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Influences of probiotics combined with sulfasalazine on rats with ulcerative colitis *via* the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: To investigate the influences of probiotics combined with sulfasalazine (SASP) on the expression of the Wnt/β-catenin signaling pathway in rats with ulcerative colitis (UC).

MATERIALS AND METHODS: A total of 60 clean level and healthy Sprague-Dawley (SD) rats were randomly divided into the normal group, model group, SASP group and combination therapy group, with 15 rats in each group. The rats in the normal group were given normal feeding, and those in the remaining three groups were subjected to the establishment of the UC model. During the modeling, the rats underwent daily gavage and were sacrificed after 4 weeks. Clinical symptoms and pathological changes in ulcer indexes and colon tissues were observed in each group. The expression levels of relative genes in the Wnt/β-catenin signaling pathway were detected by Polymerase Chain Reaction (PCR).

RESULTS: Compared with those in the model group, the pathological sections of rats in the SASP group and combination therapy group showed significant improvement in the inflammatory response. The expression levels of relative genes in the Wnt/β-catenin signaling pathway were downregulated in rats of SASP group and combination therapy group relative to those in the model group.

CONCLUSIONS: SASP and probiotics alleviate UC by reducing inflammation by inhibiting the activation of the Wnt/β-catenin signaling pathway, thereby improving intestinal function and restoring the intestinal structure.

Key Words:
Probiotics, Sulfasalazine, Ulcerative colitis, Wnt/β-catenin signaling pathway.

Introduction

As the two main types of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are mainly characterized by chronic non-specific inflammation of the colon. The incidence rate of UC is higher than that of CD, and has been on the rise every year. The incidence rate of UC in Europe and the United States is 24.3/100,000, and that in Guangzhou is 2.22/100,000, which is the highest in China[1,2]. As a common digestive system disease, UC is usually recurrent and persistent, manifested as abdominal pain, diarrhea and bloody or purulent feces, and has an adverse effect on the life quality of affected people. Repeated medical treatment also leads to high costs[3-5]. Intestinal immune dysfunction is considered to be the biological mechanism of UC development. In UC patients, a large number of immune cells (T cells, B cells, macrophages and dendritic cells) and cytokines [pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-β, interleukin (IL)-6, IL-12, IL-17, IL-21, IL-23 and integral proteins and anti-inflammatory cytokines

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such as IL-10, transforming growth factor (TGF)-β and IL-35] are abnormally expressed in the colon[6]. The imbalance of cytokines regulated by activated immune cells is an initial factor in causing diffuse superficial inflammatory lesions in UC[7]. The regulation of immune cell activity and cytokine expression is beneficial for the healing of the lining of the colon and the relief of inflammation in UC patients[8]. Several signal transduction cascades and transcription factors such as nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), Janus kinase/signal transducers and activators of transcription (JAK-STAT) and the Keap1/Nrf2 signaling pathway are involved in pathological inflammatory processes[9]. At present, more and more studies have focused on the role of the Wnt/β-catenin signaling pathway in the inflammation-related pathogenesis and inhibition of pro-inflammatory signaling pathways. In this work, the UC model in rats was established to observe the therapeutic effect of probiotics on UC rats and expressions of the Wnt/β-catenin signaling pathway in colon tissues. We aim to explore the possible mechanism of probiotics for UC treatment, thus providing a theoretical basis for the clinical treatment of UC by probiotics.

Materials and Methods

Laboratory Animals

60 male Sprague-Dawley (SD) rats weighing 180-250 g were purchased from the Shanghai Experimental Animal Center (Shanghai, China). All animals were treated according to the guidelines approved by the Animal Care and Use Committee of our hospital. This study was approved by the Animal Ethics Committee of the Shanxi Provincial People's Hospital Animal Center.

Animal Models

Animal Grouping

A total of 45/60 rats were randomly selected to prepare into rat models of UC. (1) Rats were fasted but given free access to water. After 24 h, rats were intraperitoneally anesthetized with 10% chloral hydrate. (2) A polypropylene tube (2 mm in diameter) was inserted through the anus to a depth of about 8 cm. Trinitro-benzene-sulfonic acid (TNBS, 100 mg·kg^{-1}) was slowly injected into the rat intestine. (3) Rat anus was clenched for 10 min and the solution was retained. A total of 45 UC rats were randomly divided into model group, sulfasalazine (SASP) group and SASP combined with probiotics *Saccharomyces boulardii* (SB) group (combination therapy group), with 15 rats in each group. 15 rats not treated formed the control group. The rats in the control and model group were given normal saline for gavage, while those in the SASP group received gavage with SASP tablets, which were mashed, ground into powder and mixed with normal saline, forming the mixed solution (0.3 g·kg^{-1}). Rats in the combination therapy group were given probiotics and SASP for gavage. The gavage for each group of rats lasted for 4 weeks, once in the morning and once in the evening. Finally, rats were euthanized with a volume fraction of 10% chloral hydrate.

