Effect of fecal microbiota transplantation on the TGF-β1/Smad signaling pathway in rats with TNBS-induced colitis

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Background: Traditional treatments for inflammatory bowel disease (IBD) have adverse side effects, and patients who receive such treatments have high recurrence rates. Fecal microbiota transplantation (FMT) has become an increasingly popular therapeutic option for patients with IBD. However, the mechanism by which FMT alleviates this disease remains unclear.

Methods: In this study, a rat model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis was established and used to explore whether the transforming growth factor-beta 1 (TGF-β1)/small mothers against decapentaplegic (Smad) signaling pathway plays a critical role in the FMT alleviation of IBD.

Results: After the FMT intervention, the disease activity index and histologic scores were significantly decreased. In addition, the TGF-β1 expression level in the FMT group was significantly decreased by approximately 0.72-fold relative to the level in the TNBS colitis group, whereas the Smad3, Smad4, and Smad7 expression levels had increased by approximately 1.21, 1.40, and 1.18 folds, respectively. Similarly, SB431542 inhibited the expression of TGF-β1 and promoted the expression of Smad3, Smad4, and Smad7. Further, the serum levels of the inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were significantly decreased, whereas that of the interferon-gamma (IFN-γ) was not significantly changed after the FMT intervention.

Conclusions: These results suggest that FMT inhibits the TGF-β1/Smad signaling pathway to attenuate inflammation.

Keywords: Fecal microbiota transplantation (FMT); inflammatory bowel disease (IBD); transforming growth factor-beta 1 (TGF-β1); small mothers against decapentaplegic (Smad)

Introduction

Inflammatory bowel disease (IBD) is a chronic intestinal disorder, which is associated with exacerbation of the host immune response, the disruption of the intestinal barrier, and alterations in the luminal and mucosal microbial communities (1). The 2 major chronic forms of this disease are ulcerative colitis and Crohn's disease (2). Currently, IBD remains incurable, and treatments to alleviate its effects include 5-aminosalicylic acid, steroids, antimicrobials, immunosuppressive agents, and tumor necrosis factor (TNF) blockers (3). However, the adverse side effects of these treatments and the high recurrence rates in patients

Table 1 The DAI changed in the experimental colitis rats (mean ± standard deviation)

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Blank	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
TNBS	1.20±0.73*	1.40±0.68*	1.00±0.38*	1.17±0.59*	1.17±0.59*	1.00±0.56*	0.83±0.67*	0.92±0.76	0.67±0.56	0.50±0.60
TNBS + FMT	1.67±0.58*#	1.27±0.80*#	1.17±0.60*#	1.17±0.64*	1.00±0.56*	1.00±0.56*	0.33±0.37#	0.33±0.37	0.33±0.37	0.17±0.30
TNBS + Ped	1.33±0.00*	1.53±0.38*	1.13±0.45*	1.25±0.67*	1.17±0.60*	0.67±0.56*#	0.67±0.56	0.67±0.56	0.42±0.47	0.17±0.30
TNBS + SB431542	1.73±0.72*#	1.53±0.38*#	1.27±0.37*#	1.20±0.30*	1.27±0.37*	1.00±0.67*	1.00±0.58*	0.60±0.60	0.27±0.37	0.27±0.37
F	10.549	7.532	12.244	13.300	13.776	4.328	3.372	2.139	1.697	1.031
P	<0.001	0.001	<0.001	<0.001	<0.001	0.014	0.033	0.120	0.197	0.420

Blank: normal untreated rats; TNBS: colitic rats; TNBS + FMT: colitic rats treated with fecal microbiota transplantation; TNBS + Ped: colitic rats treated with prednisone; TNBS + SB431542: colitic rats treated with the TGF-β1 inhibitor SB431542. *, $P<0.05$ *vs.* the Blank group; #, $P<0.05$ *vs.* the TNBS group. DAI, disease activity index; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

taking such drugs have necessitated the development of novel therapeutic approaches (4-6).

It is well known that the development and maintenance of IBD is associated with an imbalance of the intestinal microbiota (7). Fecal microbiota transplantation (FMT), a process by which feces from a healthy donor is transferred to a patient suffering from intestinal microbial imbalance, has become an increasingly popular therapeutic option for restoring gut homeostasis in patients with IBD (8).

