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Nutrition (original Investigation: basic research)

Enteral supplement enriched with glutamine, fiber, and oligosaccharide
attenuates experimental colitis in mice

Running head: Effects of GFO supplement on colitis.

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ABSTRACT

Objective: Ulcerative colitis (UC) is a chronic recurrent disease characterised by acute inflammation of the colonic mucosa. In Japan, a dietary supplementation product enriched with glutamine, dietary fiber, and oligosaccharide (GFO) is widely applied for total parenteral nutrition support. All three of these components have been suggested to improve intestinal health. In this study, we investigated to find if GFO has suppressive effects on mucosal damage in UC in a mouse experimental model.

Methods: C57BL/6 mice received 2.5% of dextran sulfate sodium (DSS) in drinking water for 5 days to induce colitis. They then were given 0.25 mL of GFO or 20% of glucose solution twice daily for 10 days. Another set of mice receiving unaltered drinking water was used as normal control.

Results: Body weight loss and disease activity index were significantly lower in GFO-treated mice compared to those in glucose-treated mice (p < 0.05). DSS-induced reduction of colon length was significantly alleviated in GFO-treated mice compared to that in glucose-treated mice (p < 0.01). In addition, histological findings revealed that intestinal inflammation was significantly attenuated in mice treated with GFO. Furthermore, treatment with
GFO significantly inhibited DSS-induced increase in mRNA expression of interleukin-1 beta.

**Conclusion:** These results suggest that GFO has potential therapeutic value as an adjunct therapy for UC.
Key Words

GFO, ulcerative colitis, DSS, IL-1β, glucagon-like peptide
Introduction

Ulcerative colitis (UC) is a major form of inflammatory bowel disease (IBD) characterised by acute inflammation of colonic mucosa leading to rectal bleeding and diarrhea. Patients with UC repeat chronic relapsing-remitting inflammatory conditions, and the chronic inflammation of colon is reported to promote the risk of colonic cancer [1]. Aminosalicylates and corticosteroids are applied as standard medications to reduce such intestinal mucosal inflammation [2]. However, in some cases, these medications have a variety of side effects and are not effective enough to achieve complete remission of UC [3]. More effective intervention with fewer side effects is required for better UC treatment.

GFO is a commercial enteral supplement enriched with glutamine, dietary fibers, and oligosaccharide. The medicinal supplementation product is widely applied as enteral nutrition support and perioperative nutrition management. The dietary fibers contained in GFO are polydextrose and hydrolyzed guar gum, which are water-soluble. The oligosaccharide in GFO is lactosucrose, a kind of galacto-oligosaccharide. Both the fiber and the oligosaccharide are prebiotics that stimulate growth and activity of gastrointestinal microflora [4,5]. Previous
studies suggested that abnormal regulation of the mucosal immune response toward commensal bacterial flora plays an important role in the pathogenesis of UC [6]. Some reports find that administration of prebiotics such as inulin [7], resistant starch [8], lactulose [9], oligofructose [10] and goat's milk oligosaccharides [11] effectively decrease severe clinical symptoms and prolong remission of intestinal mucosal inflammation by improving the intestinal microbial balance in rats with experimental colitis. In a human trial involving ten patients with Crohn's disease (CD), administration of a fructo-oligosaccharide significantly reduced CD activity indices [12]. These data suggest that modification of intestinal microflora by GFO might be useful adjunctive treatment for UC. Glutamine, the other of the three constituents of GFO, is a major oxidative fuel for the intestine that supports intestinal mucosal functions [13]. Previous studies have shown that glutamine has anti-inflammatory effects on both intestinal epithelial cells of rodent IBD models [14,15,16] and cell lines [17] and biopsy samples [18] of human gut. The aim of the present study was to investigate the hypothesis that GFO has multiple therapeutic effects on UC by using mice with DSS-induced colitis.
Materials and methods

Animals

C57BL/6 male mice weighting 25-30 g and 11-wk-old (Shimizu Laboratory Supplies Co. Ltd., Japan) were used in this study. The mice were housed in a specific pathogen-free environment at ambient temperature of 23 °C with a 12-h light-dark cycle. They were given a standard chow and tap water ad libitum. All procedures were approved by the Animal Care Committee of Kyoto University Graduate School of Medicine.

