JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2018, 69, 3, 359-371
www.jpp.krakow.pl | DOI: 10.26402/jpp.2018.3.03

Original articles

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OLIGONOL PREVENTED THE RELAPSE OF DEXTRAN SULFATE SODIUM-ULCERATIVE COLITIS THROUGH ENHANCING NRF2-MEDIATED ANTIOXIDATIVE DEFENSE MECHANISM

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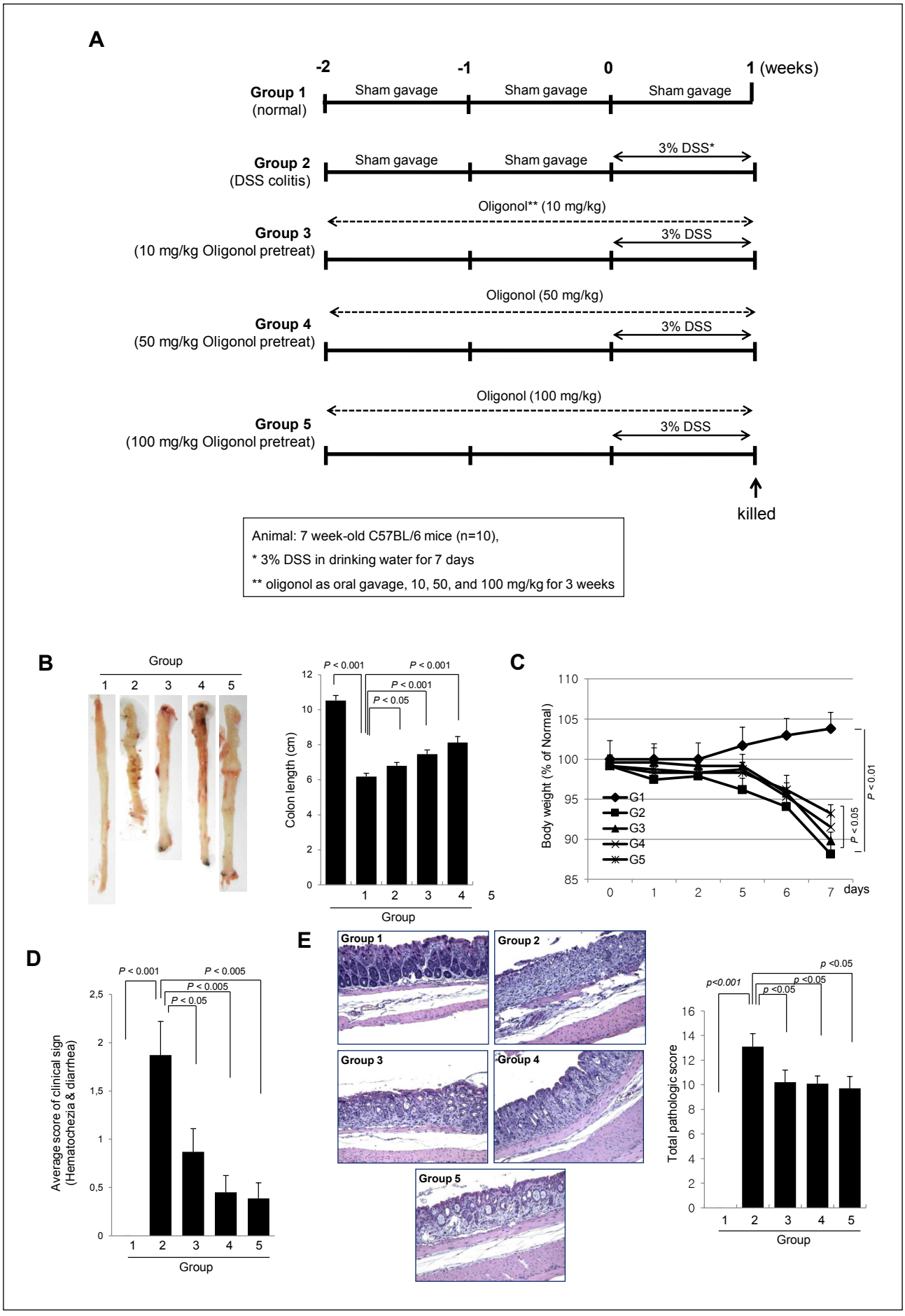
Repeated bouts of ulcerative colitis featured troublesome course of inflammatory bowel disease leading to fatal colitis-associated cancer, which is strongly associated with oxidative stress and sustained inflammation. Since oligonol, low molecular weighted polyphenol extracted from fruit lychee, showed antioxidative and anti-inflammatory actions, we hypothesized that oligonolcan prevent relapse of colitis. We compared oligonol with current gold standard therapeutics, sulfasalazine in preventive efficacy of relapse. First, dextran sulfate sodium (DSS)-induced colitis were made following pretreatment with oligonol, 10, 50, and 100 mg/kg for 7 days to measure therapeutic effect of oligonol and relapse model *via* repeated DSS administration was made following with either 50 mg/kg oligonol or 30 mg/kg sulfasalazine to explore relapse preventing action of oligonol in C57BL/6 mice. Detailed changes in colon were measured to explain molecular mechanisms. Pretreatment of 10, 50, 100 mg/kg oligonol (p.o.), significantly reduced DSS-induced colitis; total pathologic scores, colon length, and clinical symptom scores ($P < 0.05$). Oligonol pretreatment significantly decreased the levels of interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) as well as nuclear factor-κB (NF-κB), c-Fos, and c-Jun in affected colon tissues, but the expression of heme oxygenase-1 (HO-1) and NADH: quinone oxidoreductase-1(NQO-1) as well as total antioxidant concentration ($P < 0.005$) was significantly increased with oligonol. A relapse model established with repeated DSS administration led to high mortality. However, oligonol significantly ameliorated exacerbations of colitis, while sulfasalazine did not ($P < 0.01$). Significantly decreased expressions of cyclooxygenase-2 (COX-2), TNF-α, and macrophages inhibition were relapse preventing actions of oligonal, but significant action of oligonol relevant to relapse prevention was either significantly increased expressions of NQO-1 or significantly preserved mucin ($P < 0.05$). Concerted anti-inflammatory, antioxidative, and host defense enhancing actions of oligonol can be applied during maintenance therapy of IBD to prevent relapse of IBD.