A previous study has shown that the transforming growth factor-beta 1 (TGF-β1)/Small mothers against decapentaplegic (Smad) signaling pathway defected involved in IBD (9). Thus, the TGF-β1/Smad signaling pathway has potential in the development of possible novel therapies for IBD (10). Additionally, FMT has been proven to be related to the TGF-β1/Smad signaling pathway. FMT significantly decreases the expression of TGF-β1 and Smad3, and increases the levels of Smad7 (11). However, the mechanisms by which FMT regulates the TGF-β1/Smad signaling pathway to alleviate the inflammatory effects of IBD remain unclear.

This study was conducted to verify the effects of FMT on IBD and obtain insights into the mechanisms involved. First, we used 2,4,6-trinitrobenzenesulfonic acid (TNBS) to experimentally induce colitis in rats. Next, we used the colitic rats to explore whether the TGF-β1/Smad signaling pathway plays a critical role in the treatment effects induced by FMT. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3227/rc).

Methods

Ethics statement

This study was approved by the Animal Ethics Committee of Fuzhou Traditional Chinese Medicine Hospital (No. 2020-05-01-01). All animal care procedures were carried out in accordance with national policies for animal health and wellbeing. All the animal surgeries were performed under appropriate anesthesia to minimize the suffering of the rats.

Animals and experimental procedures

A total of 25 male Sprague-Dawley rats, weighing 200–250 g, purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China), were raised in a humidity-controlled room with free access to food and water. The temperature of the facility was maintained at 23±2 ℃ with a 12:12-light-dark cycle. The rats were randomly allocated into the following 5 groups (n=5 per group): the Normal group (blank control), the TNBS colitis group (TNBS only), the FMT group (TNBS + FMT), the Prednisone group (TNBS + prednisone), and the TGF-β1 inhibitor group (TNBS + SB431542) (see *Table 1*). The rats in the Normal group were used as the fecal microbiota donors for the FMT group. Fecal samples from the normal rats were collected daily at 10:00 am and pooled at equal amounts within the group. Next, 7 g of the pooled fecal samples was suspended in 15 mL of sterile 0.9% normal saline by vortex mixing. The fecal suspension was then filtered through a stainless-steel mesh and centrifuged at 6,000 ×g for 15 min,

after which the supernatant was stored immediately at 4 ℃.

All the animals, except those in the Normal group, were deprived of food for 48 h before colitis modeling. The rats were then anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg), and 1 mL of 30 mg/mL TNBS (Sigma-Aldrich, St. Louis, MO, USA) dissolved in ethanol (40%, v/v) was subsequently administered via intrarectal instillation. Thereafter, the rats in the FMT group were intrarectally administered 0.2 mL of the fecal supernatant at every 2 days for 10 days. At the same time, the rats in the Prednisone group, TGF-β1 inhibitor group, and TNBS colitis group were administered 0.2 mL of 5 mg/kg prednisone, 0.2 mL of 10.0 µM SB431542 (MedChemExpress, Monmouth Junction, NJ, USA), and 0.2 mL of 0.9% normal saline, respectively, via intrarectal instillation. The rats in the Normal group were left untreated and received a normal diet and water. After the 10 days of treatments, all the rats were sacrificed, and serum and intestinal tissue samples were collected and stored at –80 ℃.

Assessment of disease activity index (DAI) scores

Each rat was assigned a DAI score daily on the basis of weight loss, stool consistency, and bloody stool, as previously described (12).

Histologic analysis

The intestinal tissues were fixed in 4% formaldehyde and embedded in paraffin wax blocks. Next, 5-µm tissue sections were obtained and stained with hematoxylin-eosin-saffron (HES) to estimate the tissue damage caused by colitis. The sections were scored by the same pathologist, who was blinded to the samples. Epithelial necrosis, inflammatory cell infiltration, and the mucosa thickness were assessed using semi-quantitative scores that ranged from 0 to 3 for each variable (0, no inflammation; 1, very low level of inflammation; 2, moderate level of leukocyte infiltration; 3, high levels of leukocyte infiltration, vascular density, and ulceration). Leica QWin software (Leica Microsystems, Bensheim, Germany) was used for the image analysis (13).