Assessment of the Macroscopic Damage to the Colon

An 8 cm-long colon at 3 cm proximal to the anus was cut, opened longitudinally and washed with saline buffer. After weighing of the tissue samples, tissue damage visible to naked eyes was recorded. The criteria for macro assessment depended on a previously validated scoring system (0-4): 0 point = no ulcer; 1 point = only mucosal erythema; 2 points = mild mucosal edema, minor bleeding or mild erosion; 3 points = moderate edema, ulcer bleeding or erosion and 4 points = severe ulcer, erosion, edema and tissue necrosis. 3M® scaled surgical scotch tape was used to measure the ulcer area and attached to a light transparent sheet. Each cell area on the tape was 1 mm^2, and the number of cells covering the ulcer area of each sample was counted. The ulcer index was measured by adding the ulcer score to the ulcer area of each tissue sample according to the following formula: UI = UN + US + UA ×10 - 1, where UI = ulcer index, UN = ulcer number, US = ulcer score, and UA = ulcer area.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The extraction of the total ribonucleic acid (RNA) was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Cells treated with different methods were collected, and the total RNA was extracted according to the instructions. 1 μL of RNA solution was taken and placed on a microplate reader to measure the concentration and purity of the total RNA. The

ratio of the optical density at 260 nm to that at 280 nm (OD_{260}/OD_{280}) between 1.6 and 1.8 suggested qualified RNA samples. The remaining RNA solution was subpackaged and stored at -80°C for later use.

The complementary deoxyribose nucleic acid (cDNA) was synthesized using PrimeScript™ Kit (TaKaRa, Otsu, Shiga, Japan). Specific operating procedures were based on the instructions. First, 2 μg of total RNAs were added. 1 μL of Oligo (dT) primer (50 μM) and 1 μL of dNTP mixture (10 mM) were added, and the enzyme-free double distilled water was added to the 10 μL systems. They were gently mixed, heated at 65°C for 5 min in the PCR machine, and transferred onto the ice for rapid cooling. The above reaction system was added with 4 μL 5× PrimeScript Buffer, 1 μL PrimeScript RTase, 0.5 μL Rnase inhibitor and 4.5 μL enzyme-free water (a 20 μL reaction system). Subsequently, they were evenly mixed and heated in the PCR machine at 42°C for 45 min and 95°C for 5 min. Then, the system was transferred onto the ice and rapidly cooled, to synthesize the single-stranded cDNA. Finally, it was placed at -20°C for PCR amplification reaction. Primer sequences used in this study were as follows: TCF4, F: 5'-ACCAGGACGGCTCCTTACAT-3', R: 5'-CGATGTGTAAGAAGTAGGGA-3'; cyclinD1, F: 5'-GCCTAGGTACCATCCTCGACTG-3', R: 5'-GGCTGCGTGTCGTCCAGTCG-3'; c-jun, F: 5'-GTTCAGCGGCATTTGGAGGA-3', R: 5'-AATCCCGAATAGCGACAGTTCT-3'. CD44: F: 5'-CTGGCGGCAGCACATATACTACCAT-3', R: 5'-CACGGTCAGAATTTGCTAGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA). The difference between the two groups was detected *via* the *t*-test, and the difference among multiple groups was tested by the one-way analysis of variance, followed by Post-Hoc Test (Least Significant Difference). A two-sided 95% confidence interval (CI) was used in all tests, and $p<0.05$ represented that the difference was statistically significant.

Results

Influences on Clinical Symptoms of UC General Condition

Rats in the normal group were vigorous with the normal diet. The bowel movement was normal and the anus was ruddy. Rat body weight increased steadily. In contrast, rats in the model group were weak and irritated, with reduced food intake. Soft feces or stools sometimes accompanied by blood or pus, staining, and slower weight gain, were observed in UC rats ($p<0.01$). Compared with those in the model group, stool pattern and mental state were improved, and the weight gain was significant in the SASP group and combination therapy group ($p<0.05$) (Figure 1).

