Investigating the Relation between the Gut Microbiota and Inflammatory Bowel Disease in a Mouse Model

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A R T I C L E   I N F O

A B S T R A C T

Introduction: Inflammatory bowel disease (IBD) is a group of chronic gastrointestinal disorders affecting millions worldwide. Several factors are involved in developing this disease, but gut microbiota is known to be one of the most critical factors. This study investigated the relationship between gut microbiota and IBD in a mouse model. **Methods:** In this study, two methods were used: chemical induction with dextran sulfate sodium (DSS) and biological induction with stool from a human with IBD (fecal microbiota transplantation) to induce inflammation in the gut of mice. The gut microbiota populations in both groups were studied using real-time PCR. In addition, the serum levels of inflammatory cytokines and the colon tissues of the mice were analyzed. **Results:** The pathological results showed that the colon tissue in the FMT group had inflammatory changes as in the DSS group. The changes in the gut microbiota population in both FMT and DSS groups on the last day of the study also showed a similar pattern. Interleukin-1 and IL-6 also increased in the FMT and DSS groups compared to the control group. **Conclusion:** Our results showed a mutual relationship between gut microbiota and inflammatory diseases and that gut microbiota was not only the cause of IBD but may also be a consequence of this disease. In fact, by chemically inducing inflammation, the gut microbiota was altered. On the other hand, performing FMT from human stool with IBD altered the gut microbiota of mice and induced inflammatory disease in the mouse model.

INTRODUCTION

The gastrointestinal tract has a large population of commensal bacteria known as the gut microbiota. The gut microbiota is also known as the "forgotten organ" because it resembles organs in biological function and complexity [1]. Gut microbiota plays a crucial role in gut homeostasis, physiology, safety, and energy metabolism [2]. When the balance of the gut microbiota is disturbed, the so-called dysbiotic microbiome causes intestinal diseases such as inflammatory bowel disease (IBD) [3].

IBD is a pathological and chronic inflammatory disease that includes Crohn's disease (CD) and ulcerative colitis (UC) [4]. Although the cause of IBD is virtually unknown, the most widely accepted hypothesis blames the abnormal immune response to the gut microbiota for IBD. Environmental factors and host genetics also play essential roles in this disease [5-9]. Significant differences in gut microbiota between healthy and IBD patients and

inflammatory and non-inflammatory intestinal areas have been found [9-12]. Dysbiosis of the intestinal microbiota is a sign of IBD progression [10, 13]. One way to assess dysbiosis of the intestinal microbiota is to measure changes in the microbiota population. Several studies have reported a high degree of change in the microbiota between individuals and changes during disease [14-16].

Human microbiota-associated mice (HMA) are reliable tools for studying the relationship between disease and gut microbiota [17]. These mouse models usually include germ-free mice colonized with human gut microbiota. This transplantation leads to the development of gut microbiota patterns in the recipient mice with the same level of diversity as the human donor and high human microbiota transplantation [18].

This study induced IBD in a mouse model via chemical and biological inductions and investigated the relationship

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between IBD and gut microbiota by analyzing inflammatory changes in the serum and colon tissues of the mice.

MATERIAL AND METHODS

IBD patient. A stool sample was obtained from an IBD patient (diagnosed by a gastroenterologist based on radiological findings and paraclinical features) and kept refrigerated under anaerobic conditions until transferred to the laboratory. The stool sample was mixed with sterile phosphate-buffered saline (PBS) and spun at 1000 rpm for 30 sec to allow the particles to settle. The IBD patient filled out and signed the informed consent form.

Microbiota-transplanted Mice. A total of 15 BALB/c mice (eight weeks old) were purchased from the Pasteur Institute of Iran (Production and Research Complex, Iran). The mice were maintained at room temperature (25-28°C) and 50 ± 5% relative humidity with a 12-hour cycle of light and darkness. Food and water were provided ad libitum (5 mice per cage). One week after adaptation, mice were randomly divided into 3 groups (5 in each group). Group 1 (FMT group): In this group, the intestines of the mice had to be cleaned on the first day of the study for FMT. Therefore, the mice were given only water and were on fasting for one hour before the start of the intestinal cleansing. The mice were then placed in a clean cage, and 200 µl polyethylene glycol (PEG) 425g / L solution was administered by gavage four times at 20-minute intervals. The mice were placed in a clean cage four hours after the intestinal cleansing and received 200 µl of fecal suspension. This fecal transfer was performed once a week for four weeks [2]. Group 2 (DSS group): In this group, 200 µl of dextran sulfate sodium (DSS) 2% was administered daily for 28 days [19]. Group 3 (control group): They received only water and food as a healthy group. On the last day of the study (day 28), stool samples were collected from three groups of mice. Blood samples were also collected to determine inflammatory cytokines in serum, and colon tissue was collected for histopathology. This study had no exclusion criteria, and samples were taken from all mice at the end of the study. All experimental protocols were performed following the Ethics Committee of the Pasteur Institute of Iran. All methods were performed following the relevant local guidelines and regulations.