Western blot analysis

The frozen intestinal tissue samples were homogenized in phosphate-buffered saline containing a 1% protease inhibitor cocktail. The homogenates were then centrifuged (12,000 ×g, 15 min, 4 ℃) and the supernatants were collected. The protein concentration in the supernatants was determined using Bradford's colorimetric method. Next, aliquots of the supernatants containing equal amounts of protein (40 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, after which the protein bands on the gel were transferred to a polyvinylidene difluoride membrane. After blocking the membrane with tris buffered saline with tween containing 5% non-fat dry milk for 2 h, the membrane was cut into 5 pieces based on the size of the target proteins. The pieces were then incubated with primary antibodies [anti-TGF-β1, anti-Smad3, anti-Smad4, anti-Smad7, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibodies] (Hanzhou Huaan Biotechnology Co., Ltd., Hanzhou, China) at dilutions of 1:250 for 1 h at room temperature. After 3 washes with TBST, all the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary immunoglobulin G antibodies (Hanzhou Huaan Biotechnology Co., Ltd., Hanzhou, China) for 1 h at room temperature. GAPDH was used as the loading control. Immunodetection was achieved using an enhanced chemiluminescence light-detecting kit (Amersham, GE Healthcare, Chicago, IL, USA). Following their normalization to the GAPDH level, the densitometric data of the target proteins were measured using an ImageScanner II densitometer and ImageQuant TL analysis software (GE Healthcare).

Enzyme immunoassay

The rat serum concentrations of the cytokines TNF-α, interleukin (IL)-1β, IL-6 and IFN-γ were determined with the enzyme-linked immunosorbent assay using commercial kits (Chengdu Zhengneng Biotechnology Co., Ltd., Chengdu, China).

Statistical analysis

Statistical comparisons were performed using SPSS version 23.0 software (International Business Machines Corporation, USA). All the quantitative data are presented as the mean ± standard deviation. Statistical analyses were performed using a one-way analysis of variance. For non-parametric data, the Kruskal-Wallis test was performed. Differences with a P value <0.05 were considered statistically significant.
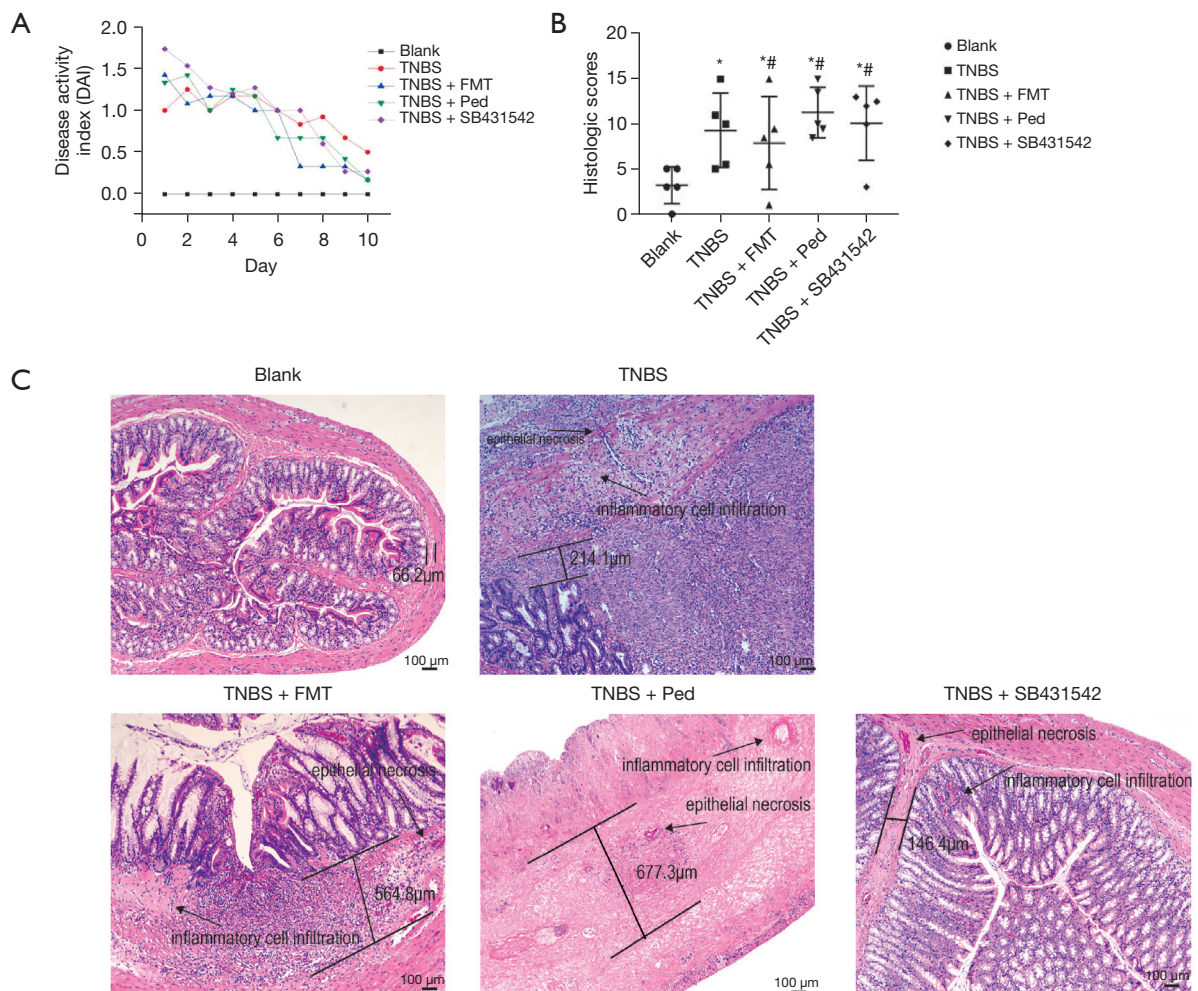
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Figure 1 The disease activity index and histologic scores changed in the rats with experimentally induced colitis. (A) Disease activity indexes of the different rat groups. (B) Histologic scores of the different rat groups. (C) HES-stained intestinal tissue from the different groups of rats. The distances between two parallel lines are mucosa thickness. Blank: normal untreated rats; TNBS: colitic rats; TNBS + FMT: colitic rats treated with fecal microbiota transplantation; TNBS + Ped: colitic rats treated with prednisone; TNBS + SB431542: colitic rats treated with the TGF-β1 inhibitor SB431542. *, $P<0.05$ *vs.* the Blank group; #, $P<0.05$ *vs.* the TNBS group. HES, hematoxylin-eosin-saffron; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