Colonic Injury Score and Morphological Changes

Compared with those in the normal group, rat colon was harder and deeper, manifesting thickening colon wall, smooth lining significant congestion and scattered ulcer in the model group. Injury score was markedly increased in the model group ($p<0.01$). Compared with those in the model group, the colonic injury was notably improved, the colon was soft pink, the intestinal wall was thickened and the colon wall was smooth and intact in the SASP group and combination therapy group. There was no

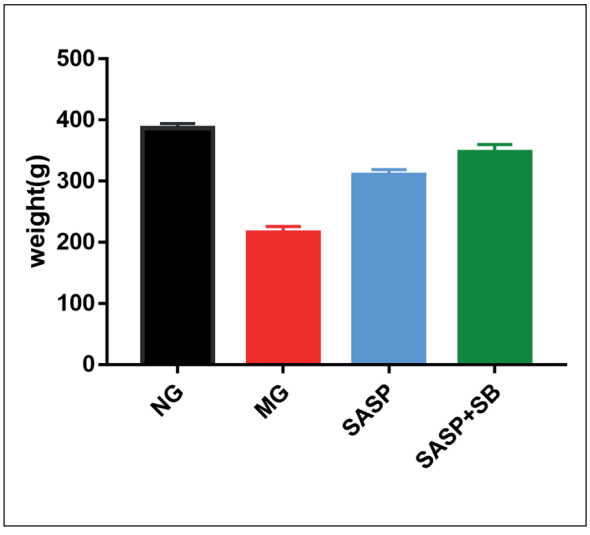

Figure 1. Change in body weight of rats in each group: NG (I): normal group, MG (II): model group, SASP (III): SASP group and SASP + SB (IV): combination therapy group. Data are expressed as mean ± SD, and there are 15 rats in each group. $p<0.01$ *vs.* normal group, and $p<0.05$ *vs.* model group.

significant ulceration, and the total injury score was evidently reduced (especially in combination therapy group) in the SASP group and combination therapy group ($p<0.05$) (Figure 2A).

Compared with those in the normal group, the histopathological score of the colonic injury was markedly higher in the model group ($p<0.01$). The colon lining was broken, the colon gland was necrotic or missing, the crypt was disordered, part of the colon gland was atrophied, and the lamina was infiltrated in the model group. The innate tissue and submucosa were composed of many inflammatory cells, such as neutrophils, eosinophils and monocytes. Compared with those in the model group, the colonic injury was remarkably improved, manifesting a substantially intact lining with no significant ulcers, but a well-structured colon gland grew up, accompanied by inflammatory polyps. The inflammation of the lamina propria and submucosa was markedly relieved (Figure 3), and histopathological scores were markedly reduced in the SASP group and combination therapy group ($p<0.01$) (Figure 2B).

Anti-Ulcer Ability

The average ulcer index of the model group was (702±8.66) mm^2, and the administration of SASP [(156.19±23.88) mm^2] or probiotics combined with SASP [(125.84±11.62) mm^2] could lower this index ($p<0.001$ *vs.* model group) (Figure 4).

A severe inflammatory response occurred in the model group (3±0.3), which was reduced after administration with SASP (1.65±0.1) or probiotics combined with SASP (1.31±0.1) ($p<0.05$) (Figure 5).

KEGG Pathway Analysis

Compared with those in the model control group and SASP group, differentially expressed cytokines regulated by probiotics combined with SASP were involved in 28 signaling pathways. The JAK/STAT signaling pathway, immunoglobulin A network, MAPK signaling pathway, immune rejection, TLR signaling pathway, chemokine signaling pathway, apoptosis, intercellular adhesion, graft *vs.* host response, T cell receptor pathway and the Wnt/β-catenin signaling pathway were included. Further bioinformatics analysis revealed that the difference in the Wnt/β-catenin signaling pathway was the most significant (Figure 6), which was further verified by experiments.

Influences of Probiotics Combined With SASP on the Wnt/β-Catenin Signaling Pathway

Influences of probiotics combined with SASP on the Wnt/β-catenin signaling pathway in UC were verified. The results demonstrated that the Wnt/β-catenin signaling pathway was activated in the model group, while probiotics and SASP were capable of down-regulating the expressions of related molecules, thereby inhibiting this abnormal activation. The inhibitory effect was more pronounced in the combination therapy group ($p<0.05$) (Figure 7).