The experimental protocols were established following the Declaration of Helsinki and approved by the Ethics Committee of the Pasteur Institute of Iran (IR.PII.REC.1398.060). All methods were performed following the relevant guidelines and regulations, and the Pasteur Institute of Iran approved experimental protocols.

Real-time PCR. DNA was extracted from 200 mg mouse stool using the FavorPrep stool DNA isolation mini kit (Favorgen, Taiwan). DNA concentration was measured using a NanoDrop 1000 UV-Vis spectrophotometer. Real-time PCR was performed using 2X SYBR Green Master Mix (Amplicon, Denmark) and ABI Step One Plus detection system (Applied Biosystems, USA). Primers were experimented with gradient PCR to earn an appropriate annealing temperature (Table 1). Data were analyzed using RQ= $2^{-\Delta\Delta Ct}$ equation, in which the readings were normalized with all bacterial genes [24]. In this method, the Ct values of the target bacterium were normalized with all bacterial genes, and their comparison was evaluated using the comparative fold change.

Table 1. Sequences of oligonucleotide primers used in this study

Target bacterial	Sequence (5'–3')	Amplicon size (bp)	References
All bacteria	F: TCCTACGGGAGGCAGCAGT R: GGACTACCAGGGTATCTATCCTGTT	466	20
Actinobacteria	F: TACGGCCGCAAGGCTA R: TCATCCCCACCTTCCTCCG	300	23
γ-Proteobacteria	F: TCGTCAGCTCGTGTAGTGA R: CGTAAGGGCCATGATG	154	23
Firmicutes	F: TGAAACTAAAAGGAATTGACG R: ACCATGCACCACCTGTC	155	23
Bacteroidetes	F: CRAACAGGATTAGATACCCT R: GGTAAGGTTCCTCGCGTAT	204	23
Lactobacillus	F: TGGATGCCTTGGCACTAGGA R: AAATCTCCGGATCAAAGCTTACTTAT	92	21
Bifidobacterium	F: GGGTGGTAATGCCGGATG R: TAAGCCATGGACTTTCACACC	278	20
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	195	20
Faecalibacterium prausnitzii	F: AGAGTTTGATCATGGCTCAG R: GGTTACCTTGTTACGACTT	191	22

Detection of interleukin levels. The levels of interleukin-1β and interleukin-6 in mouse serum were measured by ELISA using an ELISA kit (Karmania Pars Gene, Iran) according to the manufacturer's protocol. The absorbance of the samples was measured in the ELISA reader at 455 nm. The results were expressed as the concentration of interleukin per milliliter of serum.

Histopathological analysis. Colon samples from all mice (n=15 samples) were fixed and embedded in paraffin according to standard procedures. The resulting block was mounted on a microtome and cut into thin slices, which were then glued to slides, the wax removed by a solvent, and the tissue slices mounted on the slides were rehydrated. The slides were stained with hematoxylin and eosin, examined, and photographed using a Nikon Eclipse E400 microscope and a Canon DS -Fi1 camera (Japan).

Statistical analysis. All data are presented as mean ± standard deviation. Differences between the mice groups were determined with a one-way analysis of variance (ANOVA) and were considered statistically significant if the P-value was < 0.05. Tukey's multiple comparisons test was used in this study. GraphPad Prism 8.0.2 was used to perform the statistical tests.

