Natural Product Sciences
17(3) : 234-238 (2011)

Preventive Effects of Co-treatment with Fucoidan and Lutein on the Development of Inflammatory Bowel Disease in DSS Mouse Model

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Abstracts – We investigated the effects of fucoidan and lutein against dextran sulfate sodium (DSS)-induced mice colitis. Evaluations were made of the body weight, histological index such as crypt injury and inflammation score, biochemical factor such as serum amyloid (SAA) and MPO level data. The combination of fucoidan and lutein reduced the score of crypt injury and inflammation and markedly showed more decrease of the SAA and MPO levels than 5-ASA group. In addition, each sample of fucoidan and lutein was reduced the level of IL-6 which is stimulated by a lipopolysaccharide (LPS) in HT-29 cell line *in vitro*. Therefore, fucoidan and lutein may be useful as a dietary substance for preventing inflammatory bowel disease in humans.
Keywords – Fucoidan, Luetin, Dextran sulfate sodium, Inflammatory bowel disease

Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease, is a severe intestinal inflammation, the pathogenesis of which remains unclear. It is suspected that the disease is due to complex mucosal immune responses to resident enteric bacteria, because intestinal inflammation was absent from various IBD models reared under germ-free conditions (Duchmann *et al*., 1995; 1996; 1999; Kawaguchi-Miyashita *et al*., 2001; Matsumoto *et al*., 1998; Sadlack *et al*., 1993; Taurog *et al*., 1999). The large variety of animal models that has been identified so far is consonant with the view that many types of imbalance in the gastrointestinal immune system can lead to mucosal inflammation, and by extension, that human IBD is probably a common denominator for a group of clinically related disorders with multiple etiologies and distinct clinical characteristics. Interleukin (IL)-6 is one of the major cytokines secreted by lamina propria cells in the patients with IBD (Fuss *et al*., 1996; Gross *et al*., 1992; Podolsky, 1991). Strong expression of IL-6 has also been reported in murine acute bowel inflammation (Rogler and Andus, 1998). Recent studies using antisoluble-IL-6 receptor antibodies demonstrated that IL-6 plays a critical role in the development chronic colitis (Atreya *et al*., 2000; Yamamoto *et al*., 2000).

Fucoidan is a complex sulphated polysaccharide, derived from marine brown seaweed. There have been many reports on the biological effects of fucoidan on mammalian cells (Baba *et al*., 1994; Mahony *et al*., 1991). Shibata and colleagues indicated that fucoidan derived from *Cladosiphon okamuranus* Tokida blocked the adhesion of *Helicobacter pylori* to a human gastric cell line (Shibata *et al*., 1999). Matsumoto and colleagues showed that fucoidan improves murine chronic colitis by down-regulating the synthesis of IL-6 in the colonic epithelial cells. The level of IL-6 mRNA in colonic epithelial cells was lower in colitis-induced Balb/c mice fed *Cladosiphon* fucoidan than those fed a standard diet (Matsumoto *et al*., 2004). Therefore, fucoidan may be useful as a dietary substance for preventing human disease because its polysaccharide causes no toxicity or irritation.

Lutein has been used in the treatment of eye diseases and to protect visual function since the 1950s (Nussbaum *et al*., 1981). It is well known for its beneficial effects on AMD, which is a chronic, progressive, degenerative disease of the macula and is the leading cause of central vision loss among elderly people in the Western world (Slakter and Stur, 2005). Although the exact mechanisms of AMD remain unclear, inflammation may be involved in its pathogenesis and has led to the consideration of anti-inflammatory therapy as treatment for the early stages of the disease (Zarbin, 2004). Treatment with lutein reduced the concentrations of NO, TNF-α, IL-6, PGE2, MCP-1,

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and MIP-2 in aqueous humor (Jin *et al*., 2006).

The purpose of this study was to investigate the effects of the combination of fucoidan and lutein on dextran sulfate sodium (DSS)-induced mice model.