Results

The DAI and histologic scores changed in the colitic rats

Among all the groups, there were significant differences in the DAI scores from day 1 to day 7 ($P<0.05$), but there were no significant differences from day 8 to day 10 ($P>0.05$; see *Table 1*). On day 1, the TNBS colitis, FMT, prednisone, and TGF-β1 inhibitor groups all showed significantly higher DAI scores than the Normal group. However, the scores were significantly decreased in the respective groups after the different treatments (see *Figure 1A*).

The histopathological changes in the intestinal tissues were examined using HES staining. The histologic scores for the Normal, TNBS colitis, FMT, prednisone, and TGF-β1 inhibitor groups were 3.20±2.05, 9.30±4.15, 7.90±5.16, 11.30±2.80, and 10.10±4.13, respectively (see *Figure 1B,1C*). There were significant differences in the histologic scores among all the groups ($P<0.05$).

FMT inhibited the TGF-β1/Smad signaling pathway

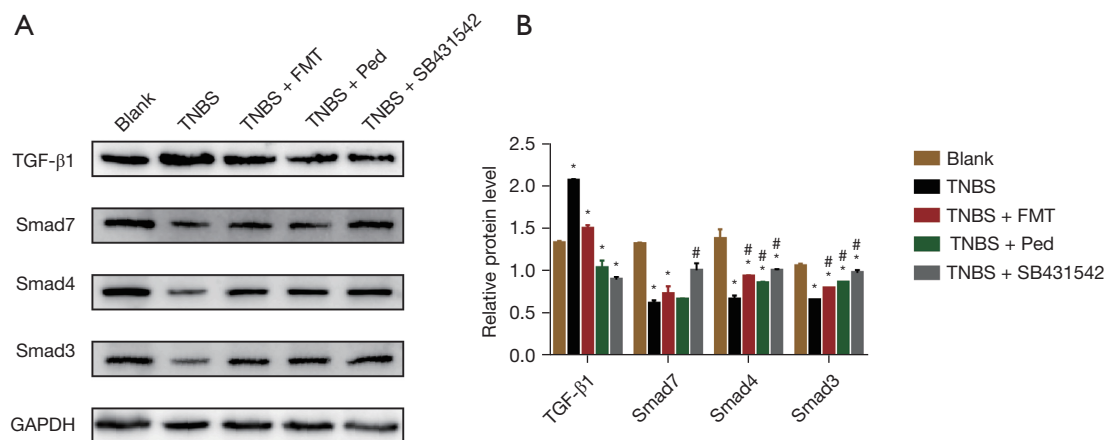To investigate whether the TGF-β1/Smad signaling

