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Beneficial and preventive effect of chitin nanofibrils in a dextran sulfate sodium-induced acute ulcerative colitis model

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ABSTRACT

Chitin nanofibrils, which are prepared from dried crab shells by a grinding method, are newly developed natural materials with uniform widths of approximately 10–20 nm. The bioactivities of chitin nanofibrils have not been investigated. In this study, we examined the preventive effects of chitin nanofibrils in a mouse model of dextran sulfate sodium (DSS)-induced acute ulcerative colitis. The results indicated that chitin nanofibrils improved clinical symptoms and suppressed ulcerative colitis. Furthermore, chitin nanofibrils suppressed myeloperoxidase activation in the colon and decreased serum interleukin-6 concentrations. Conversely, chitin powder did not suppress DSS-induced acute ulcerative colitis. Our results suggested that chitin nanofibrils have potential as a functional substance for inflammatory bowel disease patients.

1. Introduction

Chitin (β-(1–4)-poly-*N*-acetyl-D-glucosamine) is widely distributed in nature and is the second abundant polysaccharide after cellulose (Gupta, 2011). The nonspecific antiviral and antitumor activities of chitin or chitin derivatives were described 2 decades ago (Shibata, Foster, Bradfield, & Myrvik, 2000; Shibata et al., 2001). Recently, it was suggested that the size of chitin influences its effects on immune cells (Da Silva, Hartl, Liu, Lee, & Elias, 2008; Lee, Da Silva, Lee, Hartl, & Elias, 2008). Chitins in crustacean shells are highly crystalline: in the α-chitin, the microfibers consist of nanofibrils approximately 2–5 nm in diameter and 300 nm in length embedded in a protein matrix (Chen, Lin, McKittrick, & Meyers, 2008; Raabe et al., 2006). Isolated chitin nanofibrils are considered to have great potential for applications in tissue engineering scaffolds, drug delivery, and wound dressing (Muzzarelli et al., 2007).

The methods employed to prepare chitin nanofibrils include acid hydrolysis (Gopalan & Dufresne, 2003; Revol & Marchessault, 1993), and ultrasonication of squid pen β-chitin under acidic conditions for the preparation of 3–4 nm wide chitin nanofibrils of relatively lower cristallinity (Fan, Saito, & Isogai, 2008b). Recently, Ifuku et al. (2009) demonstrated that α-chitin nanofibrils with uniform widths of approximately 10–20 nm could be prepared from crab chitin flakes by a grinding method leading to fiber disassembly and high yield. However, no study has described the *in vivo* effects of chitin nanofibrils after oral administration.

Inflammatory bowel disease (IBD) is common and refers to a group of conditions characterized by inflammation in the intestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) account for the majority of the cases of these conditions (Morrison, Headon, & Gibson, 2009). Currently, some experimental animal models are used in IBD research. A model of dextran sulfate sodium (DSS)-induced colitis is one common model of IBD, in which animals develop acute and chronic colitis resembling UC (Melgar, Karlsson, & Michaëlsson, 2005).

Glucosamine hydrochloride is likely to suppress the cytokine-induced activation of intestinal epithelial cells *in vivo*, thereby possibly exerting anti-inflammatory effects in a DSS-induced rat UC model (Yomogida et al., 2008). However, no study has investigated the effects of chitin or chitin deliveries in a DSS-induced UC model. The aim of this study was to evaluate the preventive effects of chitin nanofibrils compared with those of chitin in a mouse model of DSS-induced acute UC.

2. Experimental

2.1. Reagents

DSS (molecular weight, 36–50 kDa; reagent grade) was purchased from MP Biomedicals LLC (Solon, OH, USA). Chitin powder

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Table 1
Scoring of inflammation based on clinical parameters during treatment.

Score	Weight loss (% of initial weight)	Diarrhea score	Visible fecal blood
0	<5%	Normal	Normal
1	5–10%	Slightly loose feces	Slightly bloody
2	10–20%	Loose feces	Bloody
3	>20%	Water diarrhea	Blood in entire colon

was purchased from Nacalai Tesque (Lot No.: M0A3811; Kyoto, Japan). The average diameter of chitin powder was approximately 200 μm. Chitin nanofibrils gel (1%, pH 3; hereafter referred to as chitin nanofibrils) was prepared using a previously described method (Ifuku et al., 2009). A chitin powder suspension (1%, pH 3; hereafter referred to as chitin-PS) was prepared; the percentage of deacetylated chitin-PS in the suspension was 3.9%.