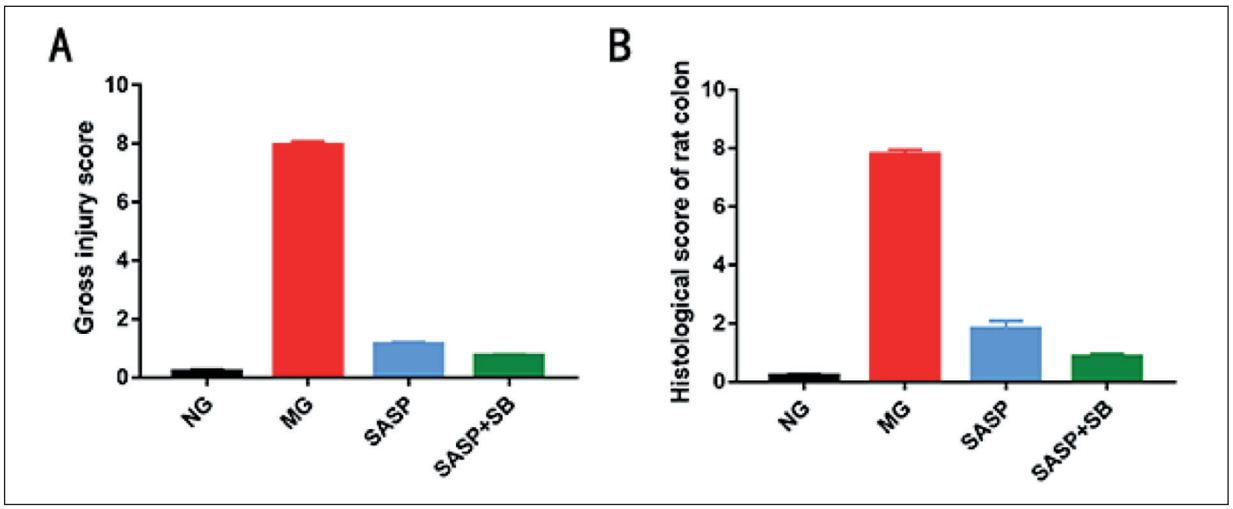

Figure 2. Histopathological observation and injury scores of colon tissues in each group. ***A,*** Total injury score of colon in each group. ***B,*** Histological score of colon in each group.