Key words: *inflammatory bowel disease, experimental colitis, oligonol, host adaptive response, nuclear factor (erythroid-derived 2)-like 2, quinone oxidoreductase-1, relapse prevention, nuclear factor-κappa B, tumor necrosis factor-alpha*

INTRODUCTION

Ulcerative colitis chronic and recurrent inflammatory bowel diseases (IBD), which is characterized by inflammatory cells infiltrate such as neutrophils, macrophages and eosinophils. These inflammatory cells are able to produce robust amounts of reactive oxygen and nitrogen species when stimulated. Therefore, considering these pathogenesis even under unknown etiology, the role of inflammatory response and robust oxidative stress was intervened extensively in IBD pathogenesis (1, 2). Therefore, a variety of therapeutic agents, including sulfasalazine, mesalazine, prednisolone, and cyclosporin have been used clinically to suppress 'inflammation prone to develop ulcer' in IBD, 5-ASA as a main treatment for mild to moderate patients and steroids as treatment for moderate to severe degree. Sulfasalazine and mesalazine were proved to have some antioxidant actions. However, though prescribed actively, still long-term medication to prevent relapse is eagerly to be improved owing to the chronic nature of IBD, occasional flares of clinical course, and fear of colitis-associated cancer especially in patients with chronic and relapsing clinical course, so called maintenance therapy (3, 4).