2.2. Animals

Sixty-eight C57BL/6 mice (female, 6 weeks old) were purchased from CLEA Japan (Osaka, Japan). The animals were maintained under conventional conditions. The use of these animals and the procedures they underwent were approved by the Animal Research Committee of Tottori University.

2.3. Study design

Mice ($n = 68$) were randomized into six groups: the control (+) group was administered only DSS ($n = 17$); the control (−) group was administered tap water ($n = 5$); the chitin nanofibrils (+) group was administered chitin nanofibrils and DSS ($n = 17$); the chitin nanofibrils (−) group was administered only chitin nanofibrils ($n = 7$); the chitin-PS (+) group was administered chitin-PS and DSS ($n = 16$); and the chitin-PS (−) group was administered only chitin-PS ($n = 6$). To induce colitis, mice were administered 3% DSS *ad libitum* for 6 days from day 0 to day 6. For 7 days before starting the administration of DSS, chitin nanofibrils (+), chitin nanofibrils (−), chitin-PS (+), and chitin-PS (−) groups were administered 0.1% chitin nanofibrils or chitin-PS dissolved in tap water *ad libitum*. Blood collection and colon sampling were done on days 3 and 5 in control (+), chitin nanofibrils (+), and chitin-PS groups (each $n = 5$) and on day 6 in all groups (each group: $n = 5$–7).

2.4. Clinical analysis

UC was evaluated using the disease activity index (DAI) as described by Melgar et al. (2005) with a slight modification by using the parameters of body weight loss, stool consistency, and bleeding (Table 1). The length and weight of the colon were measured, and tissue obtained from each colon was processed for further analysis.

2.5. Histological evaluation of colitis

Colon tissues were fixed in 10% buffered formalin. Thin sections (3 μm) were made from each sample for histological observation after hematoxylin–eosin staining. Each section was examined microscopically, and histological scoring was performed as described by Ohkawara et al. (2005). In brief, tissue damage was classified using 6 grades: 0: normal mucosa; 1: infiltration of inflammatory cells; 2: shortening of the crypt by less than half of the height; 3: shortening of the crypt by more than half of the height; 4: crypt loss; 5: destruction of epithelial cells. Histological scoring was performed in 10 fields at 100× magnification using 3 mice in each group. The mean scores for 30 fields were considered the histological score for each group.

2.6. Myeloperoxidase (MPO) staining

MPO staining, which is a marker of leukocyte invasion into tissue (Schindhelm, van der Zwan, Teerlink, & Scheffer, 2009), was performed in a routine manner as described previously (Ohtsuka, Lee, Stamm, & Sanderson, 2001). Counts of MPO-positive cells in the submucosal layer were performed in 20 fields at 400× magnification using 3 mice in each group. The mean scores for 60 fields were considered the number of MPO-positive cells for each group.

2.7. Measurements of serum IL-6 concentrations

Serum IL-6 was quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) using a Mouse IL-6 ELISA kit (Thermo SCIENTIFIC, Rockford, IL, USA) according to the manufacturer's protocol.

2.8. Statistical analysis

The data are expressed as the mean ± S.E. Statistical analyses were performed using 1-way ANOVA followed by Tukey–Kramer's test. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Effects of chitin nanofibrils on DAI in DSS-induced acute UC mice

Weight loss, loose stools, and bleeding were observed on day 3 in the control (+) and chitin-PS (+) groups and on day 4 in the chitin nanofibrils (+) group (Table 2). The chitin nanofibrils (+) group exhibited a significantly reduced DAI on days 4–6 compared with that in the control (+) group ($p < 0.05$) and on days 5 ($p < 0.01$) and 6 ($p < 0.05$) compared with that in the chitin-PS (+) group (Table 2). No change of DAI was observed in the control (−), chitin nanofibrils (−), and chitin-PS (−) groups (date not shown).