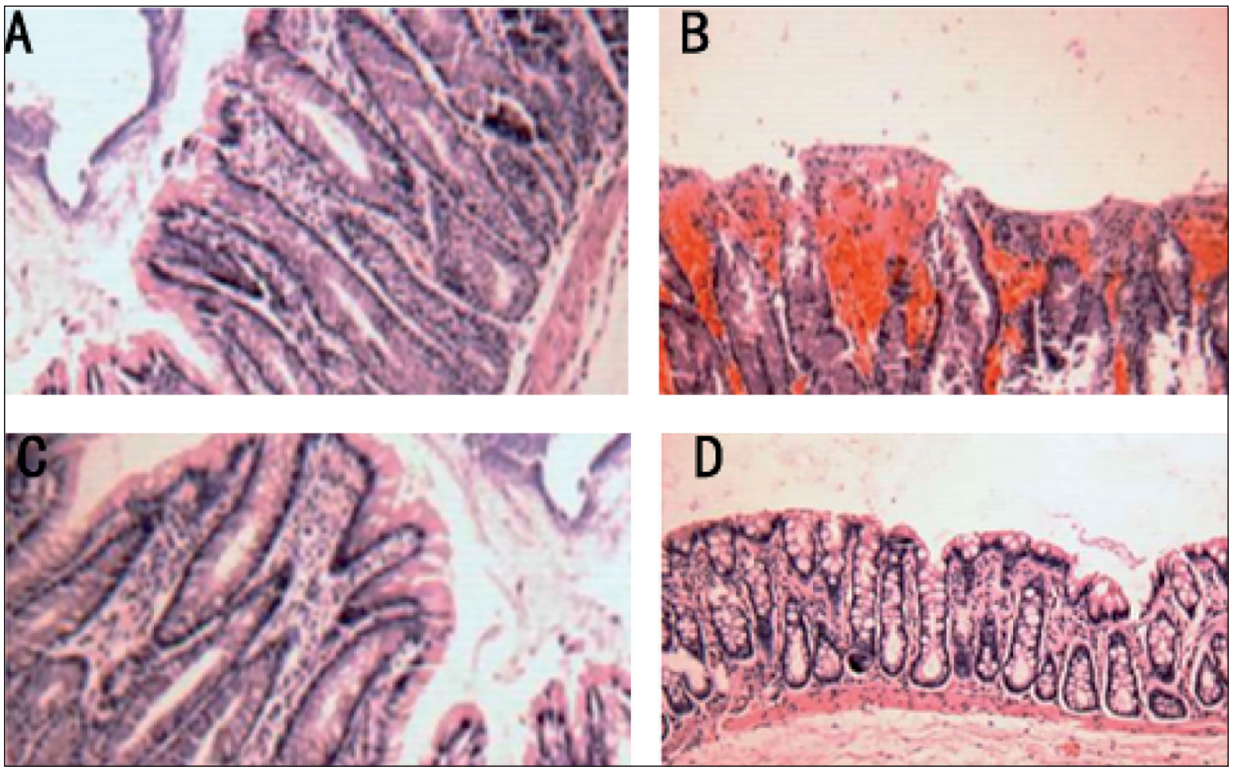

Figure 3. Colonic morphology of each group of rats shown *via* immunohistochemistry (Magnification × 40). NG (***A***): normal group, MG (***B***): model group, SASP (***C***): SASP group and SASP + SB (***D***): combination therapy group.

Discussion

The pathological manifestations of UC include epithelial barrier defects, immune responses, leukocyte recruitment and colonic flora, but the specific pathogenesis is still unclear. In particular, UC often causes damage to intestinal epithelial cells, leading to the production of endogenous and exogenous antigens, which are translocated through the portal system. Intestinal barrier defects are common in patients and are thought to increase the uptake of antigens

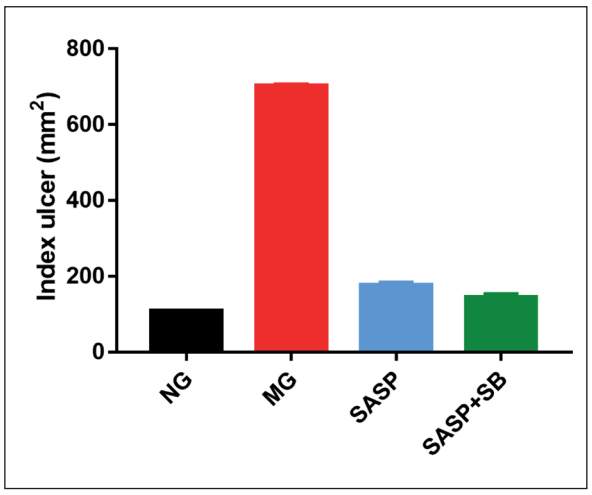

Figure 4. Index ulcer (mm^2) in different groups. Data are expressed as mean ± SEM ($p<0.01$).

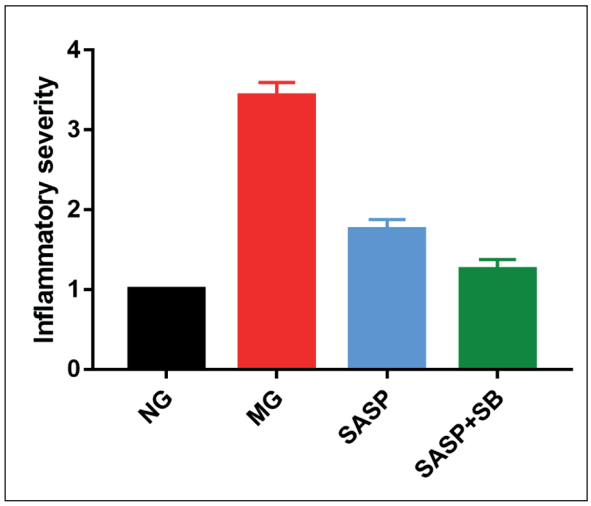

Figure 5. Inflammatory severity in different groups. Data are expressed as mean ± SEM ($p<0.05$).