Despite advancement in molecular targeted therapies and the development of biologics such as infliximab and adalimumab, there are still long-ways to achieve long-term anti-inflammatory therapy and complete healing (or cure) (5), and the patients with IBD are concerned about serious potential adverse effects of therapeutic agents. Among those with IBD, it is estimated that approximately 44% to 56% patients use some form of complementary or alternative therapy (6). For these reasons, products of natural origin that can cover the gap between


A
-2
-1
0
1 (weeks)
Group 1
(normal)
Sham gavage
Sham gavage
Sham gavage
Group 2
(DSS colitis)
Sham gavage
Sham gavage
3% DSS*
Oligonol** (10 mg/kg)
Group 3
(10 mg/kg Oligonol pretreat)
3% DSS
Oligonol (50 mg/kg)
Group 4
(50 mg/kg Oligonol pretreat)
3% DSS
Oligonol (100 mg/kg)
Group 5
(100 mg/kg Oligonol pretreat)
3% DSS
killed
Animal: 7 week-old C57BL/6 mice (n=10),
* 3% DSS in drinking water for 7 days
** oligonol as oral gavage, 10, 50, and 100 mg/kg for 3 weeks
B
Group
1 2 3 4 5
P < 0.001
P < 0.001
P < 0.001
P < 0.05
Colon length (cm)
Group
C
Body weight (% of Normal)
G1
G2
G3
G4
G5
P < 0.01
P < 0.05
days
D
P < 0.001
P < 0.005
P < 0.005
P < 0.05
Average score of clinical sign
(Hematochezia & diarrhea)
Group
E
Group 1
Group 2
Group 3
Group 4
Group 5
p<0.001
p <0.05
p <0.05
p <0.05
Total pathologic score
Group

pharmaceuticals and the effects with strong arm of safety have become an alternative option in addition to the conventional therapies that are used to treat IBD (7).

Oligonol, a low-molecular-weight polyphenols derived from lychee fruit, had been reported to inhibit TPA-induced COX-2 expression and inflammatory cytokine production by blocking the activation of nuclear factor kappa B (NF-κB) and C/EBP *via* modulation of MAP kinases, by which suppressed streptozotocin-induced diabetic rats (8), chemically induced mouse skin tumorigenesis (9), and IBD (10). Since oligonol are quickly absorbed and effective for protecting the cells from oxidative stress, treatment with oligonol exerted higher antioxidant activity than even epigallocatechin-3-gallate or catechin monomer from green tea (11). In this background, under the hypothesis that oligonol co-administration in patients with maintenance therapy might be right strategy to prevent relapse, we conducted two kinds of well-known dextran sulfate sodium (DSS)-induced colitis model, one was to document preventive effects of oligonol administration against DSS-induced colitis and the other was to document preventive effects of oligonol against repeated DSS-induced relapsing colitis. As results, anti-inflammatory, antioxidative, and host defense enhancing actions of oligonol significantly afforded either preventive action of colitis or decreasing relapse of ulcerative colitis.

MATERIALS AND METHODS

Reagents

The following materials were obtained from commercial sources: all chemical reagents from Sigma (St. Louis, MO). Oligonol (> 95% purity) was obtained from Amino Up Chemical Co., Ltd. (Sapporo, Hokkaido, Japan). Oligonol is produced by an oligomerization process that converts high-molecular weight polymeric proanthocyanidins into low-molecular weight oligomeric proanthocyanidins including monomers, dimers and trimmers, of which process is achieved by mixing proanthocyanidin polymers with tea catechins (12). Antibodies for Western blotting were purchased as follows: β-actin, p65, lamin B, HO-1 from Santa Cruz Biotechnology, (Santa Cruz, CA), iNOS from BD Biosciences (San Jose, CA), COX-2 from Thermo Scientific (Seoul, Korea), NQO-1 from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated anti-rat/rabbit/mouse IgG was purchased from Thermo Scientific Pierce (Rockford, IL).

Animals

Animals were handled in an accredited animal facility in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) guidelines under the facility named CACU (The Center of Animal Care and Use) of CHA University Laboratory Animal Research Center after IRB approval. Two experimental protocols were followed and conducted separately (*Fig. 1A* and *Fig. 5A*).

Prevention of colitis model, pre-treatment with oliogonol and induction of dextran sodium sulfate-induced colitis (Fig. 1A)

Germ-free male C57BL/6 mice (5 weeks of age, Orient Bio, Seongnam, Korea) were used for the experiments. A total of 50 mice were divided into 5 groups, 10 mice per each group, respectively; a non-colitic group that received no drug treatment and distilled water without DSS (control, Group 1); a colitic control group that received 3% DSS (molecular weight ¼ 36,000 – 50,000; MP Biomedicals) in tap water ingestion for 1 week alone (Group 2); and the other 3 pretreated groups with oligonol, which was mixed in tap water and given orally for 7 days before DSS treatment and 7 days together with 3% DSS. Oral gavage contained oligonol at a dose of 10, 50, and 100 mg/kg in each group (Group 3, 4, and 5, respectively, *Fig. 1A*). Powders of oligonol were dissolved in PBS and mice of the normal group and the DSS group were ingested with PBS as a negative control.