3.2. Effects of chitin nanofibrils on colon length and the colon weight/length ratio in DSS-induced acute UC mice

The administration of 3% DSS shortened colon length and increased the colon weight/length ratio (mg/cm) in C57BL/6 mice (Melgar et al., 2005). In the chitin nanofibrils (+) group, colon lengths were significantly longer than those in the control (+) group on days 3, 5, and 6 ($p < 0.05$ for days 3 and 5, $p < 0.01$ for day 6). Moreover, colon length in the chitin nanofibrils (+) group was significantly longer than that in the chitin-PS (+) group on days 3, 5, and 6 ($p < 0.05$ for days 3 and 5, $p < 0.01$ for day 6, Table 3a). No change of colon length was observed in the control (−), chitin nanofibrils (−), and chitin-PS (−) groups (data not shown).

The colon weight/length ratio was decreased in the chitin nanofibrils (+) group on days 5 and 6 compared with that in the control (+) group. On day 5, the colon weight/length ratios of the chitin nanofibrils (+) and chitin-PS (+) groups were significantly decreased compared with that of the control (+) group ($p < 0.05$). On day 6, the colon weight/length ratio of the chitin nanofibrils (+) group was significantly decreased compared with those of the control (+) and chitin-PS (+) groups ($p < 0.01$, Table 3b). No change of the weight/length ratio was observed in the control (−), chitin nanofibrils (−), and chitin-PS (−) groups (date not shown).

Table 2
Effect of chitin nanofibrils administration on the DAI in DSS-induced acute UC mice.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.1 ± 0.4	3.6 ± 0.3	6.9 ± 0.5
Chitin nanofibrils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1*	2.3 ± 0.3*,**	5.1 ± 0.4*,***
Chitin-PS	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.0 ± 0.4	4.0 ± 0.5	7.0 ± 0.8

* $p < 0.05$ compared with chitin nanofibrils (+) and control (+) groups.
** $p < 0.01$ compared with chitin nanofibrils (+) and chitin-PS (+) groups.
*** $p < 0.05$ compared with chitin nanofibrils (+) and chitin-PS (+) groups.

Table 3
Effect of chitin nanofibrils administration on colon length and the colon weight/length ratio (mg/cm) in DSS-induced acute UC mice.

	(−)	Day 3	Day 5	Day 6
(a)				
Control	7.4 ± 0.1	6.7 ± 0.2	5.1 ± 0.2	4.9 ± 0.1
Chitin nanofibrils	7.3 ± 0.1	7.5 ± 0.2*,†	7.0 ± 0.1**,††	6.3 ± 0.1**,††
Chitin-PS	7.4 ± 0.2	6.6 ± 0.2	5.7 ± 0.1	5.3 ± 0.2
(b)				
Control	26.8 ± 0.8	31.9 ± 1.5	50.2 ± 3.9	40.8 ± 2.7
Chitin nanofibrils	29.8 ± 0.3	29.8 ± 0.5	38.4 ± 1.2*	33.3 ± 1.2*,†
Chitin-PS	28.8 ± 1.7	27.2 ± 0.5	37.4 ± 1.4*	41.7 ± 2.5

* $p < 0.05$.
** $p < 0.01$ compared with the control (+) on the respective day.
† $p < 0.05$.
†† $p < 0.01$ compared with the control (+) on the respective day.

3.3. Effects of chitin nanofibrils on histological changes in DSS-induced acute UC mice

Damage in the intestinal mucosa was microscopically evaluated by histological scoring. No histological change was observed in the control (−), chitin nanofibrils (−), and chitin-PS (−) groups. On day 3 in the control (+), chitin nanofibrils (+), and chitin-PS (+) groups, inflammatory cell infiltration was observed. On day 5, erosions, shortening or destruction of the crypt, and edema were observed in the control (+) and chitin-PS (+) groups. Some erosions were observed on day 5 in the chitin nanofibrils (+) group; however, shortening or destruction of the crypt was markedly suppressed, and edema was slightly suppressed. On day 6 in the control (+) and chitin-PS (+) groups, severe erosions, crypt destruction, and edema were observed; moreover, some ulcers were observed. In the chitin nanofibrils (+) group, erosions, crypt destruction, and edema were markedly suppressed compared with those in the control (+) and chitin-PS (+) groups (Fig. 1).