Induction of colitis and GFO treatment

Experimental colitis was induced by oral administration of DSS (M 5000; Wako Pure Chemicals, Osaka, Japan) [19,20,21]. The mice received 2.5% (w/v) DSS in drinking water for 5 days, from day 0 to day 4, as previously described [19]. A pack of GFO (Otsuka Pharamaceutical Co., Ltd. Japan) was dissolved in 50 ml of distilled water. The calories and nutrient contents of the GFO and glucose solution are shown in Table 1. The mice received 0.25 mL of GFO or 20% glucose solution twice daily for 10 days, from day 0 to day 9. Another set of animals receiving unaltered drinking water were used for normal
control. On the morning after 10 full days, all mice were euthanized by cervical dislocation and colon samples from each of the animal groups were obtained for histological evaluation and RNA extraction.

_Determination of disease activity index_

Clinical activity of colitis was evaluated by stool consistency, hemoccult test (ColoScreen occult blood test, Helena Laboratories, Beaumont, TX, USA), and changes in body weight during the experimental period to determine the disease activity index (DAI), as described previously [20,22]. DAI scoring was as follows: weight loss (0, no change or positive change; 1, 1–5%; 2, 6–10%; 3, 11–20%; 4, over 21%), stool consistency (0, well-formed pellets; 2, loose stools; 4, diarrhea), fecal blood (0, negative hemoccult test; 2, positive hemoccult test; 4, gross bleeding). DAI ranging from 0 to 4 was the sum of scores for these parameters divided by three.

_Tissue sample preparation_

After measuring the length of the large intestine as an indirect marker of inflammation, it was washed in physiologic saline. Segments were taken from
0.5 cm of distal colon for analysis of mRNA expression levels of TNF-α (tumor necrosis factor-alpha) and IL-1β (interleukin-1 beta). Colon samples were cooled with liquid nitrogen and stored at -80 °C until use. The rest of the central colon was fixed in Bouin's fluid for histological analysis.

**mRNA assessment by semiquantitative reverse transcription-polymerase chain reaction (PCR)**

Total RNA from distal colon and proximal colon was extracted using TRIzol solution (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions. First strand cDNA was synthesized by SuperScript™ II Reverse Transcriptase system (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions. SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was prepared for real-time quantitative PCR using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR was performed for 10 min at 90 °C, followed by 50 cycles at 95 °C for 15 sec and at 60°C for 1 min. The signal of each product was standardized against the β-actin signal for each sample. Primer pairs for TNF-α, IL-1β, and β-actin are shown as follows. TNF-α (300 bp) :

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5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3' and 5'-CCG AAT TCA CTG GAG CCT CGA ATG T

A-3' and 5'-CTG CAC TAC AGG CTC CGA ATG T

3' and 5'-ATA TCG CTG CGC TGG TCG TC

Immunohistochemistry

After fixing, the samples of colon were embedded in paraffin, and 3 μm sections stained with Hematoxylin and eosin (H&E) and Periodic acid-Schiff reaction (PAS) were prepared. PAS stains the acidic mucin of colonic goblet cells a turquoise blue color. In active UC, the amount of mucin is reduced, particularly in more severely ulcerated areas [23].

Single oral administration of GFO

C57BL/6 mice were fasted for 16 h and orally administered 12.5 g/kg body weight of GFO or 1.5g/kg body weight of glucose (same carbohydrate calorie of GFO). Blood samples were collected at 0, 30, and 60 min after glucose loading or GFO ingestion and were centrifuged at 1800 g at 4ºC for 10 min. After collecting supernatant of the samples, plasma and serum were stocked at –80ºC.
The plasma glucose levels were measured by the glucose oxidase method.

Serum insulin levels were measured by enzyme-linked immunosorbent assay.

Total GIP and total GLP-1 levels were measured using mouse GIP ELISA kit (Linco Research, St Charles, MO, USA) and mouse GLP-1 ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA), respectively.

Statistical analysis

All data were expressed as mean ± SE. Statistical analysis was performed using Student’s t-test or one-way ANOVA with Fisher’s PLSD’s post-hoc test, where appropriate, by using the SPSS 11.0 software (SPSS, Chicago IL, USA).

Significant difference was considered to be present at $p < 0.05$. 
Results

Symptomatic and macroscopic changes

Changes in body weight

Control mice did not show significant changes in body weight throughout the experiment. Glucose-treated mice administered DSS showed a gradual decrease in body weight, one of the major symptoms of colitis [22]. The decrease in body weight in GFO-treated mice was significantly prevented compared to that in glucose-treated mice (Fig. 1).