Prevention of colitis relapse model; oligonol prevented the relapse of colitis provoked through repeated administration of DSS (Fig. 4A)

A total of 40 mice were divided into 4 groups, 10 mice per each group, respectively; a non-colitic group that received no drug treatment and distilled water without DSS (control, Group I); a colitic control group that received 3% DSS (molecular weight ¼ 36,000 – 50,000; MP Biomedicals) in tap water ingestion for 1 week alone (Group II); the other 2 pretreated groups with oligonol ((50 mg/kg), Group III) or sulfasalazine ((30 mg/kg), Group IV) for 2 week daily, and then 3% DSS in tap water ingestion for 1 week (*Fig. 4A*). Powders of oligonol were dissolved in PBS and mice of the normal group and the DSS group were ingested with PBS as a negative control. Clinical phenotypes such as hematochezia, rectal prolapse, diarrhea, abdominal pain, and body weight were investigated and charted daily. After 28 days after the first DSS administration, all mice were killed and colons were removed, opened longitudinally, and rinsed with PBS. The lengths of colon were measured, and isolated tissues were subjected to a histologic examination and extraction of protein.

Assessment of colonic damage

Animal body weight, the presence of gross blood in the feces, and stool consistency as well as amounts of consumed DSS-mixed water was recorded daily for each rat by an observer unaware of the treatment. None of the mice were died in all the groups. After 7 days of DSS ingestion. Once mice were sacrificed, their colons were immediately removed and rinsed

Fig. 1. Oligonol attenuated DSS-induced colitis. (A) Experimental design to evaluate therapeutic effects of oligonol on DSS-associated colitis. The experimental details are described in Materials and Methods. (B) The average of colon length and gross appearances of the representative case of groups. Average of colon length was measured. (C) The changes of body weight. Significant reductions in body weights were seen after 3% DSS administration irrespective of group ($P < 0.05$), but significant ameliorations in body weight reductions were noted in 100 mg/kg oligonol treatment ($P < 0.05$). (D) Mean scores of clinical signs including hematochezia, diarrhea, and abdominal pain. Administration of DSS provoked significant levels of colitis as reflected with gross findings that the body weight of mice and length of colon were significantly decreased accompanied with significantly increased anal bleedings or diarrhea. However, all dose of pretreatment with oligonol leads to restore in the length of colon as well as anal bleedings or diarrhea compare to DSS administration. (E) Microscopic feature of colitis and total pathologic score. Microscopically, 3% DSS administration for 1 week provoked definite colitis such as distorted glandular formation and recruited inflammatory cells especially in submucosal layer, leading to mucosal destruction. Pathologic lesion index including area affected by inflammation, extent of follicle aggregates, edema, erosion/ulceration, crypt loss and infiltration of monomorphonuclear and polymorphonuclear cells was scored. Total pathology score was all increased in DSS administration group whereas pretreatment of oligonol in all doses significantly attenuated these pathologic indices. 40 × magnification and Bar represents mean ± SD.