In addition, the severity of tissue damage was evaluated by histologically scoring hematoxylin–eosin-stained sections. The histological scores of the chitin nanofibrils (+) group were significantly decreased on day 5 compared with those of the control (+) group ($p < 0.01$) and on day 6 compared with those of the control (+) and chitin-PS (+) groups ($p < 0.01$, Fig. 2).

3.4. Effects of chitin nanofibrils on the number of MPO-positive colon cells in DSS-induced acute UC mice

The results of MPO staining on day 6 are shown in Fig. 3, and the numbers of MPO-positive cells in each group are shown in Fig. 4. In the control (−), chitin nanofibrils (−), and chitin-PS (−) groups, 0–1 MPO positive cells were observed per 400× field (data not shown). In the control (+) and chitin nanofibrils (+) groups, the numbers of MPO-positive cells gradually increased from day 3 to day 6. In the chitin nanofibrils (+) group, however, the numbers of MPO-positive cells were significantly lower than those in the control (+) group on days 3, 5, and 6 ($p < 0.01$). Moreover, significantly fewer MPO-positive cells were counted in the chitin nanofibrils (+) group than in the chitin-PS (+) group on days 3, 5, and 6 ($p < 0.01$ for days 3 and 5, $p < 0.05$ for day 6).

3.5. Effects of chitin nanofibrils on serum IL-6 concentrations in DSS-induced acute UC mice

On day 5, the serum IL-6 concentration was significantly lower in the chitin nanofibrils (+) group (85.8 ± 1.2 pg/ml) than in the control (+) group (237.1 ± 41.9 pg/ml) ($p < 0.01$).

4. Discussion

In this study, we evaluated the preventive effects of chitin nanofibrils in a mouse model of DSS-induced experimental acute UC. Chitin nanofibrils improved clinical symptoms, colon inflammation, and histological tissue injury in the DSS-induced acute UC mouse model. As MPO is a marker of oxidative stress, high MPO activities were observed in a DSS-induced UC model (Naito, Takagi, & Yoshikawa, 2007; Schindhelm et al., 2009). IL-6 is a central cytokine in IBD that contributes to enhanced T-cell survival and apoptosis resistance in the lamina propria at sites of inflammation (Mudeter & Neurath, 2007). Thereby, chitin nanofibril suppressed inflammation caused by acute UC by suppressing the MPO-mediated activation of inflammatory cells such as leukocytes and decreasing serum IL-6 concentrations.

Chitin-PS is a solid, whereas chitin nanofibrils is a gel. Solid chitin induces chemotactic neutrophil migration in a concentration-dependent manner and activates the complement system in an

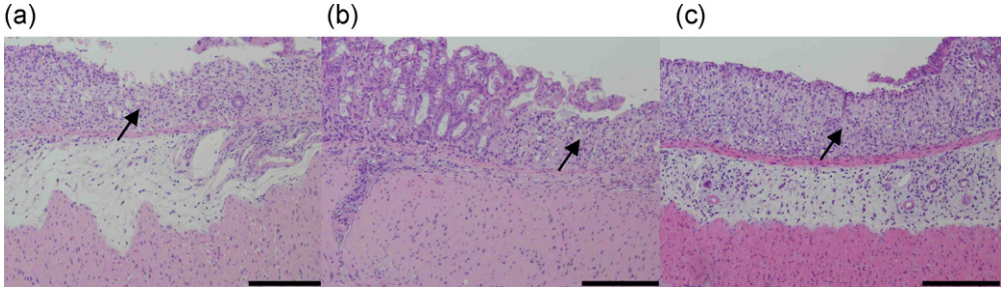

Fig. 1. Effect of chitin nanofibrils administration on histopathological changes in DSS-induced acute UC mice. The colon was fixed, and tissue sections were stained with hematoxylin and eosin. Data are presented for 1 mouse each from the control (+) (a), chitin nanofibrils (+) (b), and chitin-PS (c) groups on day 6. Erosion indicated by allow. Bar = 100 μm.

alternative pathway depending on its degree of deacetylation (Minami, Suzuki, Okamoto, Fujinaga, & Shigemasa, 1998; Usami et al., 1994). However, 50% deacetylated chitin in a homogeneous system becomes water-soluble (Kurita, Sannan, & Iwakura, 1977) and loses its effects on the complement system (Suzuki et al., 2000). Whether chitin nanofibrils can activate the complement system is unclear; however, the water solubility (dispersion) of chitin nanofibrils is resembles that of 50% deacetylated chitin. We speculate that the differences in the findings between chitin-PS and chitin nanofibrils groups are mostly due to the difference in their hydrophilicities.

