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Original Research Article

Therapeutic effect of Sijunzi decoction on ulcerative colitis: A study based on *in vitro* functional experiments

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Abstract

Purpose: *To investigate the efficacy of Sijunzi decoction (SJZD) against ulcerative colitis (UC) in vitro, and to unravel the probable mechanism of action.*
Methods: *SEC-6 cells were exposed to lipopolysaccharide (LPS) in order to establish an ulcerative colitis model, and then treated with SJZD. CCK-8 assay was employed to evaluate cell viability, while cell apoptosis was determined by flow cytometry. Enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR) were used to assess inflammation factors, viz, TNF-α, IL-1β and IL-6, whereas the expression levels of Bax, Bcl-2 and peroxisome proliferator-activated receptors (PPARs) were evaluated by Western blot.*
Results: *SJZD doses of 80 and 160 mg/L increased cell viability in LPS-induced SEC-6 cells, while Bcl2 and Bax expressions were regulated, and apoptosis inhibited at these doses ($p < 0.05$), indicating that SJZD attenuated cell apoptosis. Inflammation was also repressed by SJZD, based on the reduced expressions of TNF-α, IL-1β and IL-6 ($p < 0.05$). Furthermore, SJZD significantly increased PPARα level, thus enhancing cell viability, inhibiting apoptosis as well as inhibiting inflammation ($p < 0.05$).*
Conclusion: *SJZD lowers cell damage, and inhibits cell apoptosis and inflammation through the activation of PPARα pathway, thus suggesting that SJZD is a potential therapeutic candidate for the treatment of ulcerative colitis.*

Keywords: *Apoptosis, Inflammation, Peroxisome proliferator-activated receptors, Sijunzi decoction, Ulcerative colitis*

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INTRODUCTION

Ulcerative colitis, as an inflammatory bowel disease, poses a substantial threat to human health. Current treatments for the disease incorporate 5-aminosalicylic acid, immunosuppressants and steroids. However, clinical problems have risen recently, such as drug resistance, opportunistic infections and loss of responsiveness. Therefore, finding a suitable natural agent is crucial for the satisfactory treatment of UC [1].

PPARs including PPAR α, β, and γ belong to nuclear receptor family. It is one of the ligand-activated transcription factors. There is a lower

PPARα expression in UC patients compared to remission and control groups, and drug-treated UC patients have increased PPARα gene expression [2]. Immune cell infiltration is inhibited by PPARα in the colonic mucosa, as PPARs reduce the expression and release of pro-inflammatory markers in mice and humans [3].

Sijunzi Decoction is a traditional prescription consisting of four Chinese herbs: ginseng or *Codonopsis pilosula, Atractylodes macrocephala, Poria cocos* and licorice. This mixture has the effect of invigorating the spleen and replenishing qi, and has been used to treat gastrointestinal diseases since ancient times [4]. *Sijunzi* decoction improves dextran sulfate sodium (DSS)-induced inflammation in rats with UC [5]. *Sijunzi* decoction also has the capacity to activate PPARα [6].

However, the potential of *Sijunzi* decoction to alleviate ulcerative colitis through PPARα activation remains unclear. This study investigated the effect of SJZD on the viability and apoptosis of LPS-induced SEC-6 cells. Inflammation and PPAR pathway were also evaluated in LPS-induced SEC-6 cells treated with SJZD.

EXPERIMENTAL

Cell culture

Rat intestinal epithelial cell line IEC-6 cells were grown in Dulbecco's Modified Eagle's Medium containing 10 μg/mL insulin and 10 % fetal bovine serum at 37 °C incubator with 5 % CO_2. The cells were treated with 50 μg/mL LPS (L2630, Sigma-Aldrich) for 12 h to simulate intestinal epithelial injury in UC [7].

Cell viability assay

The LPS-induced SEC-6 cells treated with 40, 80, 1600 mg/L SJZD were cultured in fresh medium supplemented with 10 μL CCK-8 (Glpbio, CA, USA). After 3 days, the absorbance was determined at 450 nm wavelength, and cell viability ratio was calculated.

Flow cytometry

Apoptosis was measured using an apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction manual. SEC-6 cells were cultured in LPS to induce cell injury and then were treated with 40, 80, and 160 mg/L SJZD. Then, the cells stained with Annexin V-fluorescein isothiocyanate (FITC) and Propidium Iodide (PI) were analyzed by cell sorting using a FACS Calibur (BD Biosciences). The data was analyzed further by FlowJo software.