Materials and Methods

Animals – Male Balb/c mice (8 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). They were maintained under specific pathogen-free (SPF) conditions during the experiments.

Materials – Lutein (purity, > 90%) and fucoidan (purity, > 85%) were supplied as a food additive ingredient by Yixin Pharmaceutical (China) and Haewon biotech (Korea), respectively. HT-29 human colon epithelial cell (American Type Culture Collection (ATCC) HTB 38) was grown in DME (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS, 2 mM glutamine, and 25 mM HEPES.

Inhibition of IL-6 on LPS-inducted HT-29 cells – Levels of human IL-6 in the culture media were measured using enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Science, IL, USA), according to the manufacturer's instructions.

Induction of chronic colitis – Acute colitis was induced by 4% DSS (MW 25,000, TCI, Japan) dissolved in drinking water. Briefly, a total of 37 mice were randomly placed into six groups and were housed individually. The normal group (n = 5) was given distilled water, and the other groups were fed 4% DSS for 7 days. Fucoidan, lutein, fucoidan-lutein combination and 5-aminosalicylic acid (5-ASA) as positive control were orally administered daily from day 8 and day 11. The IBD scoring system assigns severity scores of 0 - 4 for three parameters: body weight, stool consistency and intestinal bleeding (Cooper *et al*., 1993).

Histological grading of colitis – Colonic tissues were removed and embedded into paraffin for histological analysis with hematoxylin and eosin staining. Crypt injury was scored as follows: grade 0, intact crypts; grade 1, loss of the bottom third of crypts; grade 2, loss of the bottom two thirds of crypts; grade 3, loss of the entire crypt with the surface epithelium remaining intact; grade 4, loss of the entire crypt and surface epithelium. Inflammation was scored from 0 to 4 as follows by the same pathologist blinded to the treatment conditions: 0, no inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, moderate fibrosis, and goblet loss; and 4, massive loss of goblet cells, extensive fibrosis, and thickening of the colon wall (Erichsen *et al*., 2005).

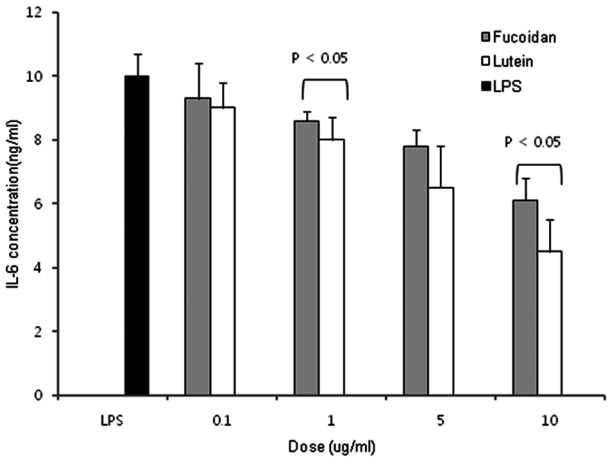

Fig. 1. Inhibitory effect of fucoidan and lutein on IL-6 synthesis in LPS-stimulated HT-29 cells. HT-29 was cultured with various doses of fucoidan and lutein in the presence of LPS for 72 h. The amount of IL-6 in the culture supernatant was measured by an IL-6-specific sandwich ELISA. Three independent experiments gave similar results. Data are mean ± S.D.

Measurement of Serum Amyloid A – Serum samples were collected for the detection of serum amyloid A (SAA) levels by a murine ELISA kit (Tridelta Development, Wicklow, Ireland) 3 and 10 days after induction of colitis according to the manufacturer's recommendations and A450 nm was measured (Uhlar and Whitehead, 1999).