It is suggested that the size of chitin determines its effects on immune cells (Da Silva et al., 2008). This can readily be observed via comparisons of large chitin polymers that are biologically inert and intermediately sized (40–70 μm) fragments that trigger inflammation and inflammatory cytokine production (Lee et al., 2008). The chitin powder used in this study has a diameter of approximately 200 μm. In our results, chitin-PS had no suppressive effects on UC model mice. It is suggested that the preparation of chitin nanofibrils confers new bioactivity to chitin. In a clinical study of CD patients, the oral administration of a chitosan and ascorbic acid mixture did not affect disease activity (Tsujikawa et al., 2003). However, to the best of our knowledge, no previous study has reported preventive or therapeutic effects of chitin or chitin derivatives on IBD.

Currently, many medical treatments are used for IBD patients: 5-aminosalicylic acid drugs such as sulfasalazine or balsalazide, immunomodulators such as thiopurines (azathioprine, 6-mercaptopurine), methotrexate, and biologic therapies that target TNF-α or IL-6 (Morrison et al., 2009; Nakamura, Honda, Mizutani, Akiho, & Harada, 2006). 5-Aminosalicylic acids drugs are expensive drugs that are well tolerated by most people with a low rate of adverse events (Morrison et al., 2009). However, 5-aminosalicylic acid has low efficacy against moderate UC (Carter, Lobo, & Travis, 2004). Immunomodulators are the mainstay of

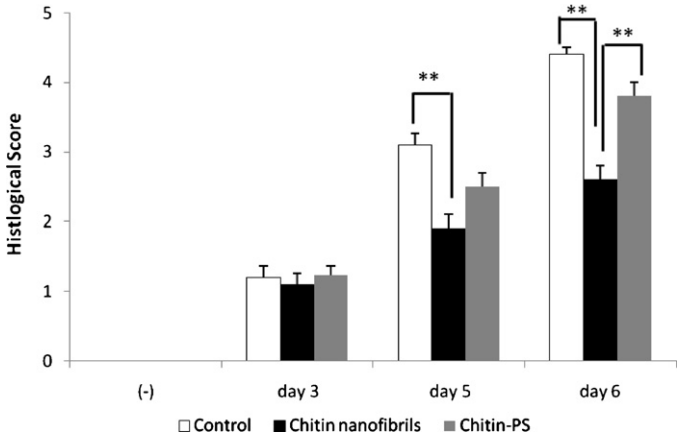

Fig. 2. Effect of chitin nanofibrils administration on the histological damage score of the intestinal mucosa in DSS-induced acute UC mice. Data represent the means ± S.E. of 30 fields/100× field in each group. Values are compared among control (+), chitin nanofibrils (+) and chitin-PS (+) groups. $^{**}p < 0.01$.

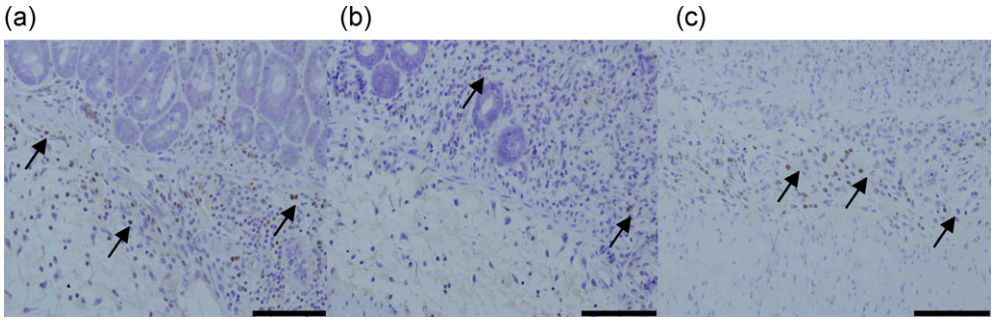

Fig. 3. Effect of chitin nanofibrils administration on the number of MPO-positive cells in the colons of DSS-induced acute UC mice. MPO positive cells indicated by arrows. Data are from one of 3 mice in the control. (+) (a), chitin nanofibrils (+) (b) and chitin-PS (+) (c) on day 6. Bar = 100 μm.