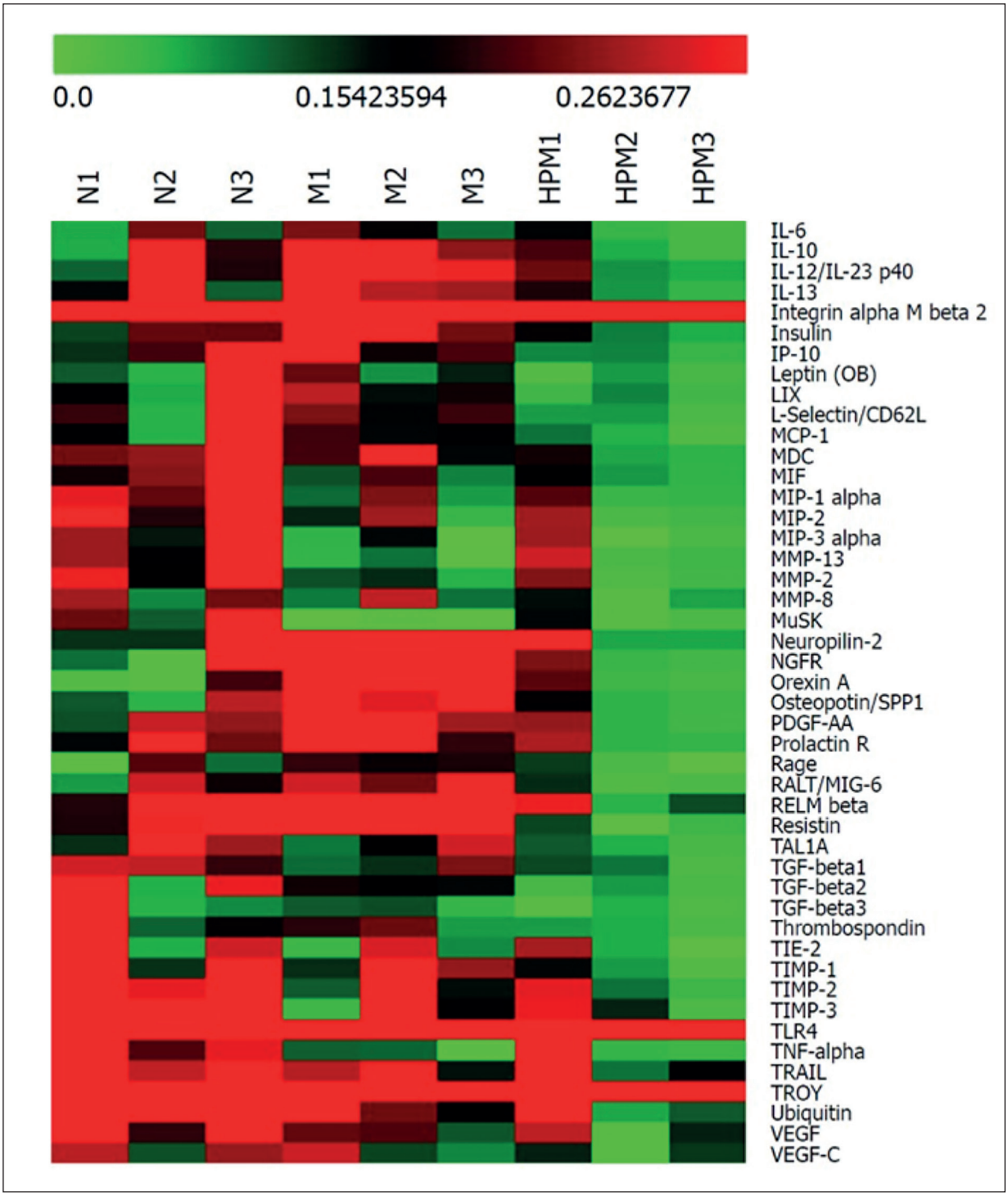

Figure 6. Cluster heat maps of colonic cytokines in each group. N: normal group, M: model group, SASP: SASP group, and SASP + SB: combination therapy group.

in the lumen by the intestinal epithelium, which triggers the development of the immune system and mucosal inflammation10. In view of the limited therapeutic effect on immune responses, improving the intestinal barrier function of intestinal epithelial cells may provide a new way for UC treatment characterized by altered barrier function. Tight junctions and adhesion