Western blot assay

Total proteins were extracted using lysis buffer (25 mM Tris-HCl pH 7.4, 250 mM NaCl, 50 mM KCl, 10 % glycerol, and 0.5 % NP-40). The lysates were subjected to gel separation and transferred to nitrocellulose membrane followed by overnight incubation at 4 ℃ with the primary antibodies against Bax (ab8227, Abcam, MA, USA), Bcl-2 (ab196495, Abcam), PPARα (ab227074, Abcam) and β-actin (20536-1-AP, ProteinTech, IL, USA). After incubation with horseradish peroxidase-labeled goat anti-rabbit IgG (B900210, ProteinTech), The target bands were visualized with ECL reagents (Solarbio, Beijing, China), and the intensity of the bands was analyzed using ImageJ software, and normalized to β-actin [8].

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using RNeasy Kits (QIAGEN, Germany), according to the kit manufacturer's protocol. An amount (500 ng) of RNA was utilized for reverse transcription, using Universal RT-PCR Kit (Solarbio, Beijing, China). ABI 7500 Real-Time PCR System was used for qRT-PCR with 2 × SYBR Green PCR Mastermix (Solarbio). The copy number of target genes was calculated with $2^{-\Delta\Delta CT}$, and normalized to β-actin level. The PCR primers used are listed in Table 1.

Table 1: Primers used in PCR

Target	Sequence (5'-3')
TNF-α	CCCAGGCAGTCAGATCATCTTC AGCTGCCCCTCAGCTTGA
IL-6	GGTACATCCTCGACGGCATCT GTGCCTCTTTGCTGCTTTCAC
IL-1β	AACAGGCTGCTCTGGGATTC AGTCATCCTCATTGCCACTGT
β-actin	GTCTGCCTTGGTAGTGGATAATG TCGAGGACGCCCTATCATGG

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNF-α, IL-6 and IL-1β in cell medium were evaluated using ELISA kits of TNF-α (ab181421, Abcam), IL-6 (ab178013, Abcam) and IL-1β (ab214025, Abcam), respectively. A 100 μL sample was added into ELISA well and incubated for 2 h followed by 100 μL Conjugate solution for 1 h. Then each well was reacted with Substrate solution, and

terminated by stop solution. The OD_{450nm} value of each well was measured for analysis.

Statistical analysis

Data are presented as mean ± standard deviation (SD) of three replicates, and statistically significant differences between any two groups were calculated by unpaired t-test. Multiple group differences were determined using ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

SJZD enhances LPS-induced cell viability

To clarify the effect of SJZD on ulcerative colitis, the viability of SJZD treated IEC-6 cells was investigated. Firstly, 40, 80 and 160 mg/L SJZD were used to treat the IEC-6 cells, and the data showed that the viability of the SEC-6 cells correlated directly with the dose of SJZD (Figure 1 a); SJZD dose < 160 mg/L did not affect cell growth. Compared with the unmodified SEC-6 cells, LPS induction reduced cell viability, meaning that LPS induced cell damage on SEC-6, while 80 or 160 mg/L SJZD improved the cell viability of LPS-induced SEC-6 cells, despite the absence of any significant difference when treated with 40 mg/L SJZD (Figure 1 b). Therefore, SJZD improved the cell viability in ulcerative colitis.

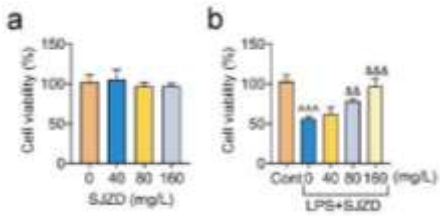

Figure 1: SJZD enhances LPS-induced cell viability. (a) The viability of SEC-6 cells was not affected by SJZD at doses of 40, 80 and 160 mg/L; (b) 80 or 160 mg/L SJZD improved cell viability of LPS-induced SEC-6 cells. Data from three repeated experiments were used for the statistical analysis. Error bar, mean ± SD; ^^^ compared to Cont, $^{\&\&/\&\&\&}$ compared to LPS group; $^{\&\&}p < 0.01$, $^{\wedge\wedge\wedge/\&\&\&}p < 0.001$

SJZD inhibited LPS-induced apoptosis

As shown in Figure 2 a, LPS-induced apoptosis was inhibited by SJZD in a dose-dependent manner. SJZD at doses ranging from 40 to 160 mg/L, significantly reduced cell apoptosis compared to the group without SJZD treatment. The results also showed that 80 and 160 mg/L SJZD lowered Bax level in LPS-induced SEC-6 cells, but increased Bcl-2 expression (Figure 2 b). Thus, apoptosis induced by LPS was suppressed by SJZD in ulcerative colitis mice.