Figure 2 Western blot analysis of TGF-β1/Smad signaling molecules in rats with experimentally induced colitis. (A) Western blot of the TGF-β1, Smad3, Smad4, Smad7, and GAPDH (loading control) protein levels in the intestinal tissues. (B) Levels of TGF-β1, Smad3, Smad4, and Smad7 standardized to the loading control. Blank: normal untreated rats; TNBS: colitic rats; TNBS + FMT: colitic rats treated with fecal microbiota transplantation; TNBS + Ped: colitic rats treated with prednisone; TNBS + SB431542: colitic rats treated with the TGF-β1 inhibitor SB431542. *, P<0.05 *vs.* the Blank group; #, P<0.05 *vs.* the TNBS group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TGF-β1, transforming growth factor-beta 1; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

pathway plays an important role in the FMT alleviation of experimentally induced colitis, a Western blot analysis was conducted to detect the expression levels of these signaling proteins in the intestinal tissues. In the TNBS colitis group, the expression level of TGF-β1 was approximately 1.56-fold higher than that in the Normal group; while the expression levels of Smad3, Smad4, and Smad7 were approximately 0.62-, 0.49-, and 0.47-fold lower (see *Figure 2A,2B*). However, after FMT treatment, the TGF-β1 expression level decreased significantly by approximately 0.72-fold relative to the level in the TNBS colitis group, whereas the Smad3, Smad4, and Smad7 expression levels increased by approximately 1.21, 1.40, and 1.18 folds, respectively. Similarly, SB431542 inhibited the expression of TGF-β1 and promoted the expression of Smad3, Smad4, and Smad7. These data strongly suggest that FMT inhibits the TGF-β1/ Smad signaling pathway to attenuate inflammation.

FMT affected the proinflammatory cytokines TNF-α, IL-1β, IL-6 but not IFN-γ

It has been previously reported that TNBS exposure leads to a significant increase in the levels of pro-inflammatory cytokines in intestinal tissues (14,15). In this study, the concentrations of TNF-α, IL-1β, IL-6 and IFN-γ were 6.36, 5.44, 5.23 and 6.68 times higher in the TNBS colitis group than that in the Normal group, respectively

(see *Figure 3*). However, after the FMT, prednisone, and TGF-β1 inhibitor interventions in the colitic rats, the concentrations of proinflammatory cytokines TNF-α, IL-1β and IL-6 in the 3 respective groups were significantly decreased (see *Figure 3A-3C*). Notably, the difference in IFN-γ concentrations between the TNBS colitis and FMT groups was not statistically significant, suggesting that the expression of this cytokine was not affected by the FMT intervention (see *Figure 3D*).

Discussion

In recent years, the therapeutic strategies for IBD have shifted away from general immunosuppressive treatment towards pathway-based approaches (16). These specific pathway-based drugs, such as anti-TNF, IL-12, and IL-23, Janus kinase inhibitors, and antisense oligonucleotides against inhibitors of the immunosuppressive cytokine TGF-β1, are fairly effective, and will increase the therapeutic options for patients with IBD (17-21). FMT has been proven to be highly successful in the treatment of recurrent and antibiotic refractory *Clostridioides difficile* infections, with a cure rate of up to 90% (22-24). Thus, FMT has become a promising therapeutic approach to correct IBD-related dysbiosis (25,26).