Myeloperoxidase assay – MPO activity was assayed using Krawisz's method (Krawisz *et al*., 1984). In brief, Colonic tissues were homogenized in ice-cold potassium phosphate buffer (pH 6.0) and centrifuged for 10 min at $6000 \times g$ at 4 °C. The suspension was sonicated on ice and then centrifuged at $1000 \times g$ for 30 min. The supernatant was mixed with an enzyme substrate buffer containing 0.167 mg of *O*-dianisidine hydrochloride (Sigma) per ml and 0.0005% hydrogen peroxide. The changes in the absorbance at 405 nm were measured.

Statistics – All data were expressed as the mean ± s.e. and evaluated by Tukey or Tukey-Kramer test for multiple comparisons. *P*-values of less than 0.05 were considered to be statistically significant.

Results

Inhibition of IL-6 production on LPS-stimulated HT-29 cells – Fucoidan and lutein showed the inhibition of IL-6 synthesis in LPS-stimulated HT-29. The inhibitory activity on IL-6 release in LPS-stimulated HT-29 cells by fucoidan and lutein were dose-dependent (Fig. 1). We did not detect any up-regulation in the release of IL-6 from

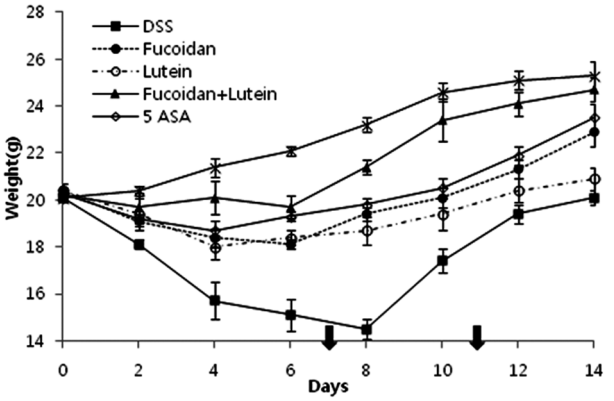

Fig. 2. Weight change of DSS-induced mice with the treatment of fucoidan, lutein and the combination of both. Treatment dose of fucoidan, lutein and the combination of both was 20 mg/kg, respectively, and 5-ASA was 100 mg/kg. ↓; Time point of treatment was at day 8 and 11.

HT-29 cells cultured in the presence of single 5-ASA (Data not shown).

Change of body weight – The body weight gain of each mouse was determined every two days by comparing the current weight with the weight on Day 0. As shown in Fig. 2, when compared with the vehicle group, the body weight of the only DSS-induced mice decreased. However, when compared with the vehicle, the body weight of mice administered with each treatment and combination of fucoidan and lutein had significant recovery. As shown in Fig. 2, at the end of the experiment, the final weight of the combination treatment group was closely reached at the weight of normal groups and the weight of the other group, fucoidan and lutein, was similar to its change of 5-ASA as positive control.

Histopathology – Histologic changes in the cecum and colon in DSS-treated Balb/c mice were examined by hematoxylin and eosin staining to evaluate the effectiveness of fucoidan, lutein and combination of two compounds against the tissue damage. Histopathological evaluation showed indications of colitis in all groups receiving DSS. Changes were most prominent in the distal colon with areas of erosions, crypt distortion, and inflammatory infiltration. In case of the combination of fucoidan and lutein, it is significantly reduced mean crypt score by 1.0 and mean inflammation score by 1.0 ± 0.3. However, no treatment group showed by 4.0 and mean inflammation score by 4.0 ± 0.1. The histopathologic results correlated well with clinical signs of colitis. Regarding histologic scores, the crypt injury score in the tissue was significantly reduced from 4.0 to 1.0 by the combination of fucoidan and lutein, even though its score was reduced from 4.0 to 2.0 by the treatment of fucoidan, lutein and 5-ASA as positive control, respectively (Table 1). The histopathologic analysis using inflammation score after induction of colitis showed infiltration of neutrophils and macrophages into the colonic mucosa and submucosa layers. In no treatment group, transmural inflammation, characterized by massive infiltration of lymphocytes, was associated with a thickening of the colon wall, ulcerations and loss of goblet cells through the colon. Treatment of combination of fucoidan and lutein improved these signs, restoring the histological appearance of the mucosa and submucosa compared with untreated mice, although the other experiment groups including fucoidan, lutein and 5-ASA-treated mice showed a minor infiltration of lymphocytes as a result of a mild inflammation (Fig. 3). As another important index of inflammation, SAA was measured in the serum. Finally, one of the most intensively studied systemic responses against an inflammatory stimulus is the hepatic synthesis of acute phase proteins, including the family of SAA. As Table 1 shows, the serum SAA levels of DSS-induced group was increased more than approximately 5000-fold (402.5 ± 5.9 ug/ml) compared with the normal group (0.007 ± 0.02 ug/ml). Treatment with combination of fucoidan and lutein induced a marked decrease in SAA serum levels.