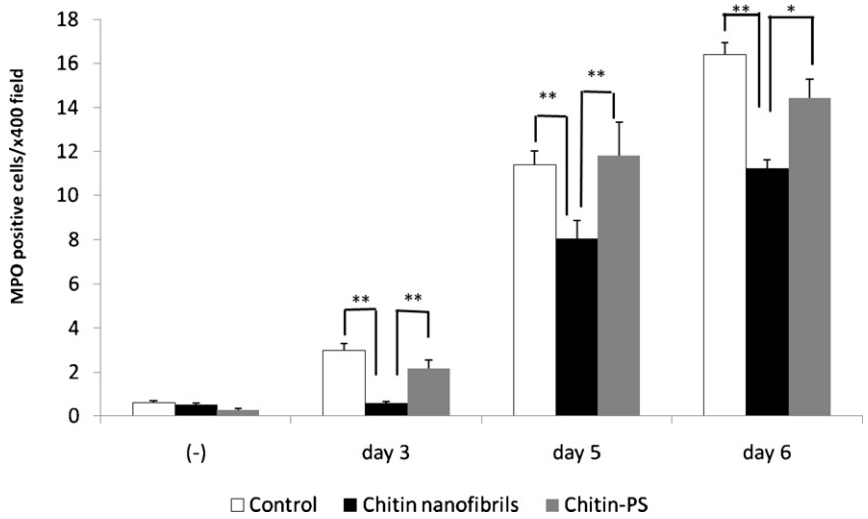

Fig. 4. Effect of chitin nanofibrils administration on the MPO-positive cells counts/400× field in the colons of DSS-induced acute UC mice. Data represent the means ± S.E. of 60 fields/400× field in each group. Values are compared among control (+), chitin nanofibrils (+) and chitin-PS (+) groups. $^{**}p < 0.01$.

treatment in maintenance therapy for patients with more than mild CD and for chronically active or frequently relapsing UC where 5-aminosalicylic acid drugs have failed (Lichenstein, Abreu, Cohen, & Tremaine, 2006). Biologic therapies are used for induction (to get the disease under control) and the long-term maintenance of moderately to severely active disease that has not responded to conventional treatment. However, immunomodulators and biologic therapies increase the risk of serious infection (Morrison et al., 2009). Optimal therapy for IBD is not still established. It was described that some nutritional supplements are beneficial for IBD including amino acids (Coëffier, Marion-Letellier, & Déchelotte, 2010), omega-3 fatty acids (Rajendran & Kumar, 2010), D-glucosamine hydrochloride (Yomogida et al., 2008), dietary fibers (Rodríguez-Cabezas et al., 2002), and probiotics (Vanderpool, Yan, & Polk, 2008). Our results suggested that chitin nanofibrils has potential as a nutritional supplement for IBD patients. In our study, chitin nanofibrils did not cause body weight losses and show side effects in gross pathology (data not shown). However, more careful evaluation of the side effects of chitin nanofibrils is necessary before its use in IBD patients. In IBD patients, mucosal lymphocytes and intracellular markers (mitogen-activated protein kinase and nuclear factor-κB) are related with mucosal inflammation (Scaldaferri, Correale, Gasbarrini, & Danese, 2010). Studies that focus on the cells and molecules in the mucosa are expected to elucidate the anti-inflammatory mechanism of chitin nanofibrils.

In conclusion, our results indicated that chitin nanofibrils improved clinical symptoms, inhibited colonic inflammation, and prevented tissue injury in DSS-induced acute UC mice. Furthermore, chitin nanofibrils inhibited mucosal inflammation by suppressing the MPO-positive cells such as leukocytes and decreasing serum IL-6 concentrations. Conversely, chitin powder was not effective in our DSS-induced acute UC model. More developmental research is necessary before the use of chitin nanofibrils as a functional food for IBD patients.

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