FMT has been proven to be efficacious in treating IBD; however, its mechanism of action remains unclear. Recently, several researchers have focused on studying the
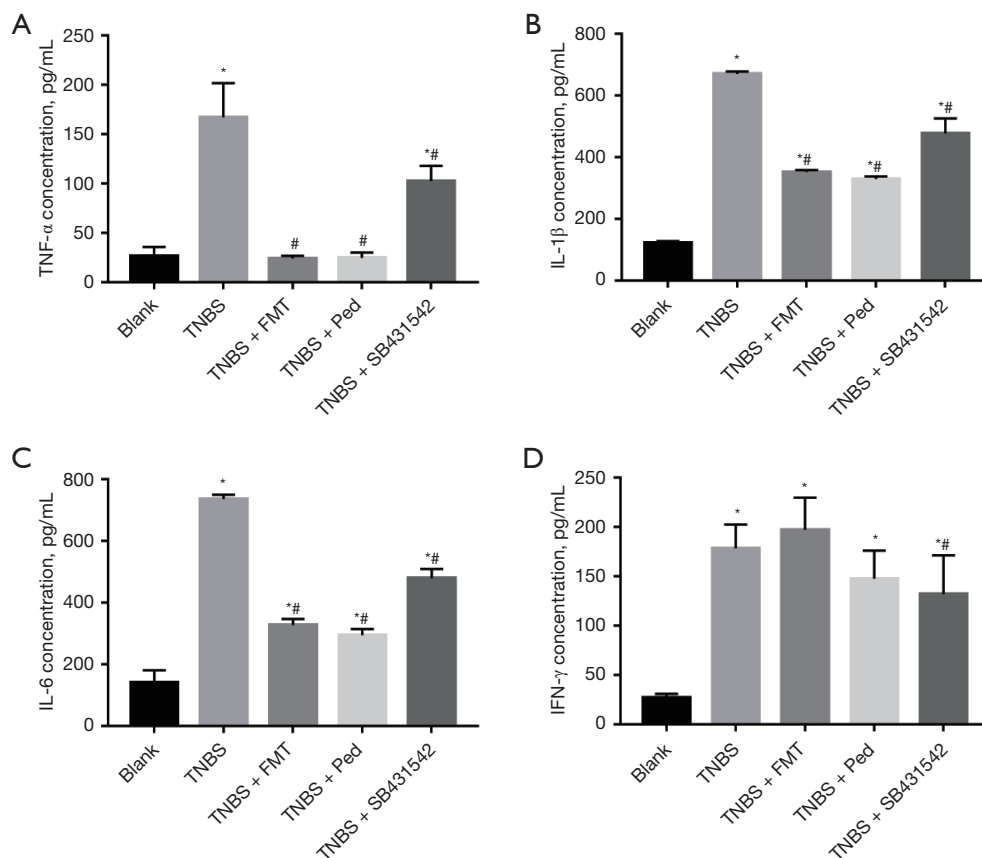
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Figure 3 Detection of the pro-inflammatory cytokines in rat sera after different interventions. (A) Detection of TNF-α in rat sera after the different interventions. (B) Detection of IL-1β in rat sera after the different interventions. (C) Detection of IL-6 in rat sera after the different interventions. (D) Detection of IFN-γ in rat sera after the different interventions. Blank: normal untreated rats; TNBS: colitic rats; TNBS + FMT: colitic rats treated with fecal microbiota transplantation; TNBS + Ped: colitic rats treated with prednisone; TNBS + SB431542: colitic rats treated with the TGF-β1 inhibitor SB431542. *, P<0.05 *vs.* the Blank group; #, P<0.05 *vs.* the TNBS group. IL, interleukin; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor α; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

mechanism. For example, Zhang *et al.* (27) found that FMT controlled experimentally induced ulcerative colitis by improving T helper 1 (Th1)/T helper 2 (Th2) and T helper cell 17 (Th17)/regulatory cells (Treg) imbalance through the regulation of the intestinal microbiota. Another study suggested that FMT could increase the abundance of the gut bacteria that produce short-chain fatty acids, which in turn decreased proinflammatory cytokine production by suppressing the nuclear factor-kappa B signaling pathway (28). In this study, we found that both the DAI and histologic scores were significantly decreased after the FMT intervention, indicating that the transplanted fecal microbiota could effectively prevent TNBS-induced colitis. The signaling molecule TGF-β1 was downregulated, whereas Smad3 was upregulated, after FMT treatment in the

colitic rats, which strongly suggests that FMT inhibits the TGF-β1/Smad signaling pathway to attenuate inflammation.

As a well-known pro-inflammatory cytokine, TNF-α induces the apoptosis of epithelial cells, disrupts the epithelial barrier, and prolongs the inflammatory reaction (29). After the FMT intervention, the concentration of TNF-α was significantly decreased in the colitic rats, which provides further evidence that this treatment inhibits the TGF-β1/Smad signaling pathway to attenuate inflammation. However, in this study, the other pro-inflammatory cytokine IFN-γ was not significantly affected by the FMT intervention.

Conclusions

In conclusion, our study verified that FMT significantly

reduced the DAI and histologic scores of rats with experimentally induced colitis. Additionally, FMT was found to inhibit the TGF-β1/Smad signaling pathway to attenuate inflammation. These findings can help to optimize the clinical use of this therapeutic option for patients with IBD.

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3227/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3227/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3227/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Animal Ethics Committee of Fuzhou Traditional Chinese Medicine Hospital (No. 2020-05-01-01). All animal care procedures were carried out in accordance with national policies for animal health and wellbeing.

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