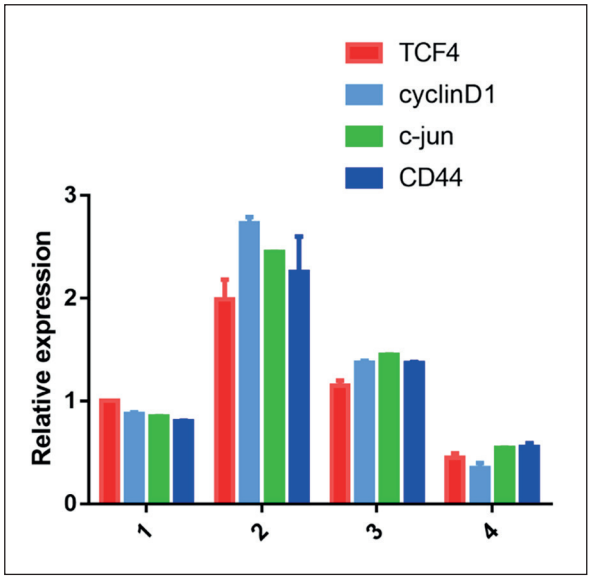

Figure 7. Wnt/β-catenin pathway-related mRNA expression in colon tissues of different groups of rats analyzed *via* PCR. 1: normal group, 2: model group, 3: SASP group, and 4: combination therapy group ($p<0.05$).

junctions are the main causes that limit and regulate intestinal permeability. Samak et al[11] found that DSS treatment increases tyrosine phosphorylation of ZO-1 in Caco-2 cells, suggesting that tight junctions are disrupted. Lahey et al[12] have shown that the promotion of tight junctions composed of occluded, claudin family proteins and intracellular ZO family proteins can help alleviate UC symptoms.

Uncontrolled release of pro-inflammatory cytokines is an essential feature of UC development. The treatment strategy of UC should be based on the reduction of inflammatory mediators and ROS[13]. In addition, UC is characterized by epithelial dysregulation of epithelial cells. These cell renewals are tightly regulated by a comprehensive signaling pathway that controls proliferation, differentiation and apoptosis. The Wnt/β-catenin signaling pathway is thought to play an indispensable role in the proliferation and differentiation of intestinal epithelial cells[14], which is active in epithelial cells of patients with UC. Wnt signals activate intracellular pathways, including the typical Wnt/β-catenin-dependent and non-typical Wnt/β-catenin-independent pathways[15]. β-catenin is a multi-tasking protein that, on the cell membrane, maintains cell adhesion to cells through the phosphorylation of protein complexes and a tightly controlled level of E-cadherin in the cytoplasm. Free and non-phosphorylated β-catenin in the nucleus bind to transcription factors and induce transcription of the target gene[16]. Colitis can induce nuclear translocation of β-catenin and affect the degree of histological differentiation of colon cancer.

In this study, a special influence of probiotics combined with SASP on the rat model of UC was found. UC was evaluated by such indicators as the severity of intestinal inflammation, ulcer area and microscopic and macroscopic scores. After the treatment with probiotics combined with SASP, the ulcer area in the model group was significantly reduced, and disease-related clinical symptoms such as diarrhea and rectal bleeding decreased. These results reflected the cytoprotective and anti-ulcer effects of probiotics.

The regulation of the Wnt signaling pathway is complex and may occur at multiple levels, including ligand/receptor, β-catenin translocation and transcriptional activation[17]. This study revealed that the expressions of Wnt ligands in the mucosa of UC rats were upregulated. In the injury response, these ligands were upregulated in both lamina propria and epithelial cells. It has been previously reported that activated macrophages promote the expression of Wnt signals in kidney and lung epithelial cells and co-cultured intestinal epithelial cells[18-20]. This work further proved these observations.

Conclusions

We showed that the over-activation of the Wnt signaling pathway promotes the development of UC, and the treatment with probiotics combined with SASP benefits the suppression of the abnormal activation of the Wnt pathway, thereby achieving anti-ulcer effects. These results can not only supplement previous studies, but also provide a basis for the establishment of new methods for the treatment of UC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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References

1) Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology 2012; 142: 46-54, e30.
2) Cosnes J, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology 2011; 140: 1785-1794.
3) Wlodarczyk M, Sobolewska-Wlodarczyk A, Stec-Michalska K, Fichna J, Wisniewska-Jarosinska M. The influence of family pattern abnormalities in the early stages of life on the course of inflammatory bowel diseases. Pharmacol Rep 2016; 68: 852-858.
4) Sun PL, Zhang S. Correlations of 25-hydroxyvitamin D3 level in patients with ulcerative colitis with inflammation level, immunity and disease activity. Eur Rev Med Pharmacol Sci 2018; 22: 5635-5639.
5) Burkhalter H, Stucki-Thur P, David B, Lorenz S, Biotti B, Rogler G, Pittet V. Assessment of inflammatory bowel disease patient's needs and problems from a nursing perspective. Digestion 2015; 91: 128-141.
6) Calzada D, Baos S, Cremades-Jimeno L, Cardaba B. Immunological mechanisms in allergic diseases and allergen tolerance: the role of treg cells. J Immunol Res 2018; 2018: 6012053.
7) de Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. Nat Rev Gastroenterol Hepatol 2016; 13: 13-27.
8) Katsanos KH, Papadakis KA. Inflammatory bowel disease: updates on molecular targets for biologics. Gut Liver 2017; 11: 455-463.
9) Dae Park D, Yum HW, Zhong X, Kim SH, Kim SH, Kim DH, Kim SJ, Na HK, Sato A, Miura T, Surh YJ. Perilla frutescens extracts protects against dextran sulfate sodium-induced murine colitis: NF-kappaB, STAT3, and Nrf2 as putative targets. Front Pharmacol 2017; 8: 482.
10) Vancamelbeke M, Vanuytsel T, Farre R, Verstockt S, Ferrante M, Van Assche G, Rutgeerts P, Schuit F, Vermeire S, Arijs I, Cleynen I. Genetic and transcriptomic bases of intestinal epithelial barrier dysfunction in inflammatory bowel disease. Inflamm Bowel Dis 2017; 23: 1718-1729.
11) Samak G, Chaudhry KK, Gangwar R, Narayanan D, Jaggar JH, Rao R. Calcium/Ask1/MKK7/JNK2/c-Src signalling cascade mediates disruption of intestinal epithelial tight junctions by dextran sulfate sodium. Biochem J 2015; 465: 503-515.
12) Lahey KA, Ronaghan NJ, Shang J, Dion SP, Desilets A, Leduc R, MacNaughton WK. Signaling pathways induced by serine proteases to increase intestinal epithelial barrier function. PLoS One 2017; 12: e180259.
13) Sanei MH, Hadizadeh F, Adibi P, Alavi SA. Inflammatory cells' role in acetic acid-induced colitis. Adv Biomed Res 2014; 3: 193.
14) Nava P, Koch S, Laukoetter MG, Lee WY, Kolegraff K, Capaldo CT, Beeman N, Addis C, Gerner-Smidt K, Neumaier I, Skerra A, Li L, Parkos CA, Nusrat A. Interferon-gamma regulates intestinal epithelial homeostasis through converging beta-catenin signaling pathways. Immunity 2010; 32: 392-402.
15) Ortiz-Masia D, Cosin-Roger J, Calatayud S, Hernandez C, Alos R, Hinojosa J, Apostolova N, Alvarez A, Barrachina MD. Hypoxic macrophages impair autophagy in epithelial cells through Wnt1: relevance in IBD. Mucosal Immunol 2014; 7: 929-938.
16) Huang P, Yan R, Zhang X, Wang L, Ke X, Qu Y. Activating Wnt/beta-catenin signaling Pathway for disease therapy: challenges and opportunities. Pharmacol Ther 2018; S0163-7258(18)30211-0.
17) Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, Red EA, Vats D, Brombacher F, Ferrante AW, Chawla A. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. Nature 2007; 447: 1116-1120.
18) Lin SL, Li B, Rao S, Yeo EJ, Hudson TE, Nowlin BT, Pei H, Chen L, Zheng JJ, Carroll TJ, Pollard JW, McMahon AP, Lang RA, Duffield JS. Macrophage Wnt7b is critical for kidney repair and regeneration. Proc Natl Acad Sci U S A 2010; 107: 4194-4199.
19) Cosin-Roger J, Ortiz-Masia D, Calatayud S, Hernandez C, Alvarez A, Hinojosa J, Esplugues JV, Barrachina MD. M2 macrophages activate WNT signaling pathway in epithelial cells: relevance in ulcerative colitis. PLoS One 2013; 8: e78128.
20) Oguma K, Oshima H, Aoki M, Uchio R, Naka K, Nakamura S, Hirao A, Saya H, Taketo MM, Oshima M. Activated macrophages promote Wnt signalling through tumour necrosis factor-alpha in gastric tumour cells. EMBO J 2008; 27: 1671-1681.