuated DSS-induced splenomegaly, but not in the Nrf2 inhibition group (Figure 2D). HE

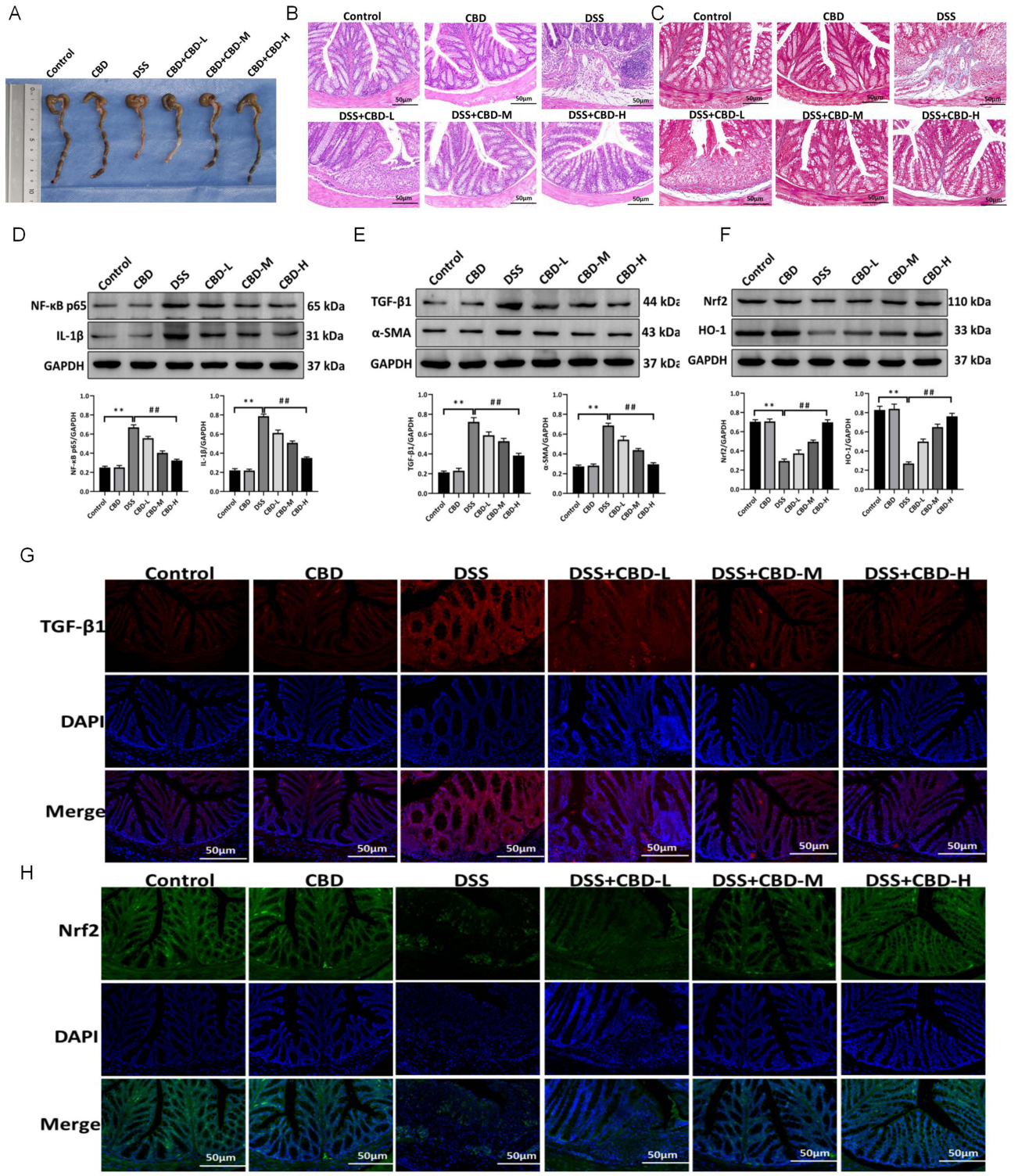

Figure 1 CBD attenuates DSS-induced inflammatory injury in the colon (Compared with the Control group, **p<0.01; Compared with CBD-H group, ## p<0.01). (**A**) Colon morphology and length on day 50; (**B**) colonic tissue sections were stained with HE; (**C**) Masson staining of colon tissue sections; (**D**) The expressions of NF-κb p65 and IL-1β were detected by WB; (**E**) expression of fibrosis markers TGF-β1 and α-SMA; (**F**) expression of Nrf2 and HO-1; (**G**)TGF-β1 IF results; (**H**) Nrf2 IF results.

and Masson staining results showed that CBD could reduce DSS-induced colonic mucosal gland structure disorder, inflammatory infiltration, and intestinal fibrosis, while CBD had no significant improvement in the Nrf2 inhibition group (Figure 2E and F). Immunohistochemical staining showed that compared with the DSS group, the expression levels of Collagen1 and α-SMA in the CBD treatment group were significantly down-regulated, while the expression levels of