RESULTS

General responses of the colon to FMT and DSS. Inflammation was induced in both FMT and DSS groups (Fig. 1). Pathological analysis in the FMT group showed hypertrophy of the muscle layer and intraepithelial neutrophils. Some intraepithelial PMNs, severe goblet cell depletion and degenerative changes were also seen in the DSS group (Fig. 2). However, the control group's colon sections showed a normal mucosa and muscle layer with normal architecture and no significant pathological changes. The ELISA test showed increased IL-1 and IL-6 levels in FMT and DSS groups sera compared with the control group (Fig. 3). There was no significant difference between the FMT and DSS groups.
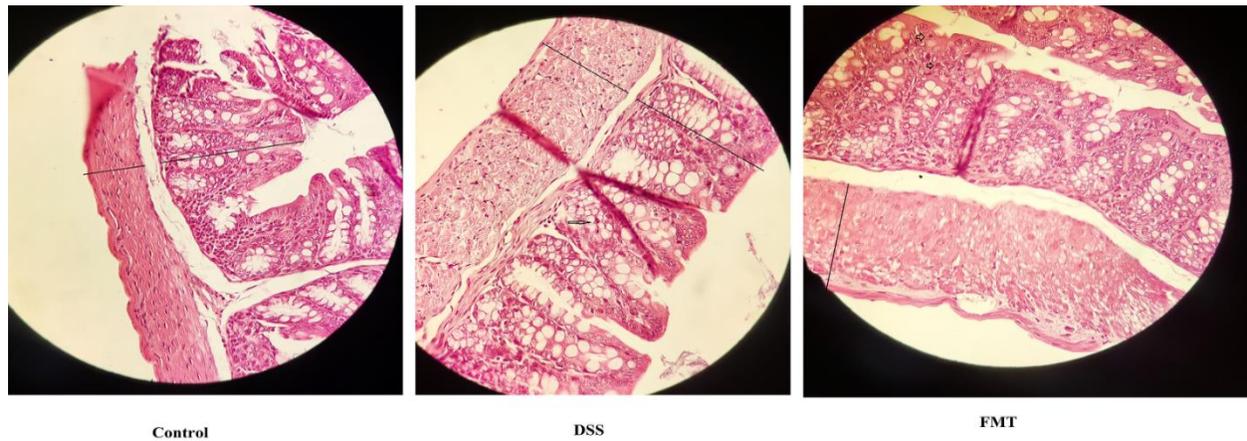

Fig. 1. Development of inflammation with dextran sulfate sodium and fecal microbiota transplantation. H&E staining of colon tissue in groups. Control group: no significant pathologic changes. DSS group: hypertrophy of muscle layer (black lines) and some intraepithelial PMNs (black arrows). FMT group: hypertrophy of the muscle layer (black line) and intraepithelial PMNs (black arrow).
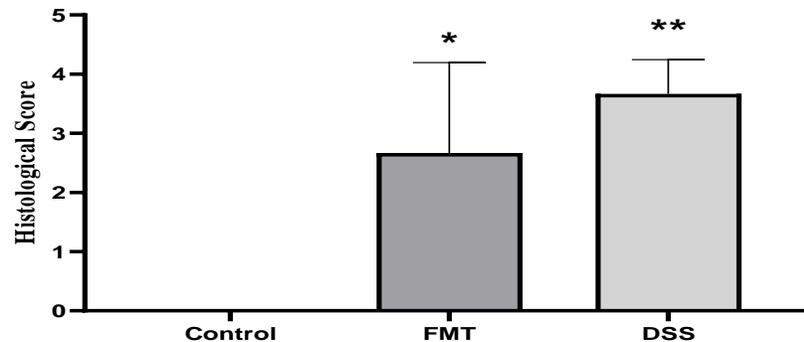

Fig. 2. Pathology changes in colon tissues in FMT and DSS groups compared to the control group. Statistical analysis was performed with one-way ANOVA test (*P < 0.05, **P < 0.001).

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Change in gut microbiota population. The results of real-time PCR showed that the population of gut microbiota in both the FMT and DSS groups changed on day 28 of the study and differed significantly from the control group (Fig. 4). In the FMT group, the population of the three phyla Actinobacteria, Firmicutes and Bacteroidetes decreased, but the population of γ-Proteobacteria increased. In the Firmicutes phyla, the populations of the three groups *Bifidobacterium*, *Lactobacillus,* and *Fecalibacterium* decreased