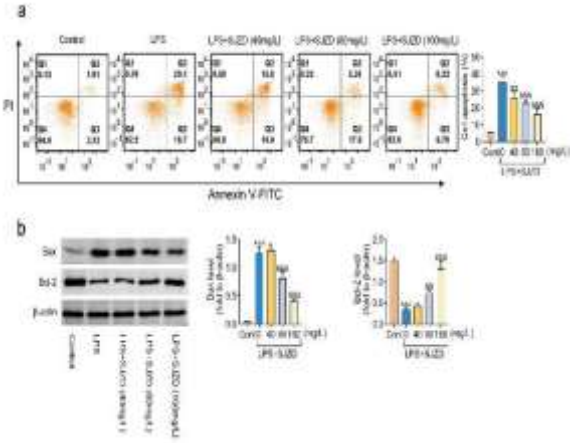

Figure 2: SJZD inhibits LPS-induced apoptosis**.** (a) LPS-induced apoptosis was inhibited by SJZD in a dose-dependent manner; (b) SJZD reduced Bax levels and enhanced Bcl-2 expression in LPS-induced SEC-6 cells. All experiments were repeated three times. Error bar, mean ± SD; ^^^ compared to Cont, $^{\&\&/\&\&\&}$ compared to LPS group; $^{\&\&}p < 0.01$, $^{\wedge\wedge\wedge/\&\&\&}p < 0.001$

SJZD inhibited LPS-induced inflammation

To investigate the effect of SJZD on inflammation, the level of TNF-α, IL-1β and IL-6 were measured [9]. SJZD doses of 80 or 160 mg/L lowered the concentrations of TNF-α, IL-1β and IL-6 in LPS-induced SEC-6 cells. LPS induction, however, increased the production of TNF-α, IL-1β and IL-6 compared to unmodified SEC-6 cells (control; Figure 3 a). The mRNA level in the LPS-modified group was elevated significantly, indicating that inflammation was induced in damaged cells induced by LPS. This inflammation was reduced by SJZD, as evidenced by the significant reduction in the mRNA levels of TNF-α, IL-1β and IL-6 in the groups given SJZD treatment (Figure 3 b). These results revealed that SJZD attenuated LPS-induced inflammation in ulcerative colitis.

SJZD activates PPARα to ameliorate ulcerative colitis

SJZD elevated the PPARα levels compared to LPS-modified group, while PPARα levels decreased to the same level as LPS-modified group after treatment with SJZD and GW6471 (Figure 4 a), indicating that GW6471 significantly inhibited PPARα production. 160 mg/L SJZD increased the cell viability lowered by SW6471 (Figure 4 b). Moreover, SJZD at a dose of 160 mg/L decreased the production of IL-6, TNF- α and IL-1β, while GW6471 upregulated their levels (Figure 4 c). Thus, PPARα was

responsible for SJZD effect on cell viability, and repression of apoptosis and inflammation in ulcerative colitis.

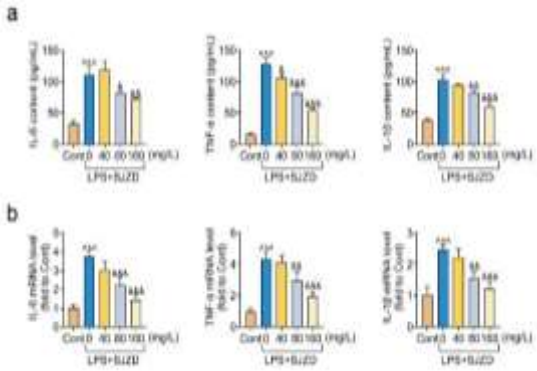

Figure 3: SJZD inhibits LPS-induced inflammation**.** (a) Levels of TNF-α, IL-1β and IL-6; (b) mRNA levels of TNF-α, IL-1β and IL-6; ^^^ compared to Cont, $^{\&/\&\&/\&\&\&}$ compared to LPS group; $^{\&}$ $p < 0.05$, $^{\&\&}$ $p < 0.01$, $^{\wedge\wedge\wedge/\&\&\&}$ $p < 0.001$