Table 1. Crypt injury and inflammation score and related peptide level in colitis model treated with the combination of fucoidan and lutein

	Crypt injury score	Inflammation score	SAA (μg/ml)
Normal	0 ± 0	0.0 ± 0.0	0.07 ± 0.02
DSS	4 ± 0*	4.0 ± 0.1*	402.5 ± 5.9*
Fucoidan	2 ± 0*	1.9 ± 0.4**	105.2 ± 4.6*
Lutein	2 ± 0*	2.0 ± 0.3**	99.1 ± 5.7**
Fucoidan + Lutein	1 ± 0*	1.0 ± 0.3*	2.4 ± 0.5*
5-ASA	2 ± 0**	1.7 ± 0.2*	50.3 ± 3.9*

Results are expressed as mean ± s.e (P, < 0.01*, < 0.05**).

MPO Activity Measurement – The MPO activity was used as an index of polymorphonuclear (PMN) infiltration. The MPO activity of vehicle, DSS control and each treatment and combination of fucoidan and lutein are shown in Fig. 4. Mice from the DSS control group demonstrated the highest MPO activity in colon, while each treatment and combination of 20 mg/kg of fucoidan and lutein significantly inhibited the increase in MPO activity.

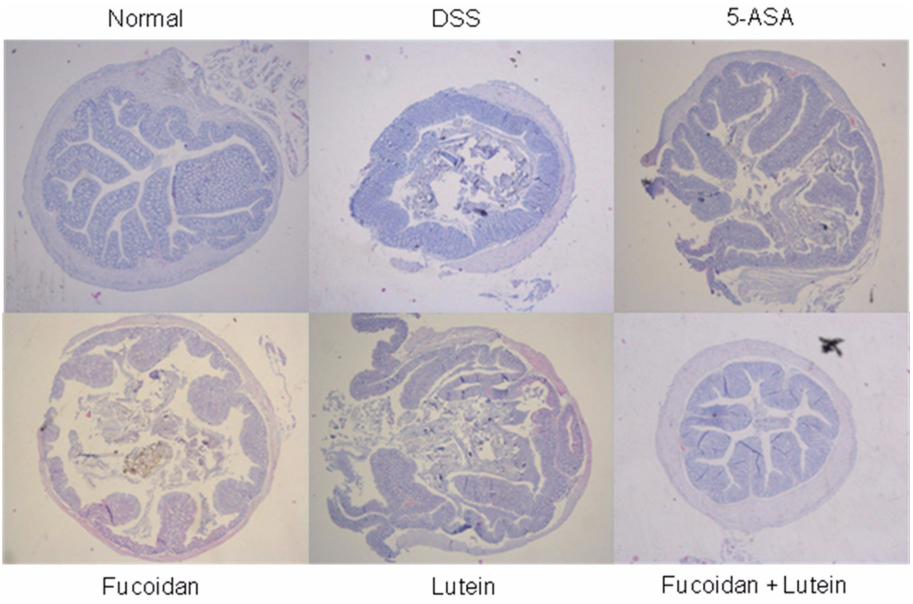

Fig. 3. Microscopic study (original magnification 50X) of colons of mice with DSS-induced colitis treated with fucoidan and lutein. Treatment dose of fucoidan, lutein and the combination of both was 20 mg/kg, respectively, and 5-ASA was 100 mg/kg.