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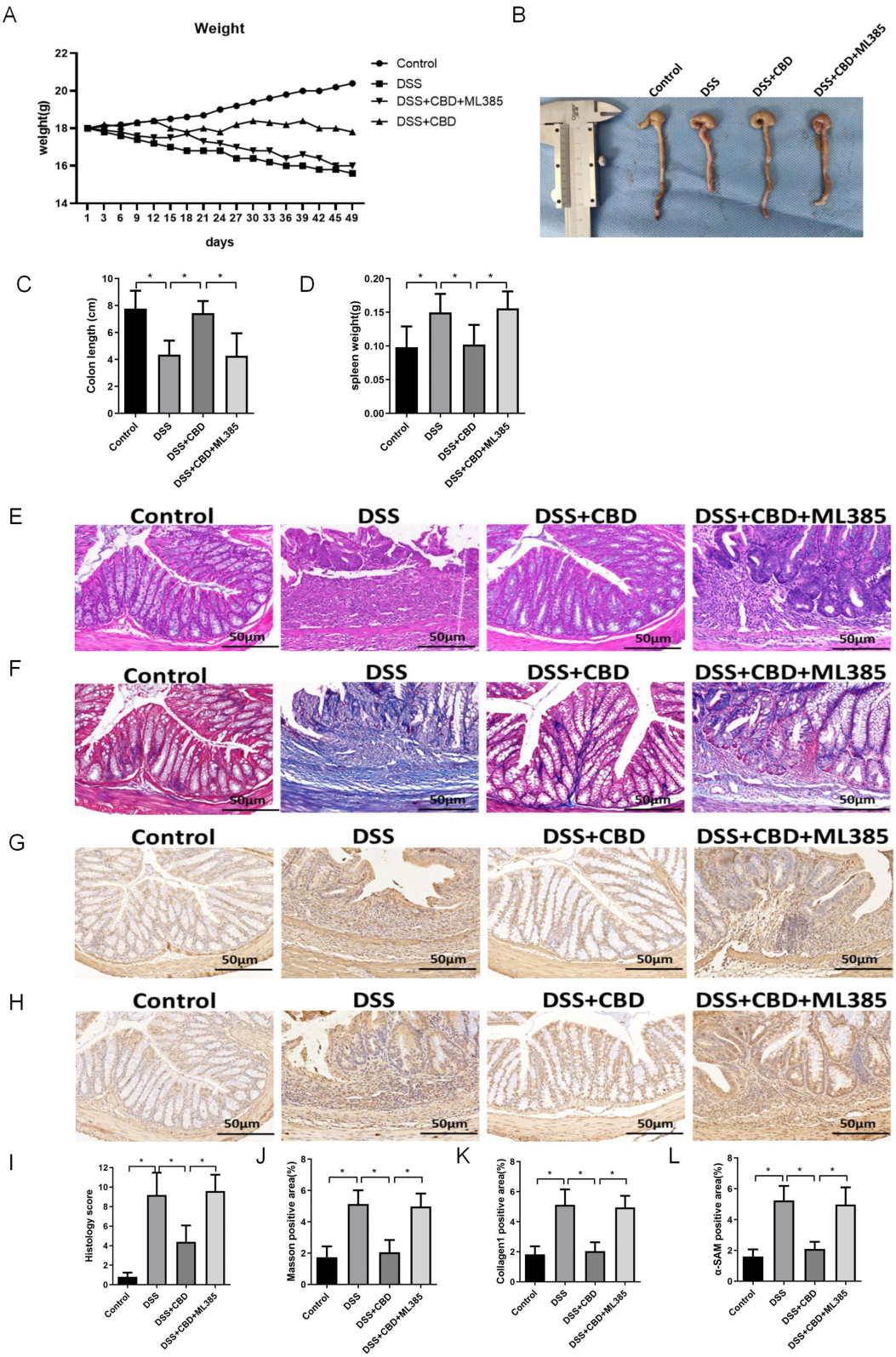

Figure 2 CBD reduces colitis disease activity by increasing Nrf2 expression (* means p<0.05 in the two groups). (**A**) changes in body weight (from day 1 to day 49); (**B**) colon morphology at day 50; (**C**) Colon length at day 50; (**D**) Spleen weight of mice in each group on day 50; (**E**) The morphology of colon tissue was observed by HE staining; (**F**) Masson staining was used to observe the fibrous components of colon tissue; (**G**) α-SMA immunohistochemical staining; (**H**) Collagen1 immunohistochemical staining; (**I–L**) Quantitative analysis of figure.

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Collagen1 and α-SMA in the Nrf2 inhibition group were similar to those in the DSS group (Figure 2G and H), suggesting that CBD inhibits colitis activity and fibrosis by increasing Nrf2 expression (Figure 2I–L).

CBD Inhibits the NF-κb Pathway by Up-Regulating Nrf2 to Improve Inflammation

After treatment with DSS, the protein expression levels of inflammatory cytokines NF-κb p65, pIKB-α, TNF-α, IL-6 and IL-1β were significantly increased (Figure 3A–F). In the CBD treatment group, the expression of the above-mentioned inflammation-related proteins was significantly reduced, indicating that CBD has a good anti-inflammatory effect. After adding Nrf2 inhibitor ML385, the therapeutic effect of CBD was significantly reduced, and there was no significant difference in the expression of each protein compared with the DSS group.

CBD Inhibits the TGF-β/SMAD Pathway by Upregulating Nrf2

After DSS-induced colitis, the protein expressions of α-SMA, Collagen1, p-smad2, p-smad3, TIMP1, and TGF-β1 were significantly increased (Figure 4A–G), while the protein expressions of MMP3, Nrf2, and HO-1 were significantly decreased (Figure 4A and H–J). This suggests that the TGF-β/SMAD pathway is activated. After adding CBD, the expression of TGF-β/SMAD pathway was significantly inhibited, but CBD lost the above regulatory effect in the Nrf2

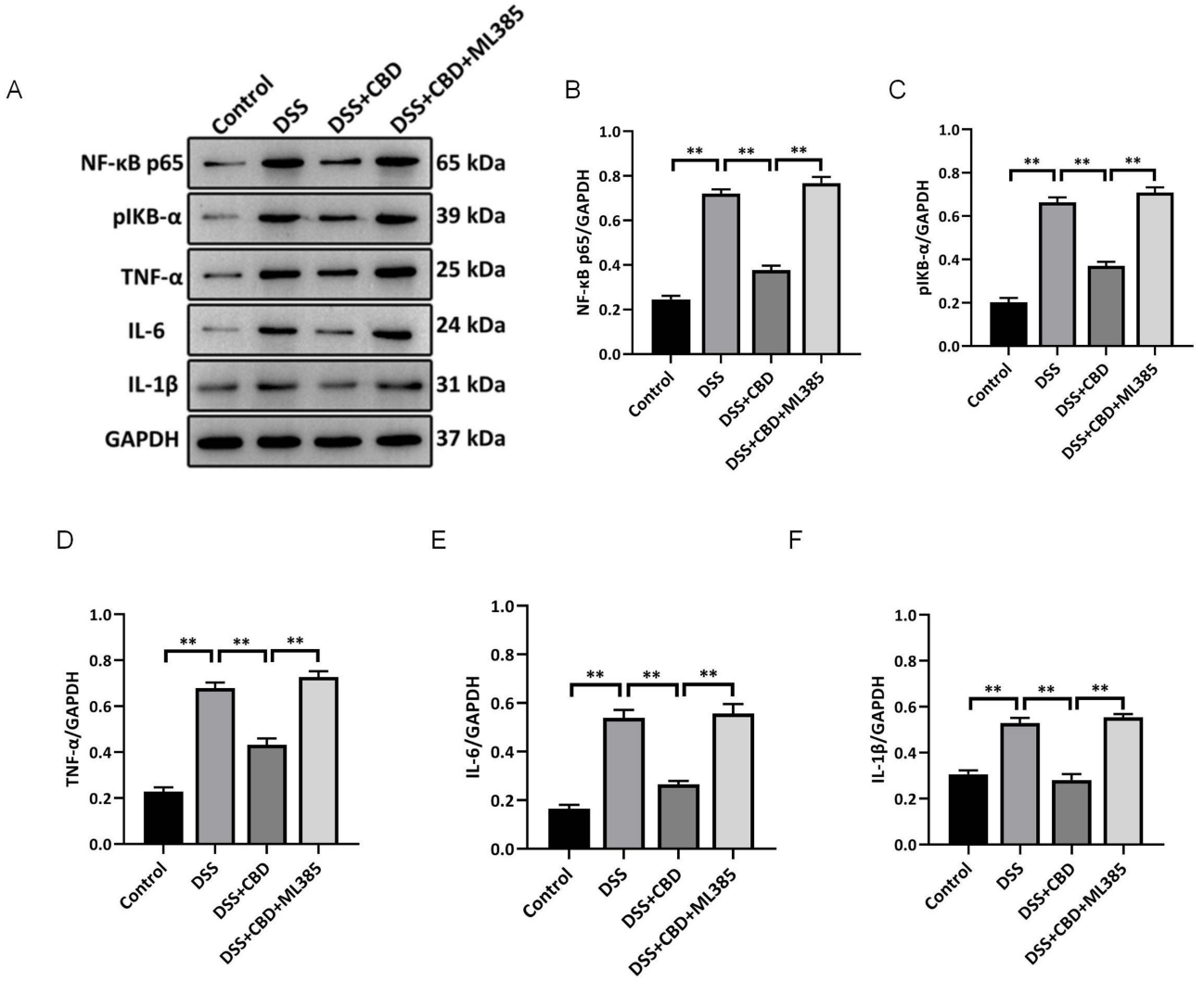

Figure 3 CBD inhibits the NF-κb pathway by upregulating Nrf2 to improve inflammation (** means p<0.01 in the two groups). (**A**) WB was used to detect the protein abundance of NF-κb p65, pIKB-α, TNF-α, IL-6 and IL-1β in colon tissue of mice; (**B–F**) Image J software was used to quantitatively analyze the protein expression in panel A.

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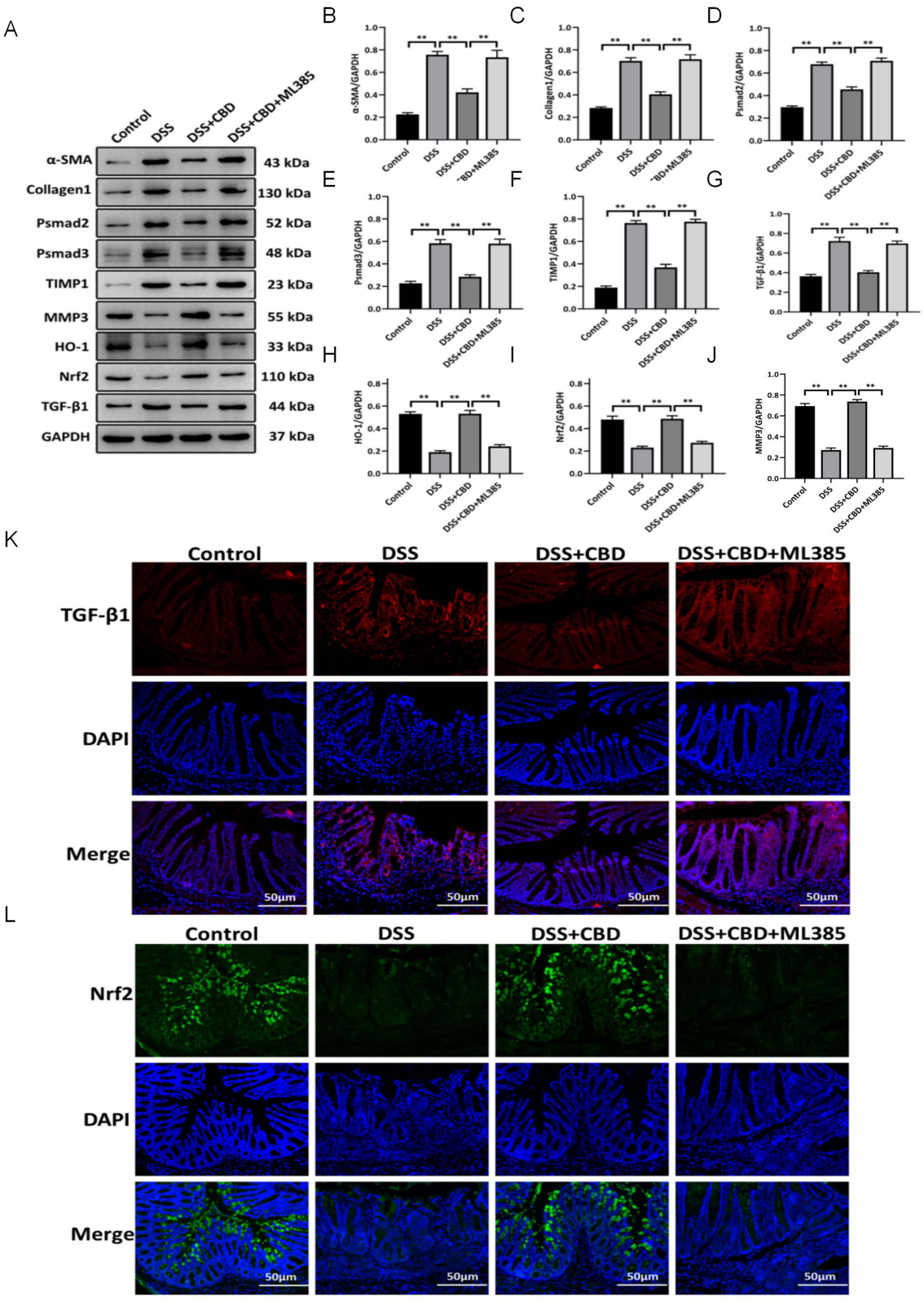

Figure 4 CBD inhibits the TGF-β/SMAD pathway by upregulating Nrf2. (**A**) WB was used to detect the protein abundance of α-SMA, Collagen1, p-smad2, p-smad3, TIMP1, MMP3, HO-1, Nrf2 and TGF-β1 in colon tissue of mice; (**B–J**) Image J software was used to quantitatively analyze the protein expression in panel A; (**K** and **L**) IF was used to detect the expression of TGF-β1 and Nrf2 in colon tissue of mice. (** means p<0.01 in the two groups).

inhibition group, and there was no significant difference in the expression of TGF-β/SMAD pathway-related proteins between the DSS group and the Nrf2 inhibition group. In addition, IF staining further confirmed that CBD inhibited TGF-β1 expression by upregulating Nrf2 (Figure 4K–L). In conclusion, CBD can improve the DSS-induced fibrosis in colon tissue by inhibiting the TGF-β/SMAD pathway through up-regulation of Nrf2.

CBD Inhibited TGF-β1-Induced CCD-18co Fibrosis

The cell activity was detected by CCK-8 assay, and the optimal concentration of TGF-β1 was 5μg/L, and the optimal modeling time was 24 hours (Figure 5A). Further exploration revealed a range of CBD administration concentrations of 5, 10, and 20μM (Figure 5B). The mRNA levels of α-SMA, Col1agen1 and TIMP-1 were significantly up-regulated in CCD-18co cells induced by TGF-β1. However, CBD could inhibit the above effects of TGF-β1, and the inhibitory effect was gradually enhanced with the increase of CBD concentration (Figure 5D). The protein expression trend of α-SMA, Col1agen1, TIMP-1 and MMP3 was found to be the same as that of mRNA by WB (Figure 5E–I). The detection of IF further confirmed that CBD could alleviate TGF-β1-induced fibrosis changes in colonic cells by up-regulating Nrf2 expression, as shown in Figure 5J–L.

CBD Inhibits the TGF-β/SMADs Pathway in CCD-18co Cells

As shown in Figure 6A and B, Nrf2 expression was not significantly increased in response to CBD stimulation alone, but was significantly upregulated in the high-dose CBD treatment group upon TGF-β1 induction. TGF-β1 stimulation significantly upregulated the expression of SMAD2 and SMAD3, while CBD at all concentrations did not improve the above results (Figure 6C and D). TGF-β1 activates SMAD2 and SMAD3 to significantly increase the expression of p-SMAD2 and p-SMAD3, while the addition of CBD can significantly inhibit their phosphorylation expression. With the increase of treatment dose, the down-regulation of p-SMAD2 and p-SMAD3 expression increased (Figure 6E and F). In addition, IF results (Figure 6G) showed that the expression of Nrf2 was significantly up-regulated in the high-dose CBD treatment group under the induction of TGF-β1. Therefore, CBD can inhibit the activation of TGF-β/SMADs pathway in CCD-18co, and can increase the expression of Nrf2 to alleviate the fibrosis induced by TGF-β1.

CBD Reduces Fibrosis by Upregulating Nrf2 and Inhibiting the TGF-β/SMADs Pathway in vitro

In this study, we used si-RNA to knock down Nrf2 expression in CCD-18Co cells (Figure 7A and B). CBD was able to remove the excess ROS induced by TGF-β1. However, after the Nrf2 knockdown, CBD lost these functions (Figure 7C). As shown in Figure 7D–F, IF detection results showed that the expression of α-SMA and Collagen1 was significantly increased under the induction of TGF-β1, while the addition of CBD could significantly up-regulate the expression of Nrf2 and inhibit the collagen deposition induced by TGF-β1. However, CBD lost the above effects after the Nrf2 knockdown. In addition, the expression of p-smad2 and p-smad3 was significantly increased under the induction of TGF-β1. CBD could inhibit the phosphorylation of SMADs by up-regulating the expression of Nrf2/HO-1 in CCD-18Co cells, thereby inhibiting the expression of α-SMA, Collagen1 and TIMP1 and reducing fibrosis (Figure 7G–N).

Discussion

The expression and potential function of CBD in DSS-induced colonic injury has not been elucidated. Previous studies have found that CBD, as one of the phytocannabinoids with pharmacological activity,[17] has good anti-inflammatory, anti-fibrosis and anti-oxidation properties.[18,19] As we all know, the NF-κb pathway has long been considered as a typical inflammatory signaling pathway[20] and also plays an important role in chronic inflammatory bowel disease. This study confirmed that CBD could inhibit the classical NF-κb pathway by regulating and inhibiting NF-κb p65 and pIKB-α, thereby reducing the expression of IL-1β and ameliorating DSS-induced colonic inflammation. IL-1β is not only a pro-inflammatory cytokine but also a pro-fibrotic factor,[21] and its decreased expression can inhibit the progression of fibrosis.[22] In addition to reducing the expression of IL-1β, CBD can down-regulate fibrogenic factors and reduce collagen deposition, thereby improving colonic fibrosis. From the results, CBD could significantly reduce the DSS-

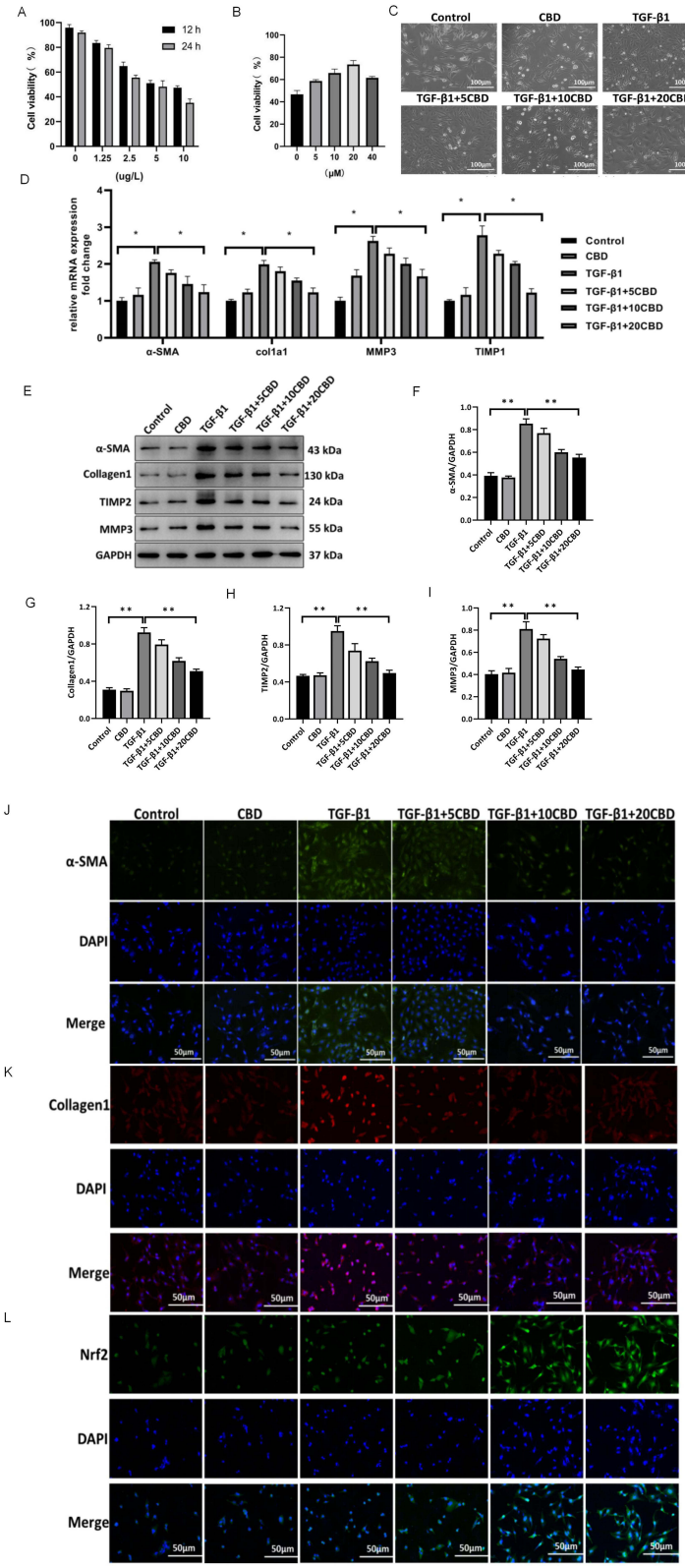

Figure 5 CBD inhibits TGF-β1-induced CCD-18co fibrosis (*means p<0.05 and ** means p<0.01 in the two groups). (**A** and **B**) CCK-8 was used to detect cell activity to explore the optimal inducing concentration of TGF-β1 and the therapeutic concentration range of CBD; (**C**) The morphology of CCD-18Co cells was observed under light microscope; (**D**) The mRNA levels of α-SMA, Col1agen1 and TIMP-1 in CCD-18co cells were detected by PCR; (**E**) The protein abundance of α-SMA, Col1agen1 TIMP-2 and MMP3 in CCD-18co cells was detected by WB; (**F–I**) Image J software was used to quantitatively analyze the protein expression in Figure E; (**J–L**) IF was used to detect the expressions of α-SMA, Collagen1 and Nrf2 in CCD-18co cells.

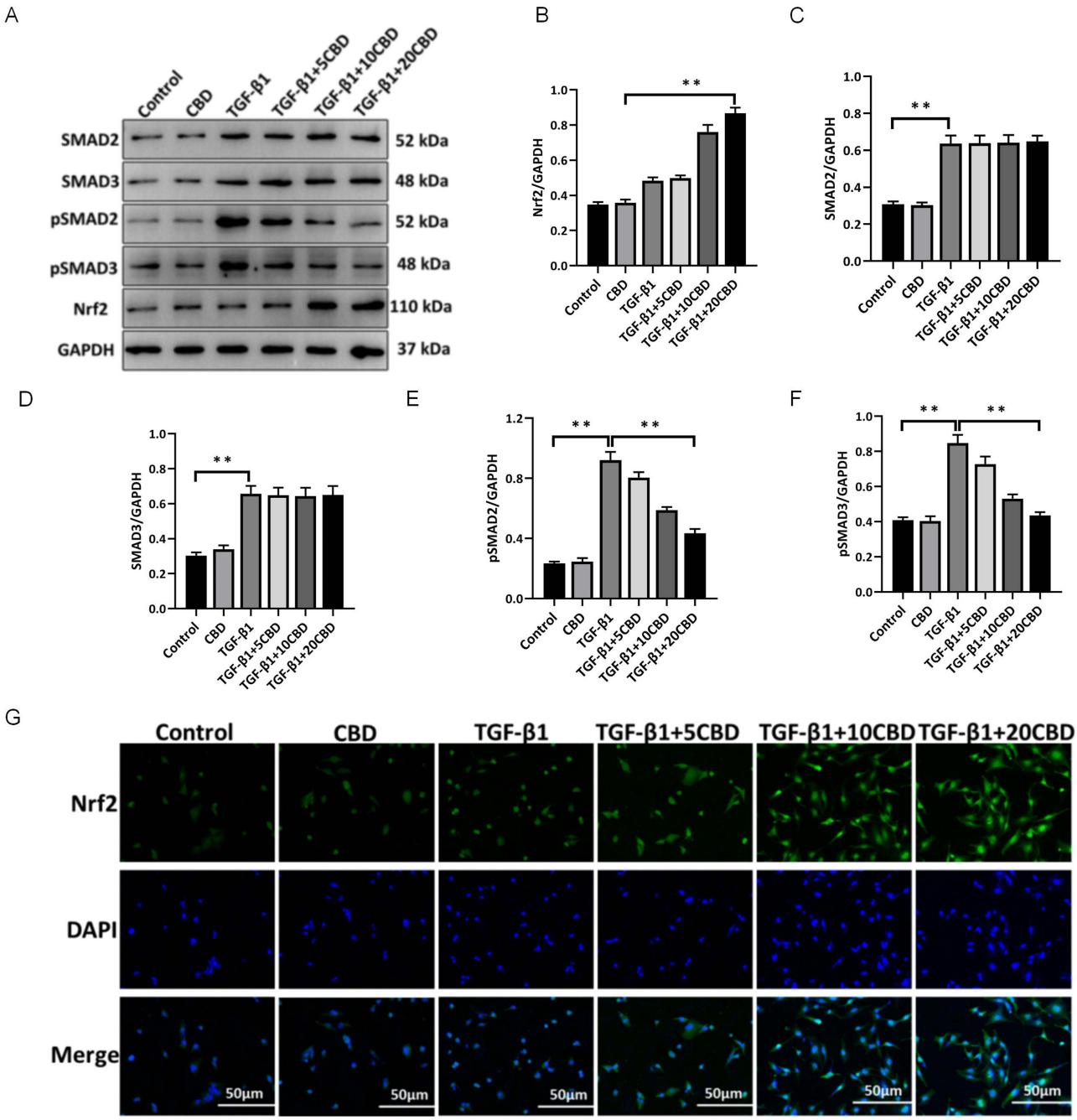

Figure 6 CBD inhibits the TGF-β/SMADs pathway of CCD-18co (** means p<0.01 in the two groups). (**A**) WB was used to detect the protein abundance of SMAD2, SMAD3, p-SMAD2, p-SMAD3 and Nrf2 in CCD-18co cells; (**B–F**) Image J software was used to quantitatively analyze the protein expression in panel A; (**G**) The expression of Nrf2 in CCD-18co cells was detected by IF.

induced colitis disease activity score, and reduce the pathological damage of intestinal tissue and the extent and severity of fibrosis. Finally, CBD also has good antioxidant activity. A number of previous studies have found that the degree of intestinal inflammation is aggravated after the administration of HO-1 inhibitors in inflammatory mouse models.[23,24] The results of this study suggest that CBD exerts antioxidant effects by positively regulating the Nrf2/HO-1 pathway, increasing Nrf2 expression and regulating HO-1 promoter activity. A large number of previous studies have shown the interaction between Nrf2 and NF-κb pathways.[25,26] The results of this study suggest that CBD inhibits the NF-κb pathway by increasing the expression of Nrf2, reducing inflammatory response and oxidative stress. The mechanism may

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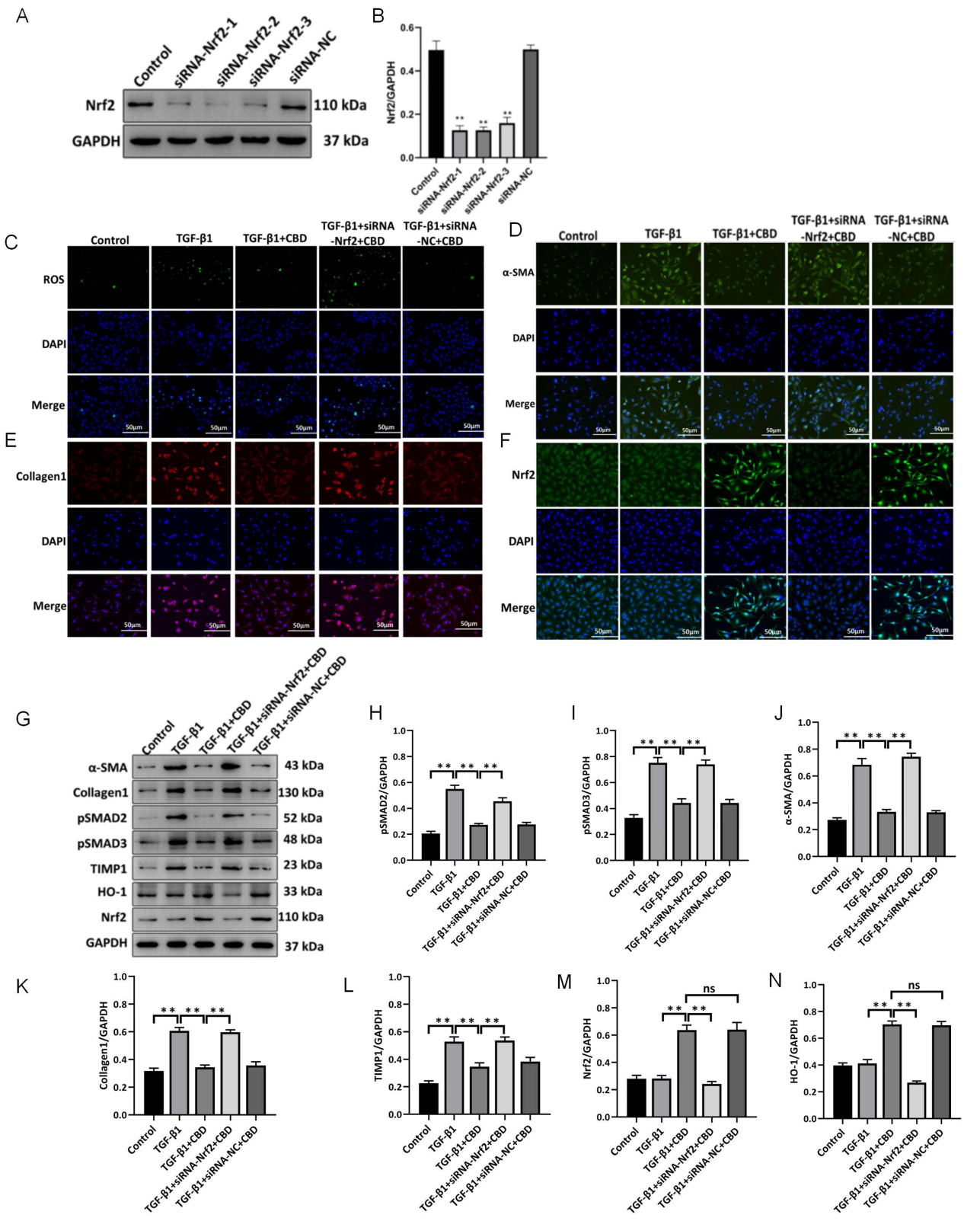

Figure 7 CBD reduces fibrosis by upregulating Nrf2 and inhibiting the TGF-β/SMADs pathway in vitro (** means p<0.01 in the two groups; ns means there was no statistical difference between the two groups). (**A** and **B**) WB was used to verify the knockdown effect of si-Nrf2; (**C**) fluorescence was used to detect the content of ROS in CCD-18Co cells; (**D–F**) IF was used to detect the changes of α-SMA, Collagen1 and Nrf2 in CCD-18Co cells; (**G**) WB was used to detect the protein abundance of p-SMAD2, p-SMAD3, α-SMA, Collagen1, TIMP1, Nrf2 and HO-1 in CCD-18co cells; (**H-N**) Image J software was used to quantitatively analyze the expression levels of each protein in Figure (**H**).

be that Nrf2 can inhibit sMaf (MafK), thereby reducing the acetylation of NF-kBp65 mediated by Nrf2 transcriptional coactivator CBP and positively regulating Nf-Kb activity.[27]

Colonic fibrosis refers to the abnormal deposition of extracellular matrix (ECM) produced by intestinal myofibroblasts in the basal layer,[28] and is considered to be the result of repeated persistent local chronic inflammation and abnormal wound repair.[29] Therefore, the management and treatment of intestinal fibrosis in inflammatory bowel disease is very important.[30] Transforming growth factor-β1 (TGF-β1) is one of the most effective cytokines to promote colonic fibrosis.[31,32] TGF-β1 mainly transmits pro-fibrotic signals through its downstream SMADS protein family, ultimately promoting the increase of ECM synthesis and accelerating the process of fibrosis.[33] The results of this study suggest that in DSS-induced colitis, p-smad2 and p-smad3 are highly expressed, TGF-β1/ Smad signaling pathway is activated, and the expressions of α-SMA and Collagen1 are increased. These results suggested that CBD could significantly reverse the above process, while knockdown of Nrf2 promoted TGF-β1/SMADs signaling in fibroblasts. These evidences suggest that CBD can increase the expression of Nrf2 by activating the Nrf2/ HO-1 pathway, while Nrf2 can reduce the expression of TGF-β1/SMADs signaling pathway, thereby inhibiting intestinal fibrosis. In addition, Nrf2 decreased TIMP1 expression and increased MMP3 expression in mouse intestinal fibrosis model and human intestinal and colonic tissue cells. The main components of ECM are collagen and fibronectin, which can be degraded by matrix metalloproteinase (MMP). Tissue inhibitor of matrix metalloproteinase (TIMP) is a specific inhibitor of MMP, which can inhibit the degradation of ECM by MMP.[34,35] This suggests that CBD can affect the balance of MMP3/TIMP1 by regulating Nrf2, thereby regulating the degradation of the extracellular matrix and inhibiting intestinal fibrosis.

Oxidative stress is commonly found in patients with inflammatory diseases and can mediate the occurrence and development of fibrosis and chronic inflammation-related cancers.[36,37] Reactive oxygen species (ROS) is an important mediator in inducing fibrosis, which can activate the TGF-β1/SMADS pathway, while Nrf2 and its downstream products have the ability to remove ROS.[38] When human intestinal fibroblasts were induced to myofibroblasts by TGF-β1, the level of ROS expression in fibroblasts was also increased, indicating that ROS was involved in the process of intestinal fibrosis. ROS and TGF-β1 can promote each other, leading to REDOX imbalance and forming a vicious cycle.[39,40] Previous studies in various fibrotic organs have found that ROS can increase the expression level of TGF-β1 and affect the pro-fibrotic effect of TGF-β1 through multiple aspects, such as the activation of fibroblasts, myofibroblast differentiation, apoptosis of epithelial and endothelial cells, and the expression of fibrosis/pro-fibrotic genes.[41,42] It was found that the expression of endogenous TGF-β1 increased during the differentiation of intestinal fibroblasts into myofibroblasts, and Nrf2 could inhibit the expression of endogenous TGF-β1. On the contrary, in Nrf2 gene knockout group, CBD could not inhibit the overexpression of α-SMA and Collagen1 induced by TGF-β1. This suggests that CBD may reduce the activation of ROS-mediated TGF-β/SMADs signaling pathway by increasing the expression of Nrf2, and significantly improve intestinal fibrosis.

Taken together, our study demonstrated that CBD affected the classical NF-κb pathway, Nrf2/HO-1 pathway, and TGF-β1/SMAD pathway by regulating Nrf2, thereby reducing intestinal inflammation, oxidative stress and intestinal fibrosis, improving intestinal function and pathological symptoms, and thereby protecting against DSS-induced colon injury. These findings provide new ideas and directions for the treatment of UC.

Ethics Statement

The animals and cells in this study have been approved by the Ethical Review Committee. This study was approved by Biological and Medical Ethics Committee, School of Basic Medicine, Jiamusi University (No. JDJCYXY20240044).

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Disclosure

The authors all declare no conflict of interest.

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