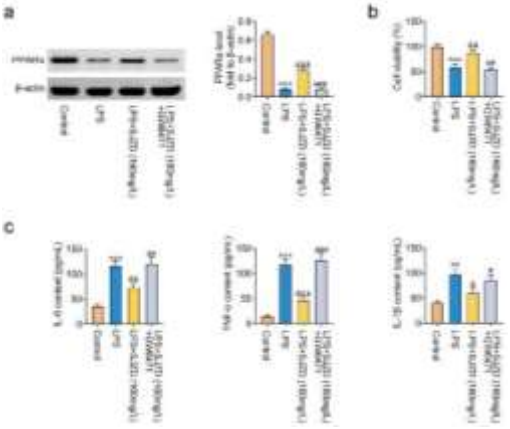

Figure 4: SJZD activates PPARα to improve ulcerative colitis. (a) PPARα expressions; (b) SJZD increase cell viability, but reduced by SW647; (c) SJZD decreased the production of IL-6, TNF- α and IL-1β, but upregulated by GW6471. ^^/^^^ compared to control, $^{\&/\&\&/\&\&\&}$ compared to LPS; $^{\#/\#\#/\#\#\#}$compared to LPS+SJZD; $^{\&/\#}p$ <0.05, $^{\wedge\wedge/\&\&/\#\#}p < 0.01$, $^{\wedge\wedge\wedge/\&\&\&/\#\#\#}$ $p < 0.001$

DISCUSSION

Ulcerative colitis is a chronic inflammatory bowel disease that injures the colon and rectum, and it seriously affects public health. SJZD is used to treat gastrointestinal diseases. For instance, it aids improvement in DSS-induced inflammation in rats with UC [5]. In this study, the ability of SJZD to relieve ulcerative colitis was determined, and LPS-induced SEC-6 cells were used as the *in vitro* model for UC, as the significant reduction in cell viability increased apoptosis and inflammation in LPS treated cells. Similar models have been widely used in numerous studies [6,10-12].

To further elucidate the mechanism of action of SJZD on cell viability of SEC-6 cells, apoptosis was determined. It has been reported that SJZD protects against cytotoxicity and apoptosis by suppressing caspase-3 activity, as well as also regulating the ratio of Bcl-2/Bax [13]. Therefore, it makes sense that SJZD attenuates apoptosis and strengthens the viability of injured intestinal epithelial cells.

Ulcerative colitis, known as inflammatory bowel disease, generally causes chronic inflammation and the formation of ulcers within the mucosal layer of the colon (large intestine) and rectum. These inflammation processes and ulcers contribute to the manifestation of symptoms such as abdominal discomfort, diarrhea, rectal bleeding, and unintended weight loss [14]. While the precise etiology of ulcerative colitis remains elusive, it is hypothesized to encompass an abnormal immune response to bacteria or other substances in the digestive tract. Genetic factors may also play a role. Treatment for ulcerative colitis usually involves medication to reduce inflammation and control symptoms. Treatments include aminosalicylates, corticosteroids, immunomodulators, and biologicals [14,15]. Dysregulated inflammatory responses facilitate the initiation, development, and progression of ulcerative colitis, and increase risk of colon carcinoma [16]. It is therefore necessary to investigate the inflammation response in LPS induced injury. Consistent with the findings above, the related data in this work showed the inhibitory role of SJZD on inflammation.

PPARs exert key functions in regulating the genes associated with both metabolism and inflammation. PPARs are involved in lipid metabolism, including the breakdown of fatty acids in peroxisomes. PPARs also regulates fatty acid metabolism, inflammation, and cell proliferation [2]. PPARs are targets for several medications used in the treatment of metabolic disorders such as type 2 diabetes and dyslipidemia, as well as for anti-inflammatory agents [3]. PPARs play a vital role in ulcerative colitis, and it has been reported that yarrow oil alleviates ulcerative colitis by regulating PPAR-γ pathway [17]. Given the inhibitory role of SJZD on inflammation, the effect of SJZD on PPARs was investigated in this study, and the results revealed that PPARα was the key molecule in SJZD responsible for enhancing cell viability, and repressing apoptosis and inflammation in ulcerative colitis.

Treatment for UC may include medications to reduce inflammation, control symptoms, and prevent complications [14]. However, the need to resolve clinical issues such as drug resistance, intolerance and loss of responsiveness has also emerged. Therefore, finding a suitable natural agent is crucial for the treatment of UC [1]. In this study, SJZD was proven to weaken inflammation and alleviate cell damage in LPS-induced UC model. More work, however, is required to determine the potential of SJZD for use in clinical practice.

CONCLUSION

This study has demonstrated that SJZD alleviates cell damage, inhibits cell apoptosis and enhances the viability of SEC-6 cells. It also attenuates inflammation by inhibition of AKT/NF-κB signaling pathway. SJZD is thus a potential therapeutic candidate for the treatment of ulcerative colitis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Guobao Zhang and Wei Li designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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