significantly in the FMT group. On the other hand, the population of Enterobacteriaceae increased significantly in this group compared to the control group. In the DSS group, the changes in the population of intestinal microbiota were the same as in the FMT group, but there was a significant difference in some bacterial groups. In group DSS, the population of Actinobacteria, Firmicutes, and γ-Proteobacteria was higher than in the FMT group, while in group FMT, the population of Bacteroidetes was higher than in the DSS group.
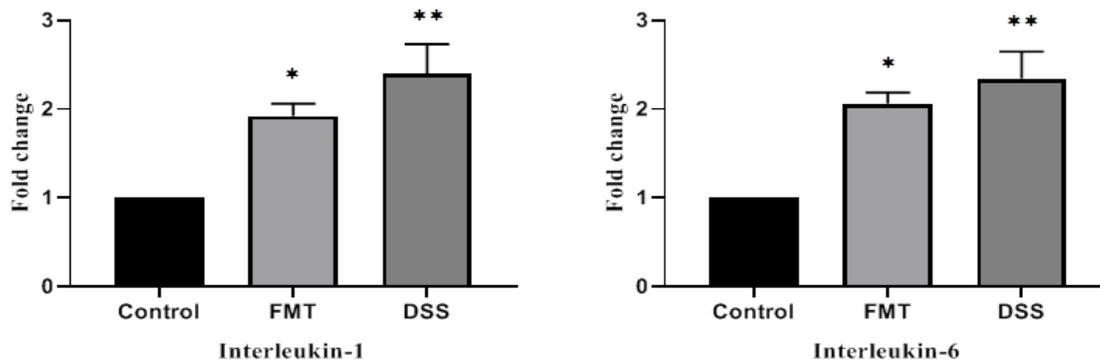
Fig. 3. Changes in serum levels of IL-1 and IL-6 in DSS and FMT groups compared to the control group. Statistical analysis was performed with one-way ANOVA test (*$P < 0.05$, **$P < 0.001$). FMT: fecal microbiota transplantation, DSS: dextran sulfate sodium.
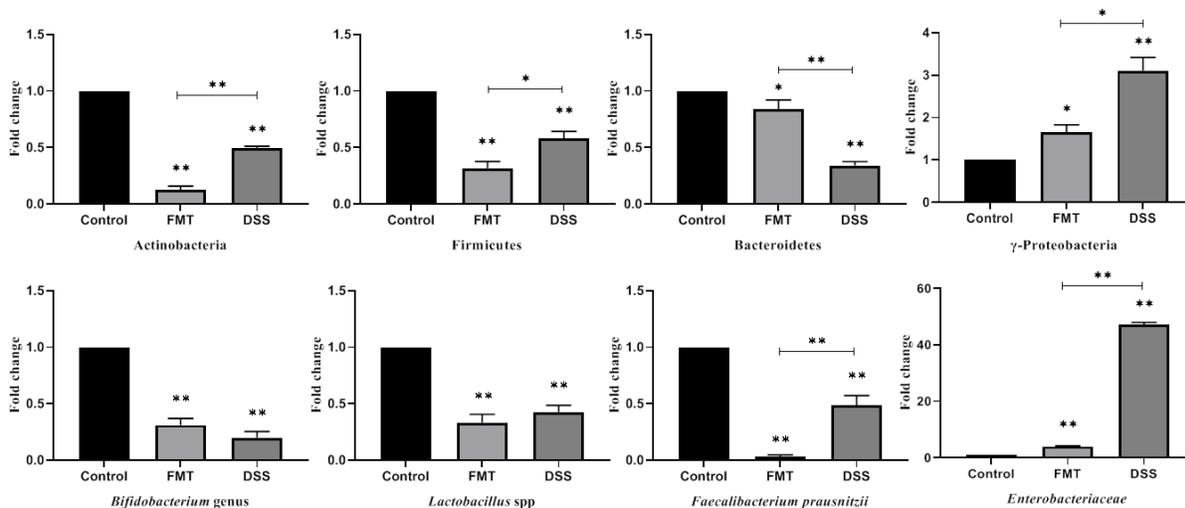
Fig. 4. Changes in the gut microbiota population in the DSS and FMT groups compared to the control group. Statistical analysis was performed using the one-way test ANOVA (*$P < 0.05$, ** $P < 0.001$).

DISCUSSION

The involvement of the gut microbiota in the pathogenesis of IBD is considered an important topic. The microbial flora of the human gut is a dynamic and diverse community of commensal bacteria, fungi, and viruses, of which bacteria constitute the majority, with over 1000 different species [14, 25-26]. More than 90% of bacterial species in the healthy human gut belong to four major phyla: Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria [27, 28, 18]. Alteration of the gut microbiota in the pathology of IBD is widely recognized. However, it is unclear whether such an alteration is the cause or a consequence of intestinal inflammation, and how these bacteria contribute to the pathogenesis of IBD remains unclear [29, 30].