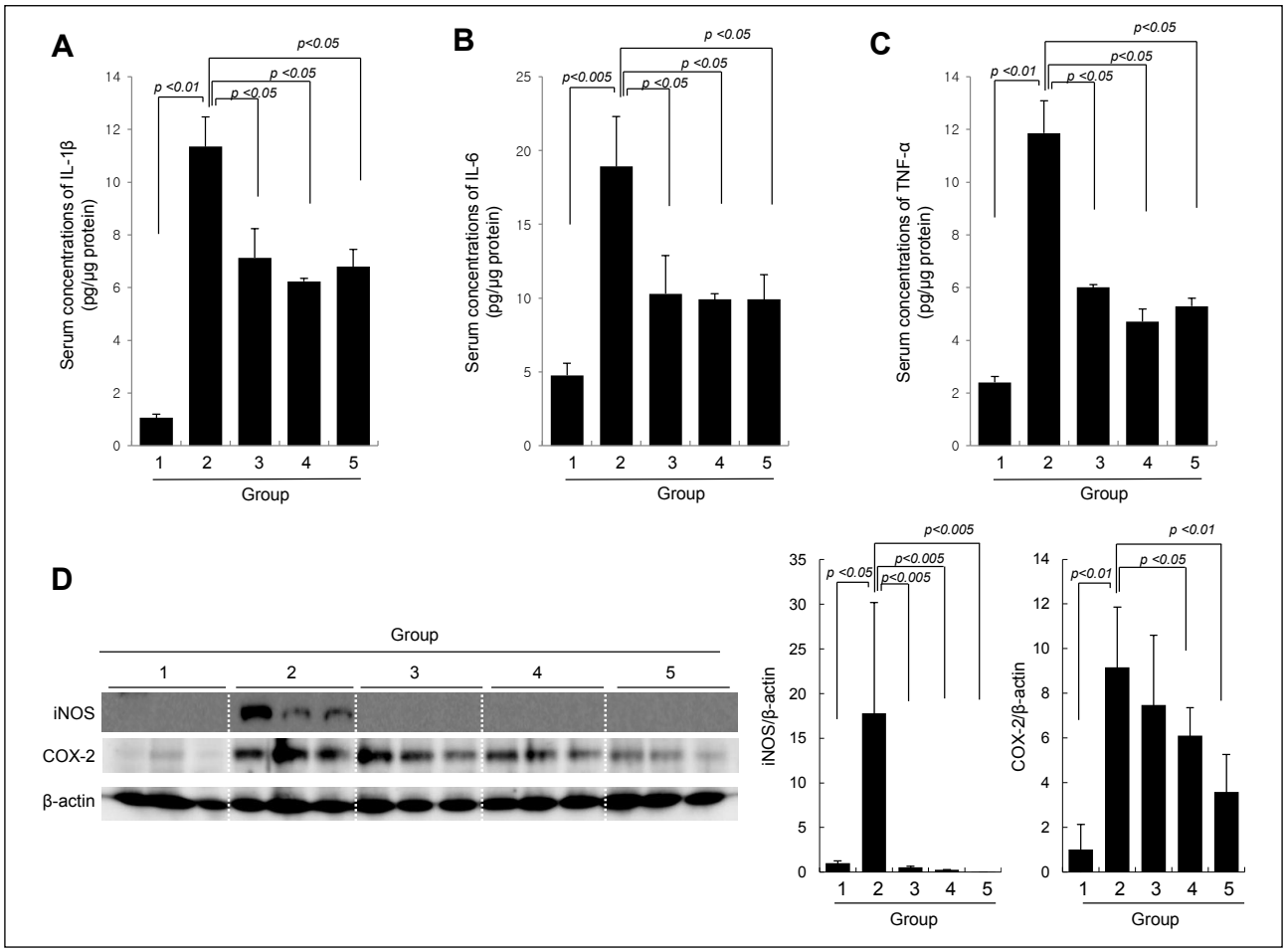


Fig. 2. Oligonol attenuated DSS-induced colitis through decreasing proinflammatory cytokine (A) IL-1β, (B) IL-6, (C) TNF-α, serum level of IL-1β, IL-6 and TNF-α were all significantly increased after DSS administration, but all dose of oligonol were significantly attenuated them ($P < 0.05$). (D) The protein expression of iNOS and COX-2 according to group. The protein expression of proinflammatory signaling pathway were detected by Western blot. Administration of DSS significantly increased the expressions of COX-2 and iNOS compared with normal group but significantly decreased in all dose of pretreatment of oligonol.

with ice-cold phosphate-buffered saline. The excised colonic segments were placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper. Each specimen was weighed and its length measured under a constant load (2 g). The colon was longitudinally opened, and a cross section from the distal diseased area was immediately fixed in 3.7% formaldehyde and embedded in paraffin for histological analysis. