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Original Article

Relevant influential factors of sanpi decoction in an ulcerative colitis rat model

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Abstract: Objective: To discuss the anti-inflammatory effect effect of sanpi decoction in ulcerative colitis (UC) mice and its likely therapeutic mechanism. Method: Forty BALB/C mice were divided randomly and equally into normal group, model group, sulfasalazine (SASP) group, and sanpi decoction group, ten mice in each group. After set up the oxazolone colitis, SASP group and sanpi decoction group were treated with sulfasalazine and sanpi decoction respectively, to observe general state of health every day. Killed on the tenth day, separated the spleen and weight it, got the colon and evaluated the histological changes of the damaged colonic tissues, and measured the interleukin IFN-γ and IL-4 concentrations in serum and spleen, Tumor necrosis factor α (TNF-α) in colon supernate by using the ELISA. Expression of TNF-α mRNA in colonic mucosa was detected by real-time fluorescent quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Results: The symptom of sanpi decoction group and SASP group mice had great amelioration compared to model group mice, the immune organ spleen of the model group mice was conspicuous shrunk, and compared to model group, the weight of spleen in sanpi decoction group and SASP group was increased, the concentration of IFN-γ and the colon mucosal TNF-α in sanpi decoction group was lower than in model group ($P<0.01$), and the concentration of IL-4 was higher than in model group ($P<0.01$). Conclusion: Sanpi decoction can certainly treat the UC and decrease the concentration of IFN-γand heighten the IL-4 concentration, reduce the expression of TNF-α in local colon mucosa, to regulate the balance of the cytokines, to relieve the inflammation in the colon.

Keywords: Sanpi decoction, ulcerative Colitis, cytokines, oxazolone

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) that is characterized by chronic inflammation of the colon, and its etiology remains unclear. In recent years, studies have demonstrated that immune responses have been implicated in the fundamental pathogenesis of UC, the effect of cytokines have significantly influenced the etiology and pathogenesis of UC, and the imbalance between Th1 and Th2 has been considered an important mechanism [1] of IBD. Professor Li Jiabang has summarized that Sanpi decoction is an effective prescription for the long term treatment of UC in clinical practice, which has been proven as markedly more superior to Salazosulfapyridine (SASP) [2]. In this study, oxazolone was applied to establish a UC rat model to observe the influence of Sanpi decoction on interferon-g (IFN-g) [3], interleukin-4 (IL-4) [4] and local TNF-α [5] changes in rat colonic mucosa; exploring the effects and possible mechanisms of Sanpi decoction in the treatment of UC in mice [6].

Materials and methods

Experimental animals and reagents

Forty 6-8 week male SPF grade BALB/c mice weighing 20-25 g were provided by the Animal Experimental Center of Central South University. Oxazolone was purchased from Sigma Company; Sanpi decoction, which comprised of pomegranate bark, ash bark, cortex ailanthi, honeysuckle stem and other traditional Chinese medicines, were provided by the Pharmacy Department of Xiangya Hospital, Central South Uni-

Table 1. Disease activity index score standard

Body weight reduction (%)	Stool Property	Hematochezia	Fraction
None	Normal	Occult blood (-)	1
1-5	Loose	Occult blood (+)	2
5-10	-	-	3
10-15	Loose stools	Bloody Stool detected by visual observation	4
>15	-	-	5

versity; and Salazosulfapyridine was provided by Shanghai Sunve Pharmaceutical Co., Ltd. IFN-g, IL-4 and TNF-α ELISA reagent kits were purchased from Wuhan Boster Biological Technology Co., Ltd. TRIzol (Gibco BRL, NY, USA), Rnasin and M-MLV were obtained from (Promega), while Tag Enzyme was obtained from Shanghai Sangon Company. Primers were synthesized by Shanghai Sangon Company. DNA marker (SD002) was purchased from Beijing Dingguo BioTech Co., Ltd., China; CO_2 Incubator was purchased from Queue System, PCR Instrument (PE480) was purchased from PerkinElmer (USA); UV Spectrophotometer (DU530) was purchased from Beckman (USA); and Eagle Eye 11 image analysis system was purchased from the USA.

Methodology

Establishment of an ulcerative colitis rat model

The UC rat model was established according to the method developed by Heller *et al* [7]. Mice are transiently anaesthetized with ether, a 2 × 2 cm area of fur on the abdomen was removed, and the area was smeared with 200 mL of 3% (w/v) oxazolone in 100% ethanol solution, which was repeated once the next day. After five days, a fine catheter was inserted into the anus at a depth of 4 cm, and 150 mL of oxazolone solution (1% of oxazolone dissolved in 50% ethanol) was slowly injected into the anus. Mice were lifted upside down by the tail for 30 seconds in order to ensure that the injected oxazolone solution would diffuse within the large intestine after injection. On the next day, five mice were randomly selected and sacrificed. Then, colon lesions were fixed with 10% formaldehyde, embedded in paraffin, sliced, and stained with hematoxylin and eosin for histopathology examination. The spleen was cut off and weighed.

Animal grouping and drug administration

Forty animals were randomly divided into four groups: normal group, model group, Sanpi decoction group, and SASP group (n=10, each group). Mice in the normal group were administered with 50% ethanol solution for enema without drug administration. Three days after the model was successfully established, mice in each group were administered with the drug at a dose of 50 units per kg of body weight, daily. The water was boiled twice and condensed corresponding to 1 g/ml of crude drug of Sanpi Decoction, and mice were gavaged daily at 6.5 g/kg. Western medicine SASP was grinded into powder, prepared with CMC into a suspension with a concentration of 20 mg/mL, and mice were gavaged with 0.3 g/kg doses, once daily. Mice in all groups were gavaged for a period 21 days.

Disease activity index (DAI) score

After gavage, body weight of the mice was observed daily, as well as stool property and hematochezia; and DAI score [8] was obtained according to **Table 1**. DAI = (body weight decrease score + stool property score + Hematochezia score + stool occult blood examination)/3. Normal stool was dry with particle shapes, semi-loose stool was paste-like and did not adhere to the anus, and loose stool was liquid-like and adhered to the anus. Benzidine was used to detect stool occult blood [9].

Histology damage score

After refining according to Ekstrom [5] score criteria, histology damage scores were obtained for the colon lesions, as shown in **Table 2**. All scores were added together to arrive at the total score.

Detection of cytokines IFN-g and IL-4 in serum

Blood was obtained from mice by the eyeball method, deposited for two hours at room temperature, centrifuged at 2,000 rpm for 20 minutes at 4°C, and the supernatant was obtained and preserved in a refrigerator at -20°C. Spleen tissues were taken from mice and weigh, shred-

Table 2. Ulcerative colitis histology damage score standard

Items	0	1	2	3
Epithelium damages and ulceration formation	None	Erosion	Ulceration	-
Depth of Ulceration	-	Submucosa	Muscular layer	Placenta percreta
Edema	None	Mild	Moderate	Severe
Lymphocyte, monocyte, and plasmocyte infiltration	None	Mild	Moderate	Severe
Depth of Infiltration		Submucosa	Muscular layer	Placenta percreta
Neutrophil granulocyte infiltration	None	Mild	Moderate	Severe
Depth of Infiltration		Submucosa	Muscular layer	Placenta percreta
Eosinophilic granulocyte infiltration	None	Mild	Moderate	Severe
Depth of Infiltration		Submucosa	Muscular layer	Placenta percreta

Table 3. Disease activity index scores of mice in different time segments in various groups ($\bar{x} \pm SD$)

Groups	*n*	Day 1	Day 4	Day 10	Histology Scores
Normal group	10	0.26 ± 0.43▲	0.13 ± 0.18▲	0▲	0.8 ± 0.45▲
Model group	10	2.20 ± 0.84	2.40 ± 0.64	1.20 ± 0.18	10.8 ± 1.48
SASP group	10	1.93 ± 0.83	1.00 ± 0.33▲	0.40 ± 0.28▲	5.4 ± 0.55▲
Sanpi decoction group	10	2.00 ± 0.41	0.87 ± 0.18▲	0.33 ± 0.24▲	4.2 ± 0.84▲

Compared with the model group, ▲P<0.01.

ded, fixed with normal saline, prepared into a 20% homogenate with glass homogenizer, centrifuged at 2,000 rpm for 15 minutes at 4°C, and the supernatant was obtained and preserved in a refrigerator at -20°C.

An ELISA Reagent Kit was used to determine serum IFN-g and IL-4 content, according to manufacturer's instructions [11]. A microplate reader was used to detect the absorbance of the sample at 450 nm, standard curves were drawn according to the absorbance of samples in different concentrations, and the corresponding concentrations of the standard curves were analyzed by the absorbance of the samples [12].

Colon mucosa tissue preservation and supernatant preparation, and TNF-α Detection by ELISA

The colon section was separated and cut off 4-7 cm away from the anus. The colon section was vertically cut off into two parts, rinsed with iced normal saline, and weighed with an electronic balance. Iced PBS buffer (pH 7.4) was applied on one portion of the colon to homogenize (0.1 g/ml, 2,000 r/min, 10 seconds), centrifuged at 4°C (3,500 r/min, 10 minutes), separated the supernatant, and preserved the supernatant for later use at -20°C. TNF-α was detected according to test kit instructions and was analyzed with the method mentioned above.

Real-Time colon mucosa tissue fluorescent quantitative reverse transcription polymerase chain reaction (RT-PCR) to detect TNF-α

Total RNA was isolated from intestinal tissues of mice using TRIzol reagent according to manufacturer's instructions. The RNA deposit obtained was dissolved in 20 mL DEPC water; then, 2 mL of the RNA sample was obtained, added into 1% agarose gel electrophoresis, and a UV spectrophotometer was used to determine RNA concentration and purity. Then, 2 μg of RNA was obtained, reverse transcribed into cDNA by a reverse transcription reagent kit, and preserve at -70°C for use. Primer design was referred to GenBank sequences, Primer Express 2.0 software was used for the design, and the determined sequences were synthesized by Shanghai Bio-engineering Technology Service Co., Ltd.; in which 5 mL of reverse transcription products were applied for PCR amplification reaction with β-actin as the internal control. The 5 mL PCR product underwent 1.5% agarose electrophoresis and dyed with ethidium bromide. Then, the optical density scanning value of the target electrophoresis bands were read with the image analysis software.

Table 4. Comparison of serum IFN-g, IL-4 and TNF-α content in colon supernatants and TNF-α mRNA expressions in various groups ($\bar{x} \pm$ SD)

Groups	*n*	IFN-g (pg/ml)	IL-4 (pg/ml)	TNF-αpg/ml	TNF-αmRNA (OD value)
Normal group	10	85.87 ± 13.38▲	219.35 ± 44.45▲	40.65 ± 5.98▲	0.582 ± 0.074▲
Model group	10	329 ± 63.83	93.65 ± 18.44	57.14 ± 10.42	0.791 ± 0.065
SASP group	10	176.79 ± 27.50▲	154.85 ± 32.26▲	48.56 ± 5.89	0.694 ± 0.087
Sanpi decoction group	10	189.68 ± 33.78▲	155.45 ± 30.15▲	44.77 ± 6.35△	0.687 ± 0.077△

Compared with Model group, ▲P<0.01, △P<0.05; OD, optical density.

Table 5. Spleen tissue weight and comparison of spleen tissue IFN-g and IL-4 content ($\bar{x} \pm$ SD)

Groups	N	Spleen tissue weight (g)	IFN-g (pg/ml)	IL-4 (pg/ml)
Normal group	10	0.1304 ± 0.0039▲	85.87 ± 13.38▲	209.35 ± 48.45▲
Model group	10	0.0586 ± 0.0065	267.18 ± 33.83	88.65 ± 20.04
SASP group	10	0.0872 ± 0.0165▲	156.79 ± 27.50	137.85 ± 58.26▲
Sanpi decoction group	10	0.098 ± 0.0085▲	179.68 ± 33.78▲	138.45 ± 66.15▲

Compared with model group, ▲P<0.01.

The ratio between the target band values was applied, and the β-actin band value was used as the expression volume for the target gene mRNA (Target gene mRNA expression volume = Target Band OD value/β-actin band OD value).

Statistical analysis

All data were analyzed by SPSS 10.0 statistical software. Data are expressed as M ± SD between different data groups. SNK-q test and Dunnett's test was used for comparison. P< 0.05 was considered statistically significant.

Results

General conditions and DAI score

Mice in the normal group are generally normal after they woke up from gavage, while mice in other groups presented with reduced body weight, diarrhea, drowsiness, hogback and anorexia. Mice in the model group all had loose stools, and bloody stools were detected in five mice by visual observation. There were significant differences in DAI scores between mice in the model and normal groups (P<0.01). Symptoms of mice in the Sanpi decoction and SASP groups decreased from day four with a gradual increase in body weight. There were significant differences in DAI score between the Sanpi decoction and SASP groups and the model group (P<0.01). On day 10 of treatment, mice in both treatment groups (Sanpi decoction and SASP groups) exhibited marked improvements in symptoms and improved spirit. Furthermore, mice were observed to have an increased appetite, no mucous bloody stools were detected, and occasional loose stools were observed. In addition, symptoms of mice in the Sanpi decoction group significantly improved compared with the SASP group, and there was no significant difference in DAI scores between these two groups (P>0.05). DAI scores of mice in various groups in different time segments are shown in **Table 3**.

Colon histopathology

General morphology of the colon by visual observation: Obvious congestion and edema could be observed in the intestinal wall of mice in model group. When the intestine was cut off along the mesentery, scattered ulcers and erosions on the mucosa of the intestinal wall were detected. Lesions were mainly located in the distal colon, in which lesions in the rectum were the most severe. Lesions in the intestinal mucosa of mice in the Sanpi decoction and SASP groups markedly improved compared with mice in the model group.

Pathological morphology under a light microscope: Erosion and ulceration in the intestinal mucosa of mice in the model group were detected. Lesions were limited to the mucosal and submucosal layer, which are infiltrated with large amounts of neutrophil granulocytes and

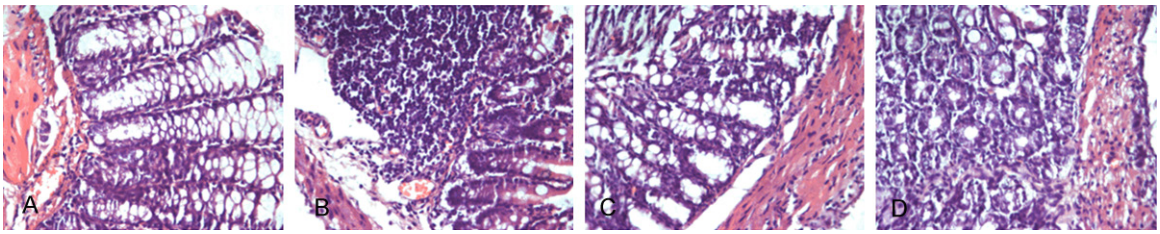

Figure 1. Histopathology slides of colons in various groups (Hematoxylin and eosin staining, 40X): (A) Normal group, (B) Model group, (C) Sanpi decoction group, (D) SASP group.

lymphocytes. Histology scores of mice in the model group were markedly higher than mice in the normal, Sanpi decoction and SASP groups; and there was a statistically significant difference ($P<0.01$). Colon lesions of mice in the Sanpi decoction group markedly improved, in which there was no mucosa and intestinal mucosal ulceration and only a small amount of inflammatory cell infiltration occurred. Erosion and ulceration in the intestinal mucosa of mice in the SASP group were not detected. However, a small amount of inflammatory cell infiltration could be detected. There was no statistically significant difference between both treatment groups (Sanpi decoction and SASP groups). Histopathology scores of colon lesions of mice in various groups are shown in **Table 3**, and histopathology slides are shown in **Figure 1**.

Discussion

UC is a form of inflammatory bowel disease (IBD), whose etiology and pathogenesis remains unclear. At present, it is generally believed that [13] the pathogenesis of this disease is mainly associated with genetic factors, which initiate intestinal immunity and non-immunity systems through infectious and environmental factors [14]. This causes the intestinal mucosa to be highly sensitive to antigens and disrupt its immunity adjustment function, resulting in the occurrence of intestinal mucosa inflammation and tissue damage. Furthermore, system imbalance between cytokines Th1 and Th2 is considered as an important pathogenesis mechanism [15] of UC. When UC occurs, the disruption of the intestinal mucosa is accompanied by the disruption of the systemic immunity function [16]. This promotes the upper regulation of inflammation promoting cytokines such as IFN-g and IL-2, which induces and promotes the occurrence of inflammation; while anti-inflammatory cytokine IL-4 [17] is downregulated, expressing as an inhibition of Th2 [18]. Therefore, UC is mainly an immunity suppressive disease of Th2, in which the imbalance in the expression and secretion between cytokines Th1 and Th2 are positively associated with the degree of inflammation of UC diseases [19, 20].

In this study, oxazolone was applied to successfully establish an UC rat model to evaluate the weight of the spleen tissue, histologically observe colon lesions, as well as to detect serum and spleen tissue cytokine IFN-g and IL-4 content, and local colon mucosa TNF-α content and mRNA expressions [21]. Simultaneously, the symptoms of mice in the Sanpi decoction and SASP groups markedly improved after treatment for 21 days, compared with the model group. Serum cytokine IFN-g and IL-4 content of mice in the SASP group were better than mice in the Sanpi decoction group, while the TNF-α content of mice in the Sanpi decoction group was superior to that of mice in the SASP group. However, there were no significant differences between these two groups. Results suggest that both Sanpi decoction and SASP treatments improved the symptoms of mice in the model group established by oxazolone [22].

At present, the clinical treatment of UC is mainly dominated by aminosalicylic acid, corticosteroids, etc. However, the application of these types of drugs both induce the problems such as relapse after suspension, multiple side effects for long term medication, and poor tolerance by patients. Furthermore, in UC treatment, these drugs are insufficient for adjusting the complicated imbalance of the immune system mediated by Th1/Th2, which is the main pathogenesis. However, traditional Chinese medicines are composed of multiple ingredients that have been proven to provide excellent adjustment functions in immunoregulation st-

udies. In the current study, Sanpi decoction has been shown to be an effective formula prescription summarized by Professor Li Jiabang at our department for the long term clinical treatment of UC. This formula includes pomegranate bark, cortex ailanthi, ash bark, and honeysuckle stem. In the formula, pomegranate bark, cortex ailanthi, and ash bark can be applied for heat-clearing and damp-drying, relieving toxicity and transforming turbid, and stopping bleeding and diarrhea. The addition of the honeysuckle stem makes the treatment pungent-cool and detoxifying, and helps Sanpi to clear damp-heat toxins, which are accompanied by medicines for conducting Qi and removing stagnancy to remove heaviness afterwards, "Regulate the Qi and heaviness afterwards are removed"; and the combined efficacy of many medicines achieves the purpose of heat-clearing and damp-drying, cooling the blood and detoxification, and conducting Qi and stopping dysentery. These are suitable for conditions of damp-heat retention in the interior during the UC activation period. Modern pharmacological research has demonstrated that the pomegranate bark, cortex ailanthi, and ash bark could be combined together to inhibit and adjust intestinal flora. Furthermore, pomegranate bark contains large amounts of tannin that could precipitate or solidify local protein when it goes in contact with the mucosa wound, forming a denser protection layer conducive for healing the surface of the ulceration [23]. These results have demonstrated that the UC rat model induced by oxazolone was mainly Th2 type inflammation mediated by IL-4, which was predominant as IL-4 levels were reduced. Sanpi decoction could improve local abnormal TNF-α expressions in the colon mucosa, recover the balance between Th1 and Th2, and relieve intestinal inflammation reaction through the marked increase in IL-4 levels and reduction of IFN-g levels, thereby achieving the purpose of the treatment [24].

Disclosure of conflict of interest

None.

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