Disease activity index (DAI)

Mice exposed to DSS during 5 days developed colitis characterized by decreased body weight (Fig. 1), loose stools, and fecal blood. DAI scores throughout the experimental period are shown in Figure 2. DAI scores were significantly lower in GFO-treated mice compared to those in glucose-treated mice on day 6, day 7 and day 9.

Colon length

Shortening of colon is generally considered a hallmark of DSS-induced
colonic damage. Colon shortening following DSS-induced colitis was reduced in GFO-treated mice compared to that in glucose-treated mice (Fig. 3).

**Histological appearance**

*Hematoxylin and eosin (H&E) stain*

Control mice showed normal colonic structure (Fig. 4A and 4D), whereas the colon of glucose-treated mice administered DSS showed developed colonic inflammation characterized by edema, massive inflammatory cell infiltration (mononuclear cells, neutrophils, and eosinophils), mucosal ulceration, and crypt destruction (Fig. 4B and 4E). In contrast, although there was mild thickening of the colon wall, GFO-treated mice administered DSS showed much less infiltration of inflammatory cells and exhibited a relatively maintained mucosal structure, indicating that colonic inflammation was attenuated in GFO-treated mice (Fig. 4C and 4F).

*Periodic acid-Schiff (PAS) stain*

Control mice exhibited a high intensity of PAS staining of goblet cells and epithelial surface mucin through the length of the colon samples (Fig. 5A and
5D). On the other hand, glucose-treated DSS mice showed strongly reduced PAS-positive cells (Fig. 5B and 5E). In contrast, the intensity of PAS staining was substantially similar in control and GFO-treated DSS mice, indicating that GFO attenuated mucosal damage and maintained the architecture of normal colonic mucosa (Fig. 5C and 5F).

**Expression of proinflammatory cytokines**

mRNA expressions of proinflammatory cytokines, IL-1β and TNF-α, in colonic mucosa were evaluated by real-time quantitative PCR (Fig. 6). IL-1β mRNA levels in GFO-treated mice were significantly decreased compared to those in glucose-treated mice ($p < 0.01$). mRNA expression levels of TNF-α tended to be lower in GFO-treated mice compared to those in glucose-treated mice, although the difference was not significant.

**Effect of GFO on GIP and GLP-1 secretion**

Fasting concentrations of glucose, insulin, and GIP and GLP-1 were similar in glucose-administered mice and GFO-administered mice. After administration of glucose and GFO, the levels of glucose, insulin and GIP were significantly
higher in glucose-administered mice than in GFO-administered mice (Fig. 7A, 7B and 7C). In contrast, GLP-1 levels were significantly higher in GFO-administered mice than in glucose-administered mice (Fig. 7D).


**Discussion**

In the present study, we show therapeutic effects of GFO, a dietary supplementation product enriched with glutamine, dietary fiber, and oligosaccharide, on intestinal damage in mice with DSS-induced colitis. DSS has been utilized to experimentally induce acute and chronic models of colitis. Following administration of DSS solution, the mice show signs of diarrhea, gross rectal bleeding, weight loss, and distinctive histological phenotypes similar to those of UC in human subjects [21]. In this study, body weight loss, reduction of colon length, increased disease activity index, and histological abnormalities in DSS-treated mice were significantly suppressed in GFO-treated mice compared to those in vehicle-treated mice, demonstrating that GFO prevents the development of experimental UC.

GFO is composed of glutamine, dietary fiber, and oligosaccharide. Each component has been reported to have beneficial effects on intestinal condition [4,5,13,24]. It is well known that both fiber and oligosaccharide are prebiotics that produce short-chain fatty acid (SCFAs) such as acetate, propionate, and butyrate in colon by anaerobic bacterial fermentation [25]. One of the most abundant SCFAs, butyrate, has been considered a major fuel source for the
colonocytes [26] and has been shown to increase mucosal growth and epithelial proliferation in both small and large intestine [27,28]. Several studies have reported that IBD is associated with impairment of SCFA production [29], and that the butyrate enema is highly effective in treating active UC [30]. Therefore, it is possible that GFO has a therapeutic effect by reducing mucosal damage through an incremental increase of SCFA production. In addition, glutamine is a major oxidative fuel for the intestine that supports intestinal mucosal functions [13]. Baskerville et al. reported that animals receiving intravenous infusion of glutaminase developed diarrhea, mild villous atrophy, mucosal ulcerations, and intestinal necrosis [31]. Additionally, supplement of glutamine to total parenteral nutrition solution significantly maintained height of intestinal villi, thicknesses of mucosa, and integrity of the intestinal wall in endotoxemic rats [32].

UC is closely associated with increased activation of intestinal immune cells, and most of the medications are focused on reducing the inflammation [2]. Pro-inflammatory cytokines, such as TNF-α and IL-1β, were increased in the colonic mucosa of UC patients [33,34]. Some reports find that administration of prebiotics, including lactulose, oligofructose, and goat’s milk oligosaccharides,
produce intestinal anti-inflammatory effects in rats with experimental colitis [9,10,11]. Glutamine also has been shown to have anti-inflammatory effects in intestinal epithelial cells of rodents in vivo [14,15,16], in a human intestinal cell line [17], and in primary human intestinal epithelial cells [18]. In the present study, GFO treatment significantly inhibited the DSS-induced increase in mRNA expression of IL-1β. Moreover, histological findings indicate that infiltration of lymphocytes into mucosal tissue was significantly attenuated in GFO-treated mice. These findings suggest that GFO attenuates mucosal damage of UC partly through its anti-inflammatory effects.

When nutrients pass through the intestine, enteroendocrine cells secrete many kinds of gastrointestinal hormone including gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and GLP-2 [37]. Both GLP-1 and GLP-2 are proglucagon-derived peptides, and are simultaneously secreted in response to meal ingestion from intestinal L-cells, which are located mainly in the distal ileum and colon [37]. GLP-1 binds to its receptor on the surface of pancreatic β-cells to stimulate insulin secretion. On the other hand, GLP-2 has various biological effects on the intestine, such as stimulation of intestinal mucosal growth [38], intestinal nutrient transport [39], intestinal blood flow [40],
and inhibition of crypt cell apoptosis [41]. In addition, human GLP-2 is reported to reduce the severity of colonic injury in a murine model of DSS-induced colitis [42]. *In vivo* animal studies show that some kinds of dietary fiber and SCFAs are potent stimulators of GLP-2 secretion [28, 43]. It is also accepted that glutamine promotes GLP-1 secretion [35, 36]. In this context, we evaluated the effect of single oral administration of GFO on plasma GLP-1 levels and plasma GIP levels in mice. GIP levels were significantly higher in glucose-administered mice than in GFO-administered mice probably because absorption of oligosaccharide contained in GFO is slower than that of glucose. In contrast, GLP-1 levels were extremely higher in GFO-administered mice as compared to those in glucose-administered mice at 30 min and 60 min after the administration, indicating that glutamine contained in GFO stimulates GLP-1 secretion. Therefore, we may speculate that GFO stimulates GLP-2 secretion concomitantly with GLP-1 secretion, and attenuates the development of mucosal damage of UC via enhancement of GLP-2 secretion, although direct measurement of plasma GLP-2 levels is required in future.
Conclusion

We demonstrate that GFO supplement (combination of glutamine, dietary fiber, and oligosaccharide) has therapeutic effects on DSS-induced experimental colitis in mice. Although further studies are needed to clarify the mechanism of GFO action, GFO may be a useful adjunct therapy for UC.
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References


Hubert-Buron A, Leblond J, Jacquot A, Ducrotte P, Dechelotte P, Coeffier M. Glutamine pretreatment reduces IL-8 production in human intestinal


[28] Bartholome AL, Albin DM, Baker DH, Holst JJ, Tappenden KA. Supplementation of total parenteral nutrition with butyrate acutely increases structural aspects of intestinal adaptation after an 80% jejunoileal resection in


[40] Drucker DJ, Yusta B, Boushey RP, DeForest L, Brubaker PL. Human


Figure legends

Figure 1 | The effects of GFO on body weight change in mice with DSS-induced colitis.

Serial changes in body weight throughout the experiment in normal control mice (n = 4; closed diamonds), glucose-treated mice with DSS-induced colitis (n = 5; closed triangles) and GFO-treated mice with DSS-induced colitis (n = 6; open triangles). Each value represents the mean ± SE. **p < 0.01 between control mice and glucose-treated mice with DSS-induced colitis. †p < 0.05 and ‡p < 0.01 between control mice and GFO-treated mice with DSS-induced colitis. #p < 0.05 and ##p < 0.01 between glucose-treated and GFO-treated mice with DSS-induced colitis.

Figure 2 | Disease activity index (DAI) of normal control mice and DSS mice treated with or without GFO.

DAI combining the scores of bleeding, weight loss and stool consistency is divided by 3. Normal control mice (n = 4; closed diamonds), glucose-treated mice with DSS-induced colitis (n = 5; closed triangles) and GFO-treated mice with DSS-induced colitis (n = 6; open triangles). Each value represents the mean
± SE. *\( p < 0.05 \) and **\( p < 0.01 \) between control mice and glucose-treated mice with DSS-induced colitis. †\( p < 0.01 \) between control mice and GFO-treated mice with DSS-induced colitis. #\( p < 0.05 \) between glucose-treated and GFO-treated mice with DSS-induced colitis.

**Figure 3** | Colon length of normal control mice and DSS mice treated with or without GFO.

Normal control mice (\( n = 4 \)), glucose-treated mice with DSS-induced colitis (\( n = 5 \)) and GFO-treated mice with DSS-induced colitis (\( n = 6 \)). Each value represents the mean ± SE. **\( p < 0.01 \) between control mice and glucose-treated mice with DSS-induced colitis. #\( p < 0.01 \) between glucose-treated and GFO-treated mice with DSS-induced colitis.

**Figure 4** | Histological findings of colon stained with Hematoxylin and eosin (H&E).

Normal control mice (A and D), glucose-treated mice with DSS-induced colitis (B and E), and GFO-treated mice with DSS-induced colitis (C and F). Original magnification \( \times 8 \) (A, B, and C) and \( \times 48 \) (D, E, and F).
**Figure 5** | Histological findings of colon stained with Periodic acid-Schiff (PAS). Normal control mice (A and D), glucose-treated mice with DSS-induced colitis (B and E), and GFO-treated mice with DSS-induced colitis (C and F). Original magnification; \( \times 8 \) (A, B, and C) and \( \times 48 \) (D, E, and F).

**Figure 6** | Real-time quantitative PCR analysis of IL-1\( \beta \) and TNF-\( \alpha \) mRNA expressions in the colon.

(A) IL-1\( \beta \) mRNA expression in distal colon. (B) TNF-\( \alpha \) mRNA expression in distal colon. Each value represents the mean ± SE (\( n = 3-6 \)). ##\( p < 0.01 \) between glucose-treated and GFO-treated mice with DSS-induced colitis.

**Figure 7** | The effects of GFO on GIP and GLP-1 secretion

The levels of plasma glucose (A), Serum insulin (B), plasma total GIP (C) and plasma total GLP-1 (D) after oral ingestion of glucose (\( n = 7 \); closed circle) and GFO (\( n = 7 \); open circle). Each value represents the mean ± SE. *\( p < 0.05 \) and **\( p < 0.01 \) between glucose-administered mice and glucose-administered.
Figure 1

Body weight (g)

- control
- glucose
- GFO

Days

**

#
Figure 2

The figure shows a line graph comparing DAI scores across different groups over a period of 9 days. The groups include control, glucose, and GFO. The x-axis represents the number of days, ranging from 0 to 9, while the y-axis represents the DAI score, ranging from 0 to 4.

- Control group shows a steady increase in DAI score with a peak around day 5, followed by a decline.
- Glucose group has a sharp increase around day 4, followed by a plateau and a slight decline.
- GFO group demonstrates a gradual increase with a peak around day 6, followed by a decline.

Significant differences are indicated by asterisks and hashtags. Two asterisks (*) indicate a significant difference, while a hashtag (#) indicates a trend.
Figure 3

Colon length (cm)

- Control
- Glucose
- GFO

Significance levels: ** and ##
Figure 6

A

IL-1β (ratio to β-actin)

control glucose GFO

B

TNF-α (ratio to β-actin)

control glucose GFO
Figure 7

A. Plasma glucose (mg/dl) over time (0, 30, 60 minutes).

B. Insulin (pg/ml) over time (0, 30, 60 minutes).

C. GLP (pg/ml) over time (0, 30, 60 minutes).

D. GLP-1 (pg/ml) over time (0, 30, 60 minutes).