To address these unanswered questions, scientists are increasingly using HMA mice, where the human fecal microbiota is established by microbiota transplantation into germ-free mice [2]. The HMA mouse model is used to test the contribution of a dysbiotic microbiome to a particular pathology by comparing the disease phenotypes of germ-free mice colonized with the fecal microbiota of patients to those of mice colonized with the microbiota of a healthy control [17]. These animals can maintain the human gut's bacterial population and the microbiota's composition and metabolic activities similar to the human gut [31, 32].

In this study, we used conventional mice to create the HMA mouse model. The reason is that germ-free mice have biological limitations, and the gut microbiota is essential for the complete maturation of the gut and host immune system [33-35]. In addition, germ-free mice have atrophic Peyer's patches and fewer B and T cells and IgA-secreting plasma cells in the gut than normal mice [36]. Therefore, we used the PEG solution to empty the intestinal contents of the mice and prepare them for transplantation of human stool. PEG eliminates about 99% of intestinal bacteria in rats [2] and is better than antibiotics in clearing the intestine of microbiota because antibiotics can affect many biological pathways and reduce the number of T lymphocytes in the intestine [37]. Metronidazole impairs goblet cell function by reducing Muc2 production, resulting in a thinner inner mucosal layer. The mucosal layer plays a vital role in some diseases, including inflammatory bowel disease [38].

In this study, we compared two inflammation induction methods. DSS activates inflammatory cells by damaging DNA and inhibiting the repair of intestinal epithelial cells [39]. In biological induction by FMT, the gut microbiota from an IBD patient are established in the mice's gut. The results of this study showed that the induction of IBD in the group receiving DSS changed the population of the intestinal microbiota of mice. On the other hand, the human microbiota with IBD settled in the intestine of mice in the group FMT, and inflammatory disease developed after four weeks. These results suggest a reciprocal relationship between gut microbiota and IBD.

Thus, alterations in the gut microbiota population can cause disease, and IBD can also alter the gut microbiota population. Moreover, the gut microbiota population in both FMT and DSS groups showed almost the same pattern on day 28 of the study, and with the decrease of Actinobacteria and Firmicutes, the population of γ-Proteobacteria increased. Similar to our results, studies have shown that the intestinal microflora of patients with IBD has an increased number of bacteria from the Proteobacteria phyla and a decreased number of bacteria from the Firmicutes and Bacterioidetes compared to healthy individuals [40]. Also, other studies have shown a decrease in bacteria with anti-inflammatory properties and an increase in bacteria with inflammatory properties in patients with IBD compared with healthy individuals [27, 41]. In this study, the FMT group significantly reduced the *F. prausnitzii* population, which plays an anti-inflammatory effect through butyrate production [42].

Additionally, the population of Enterobacteriaceae increased in both FMT and DSS groups, while the population of *Lactobacillus* and *Bifidobacterium* decreased. Enterobacteriaceae can increase intestinal permeability and inflammation by stimulating the secretion of cytokines IL -8, TNF, IL -1β, and the destruction of mucosal junctions [43]. However, IL -1β and IL -6 levels increased in the FMT and DSS groups compared with the control group. Moreover, a study has shown *L. acidophilus* can reduce IL-1β, IL-6, IL-12p70, TNF-α, IL-23, IFN-γ, IL-17A, and IL-21 and increase IL-10 expression levels in mice with chronic colitis [44]. This study showed that the intestinal microbiota population plays an essential role in the level of inflammatory cytokines. With the increase in the population of γ-Proteobacteria phyla, especially the Enterobacteriaceae group, inflammatory cytokines also increased and caused inflammation in FMT and DSS groups.

This study had some limitations: We did not have NGS facilities to compare the gut microbiota population of the FMT group with the gut microbiota of the human donor. In addition, we did not compare our gut cleansing strategy (PEG) with the other models (germ-free or antibiotic treatment).

In conclusion, gut microbiota plays a vital role in inflammatory diseases such as IBD. HMA mice are an excellent model to study the relationship between IBD and gut microbiota, and in this study, we could induce inflammation in this animal model using human stool with IBD.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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