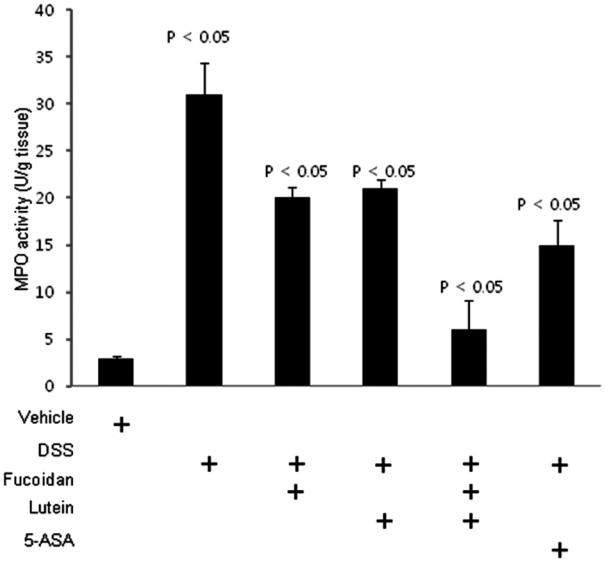

Fig. 4. MPO activity of the colonic tissue in DSS-induced mice treated with fucoidan and lutein. Treatment dose of fucoidan, lutein and the combination of both was 20 mg/kg, respectively, and 5-ASA was 100 mg/kg.

Discussion

In this present study, we examined the inhibitory effects of the combination of fucoidan and lutein on the production of IL-6 in an LPS-stimulated HT-29 cells and also examined the ameliorating effects of the combination of both on the DSS-induced inflammation.

IL-6 has been shown to play important roles on the pathogenesis of murine Th-1-mediated colitis (Yamamoto *et al*., 2000). Intestinal epithelial cells and lamina propria lymphocytes are a major source of IL-6, which is released by the cultured intestinal epithelial cell line T84, induced an intracellular (Ca^{++}) flux and degranulation in neutrophils, in inflammatory bowel disease (Hungness *et al*., 2000; Sitaraman *et al*., 2001). These results showed that secretion of IL-6 by intestinal epithelial cells plays a major role in the pathogenesis of IBD and the prevention of this secretion may be useful in the treatment of inflammatory bowel disease. To test this possibility, we examined an inhibitory effect of the combination of fucoidan and lutein on IL-6 secretion in LPS-stimulated HT-29 and our results was consistent with the results of previous study (Jin *et al*., 2006; Matsumoto *et al*., 2004). In inflammation, one of the most intensively studied systemic responses against an inflammatory stimulus is the amyloid A (SAA) of the family of hepatic synthesis of acute phase proteins. In general, SAA is an acute-phase protein measured for clinical monitoring of Crohn's disease and is potently induced in response to proinflammatory stimuli that synergize with IL-6 cytokines (Preciado-Patt *et al*., 1996). In our results, SAA was significantly reduced by the combination of fucoidan and lutein on DSS-induced mice disease model. These change of biochemical factors showed the correlation with the disease parameters such as disease activity index and the MPO activity in the colonic tissue that compared with these parameters in the normal mice.

In conclusion, we discovered that fucoidan and lutein inhibited the synthesis of IL-6 in an LPS-stimulated colonic epithelial cell line *in vitro* and DSS-induced colitis *in vivo*. Moreover, we observed a positive effect of the combination of both in murine chronic colitis. Therefore, fucoidan and lutein may be useful as a dietary substance for preventing inflammatory bowel disease in humans.

Acknowledgements

The present research had been conducted by the Research Grant of Seoil University in 2010.

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Received May 12, 2011
Revised August 30, 2011
Accepted September 2, 2011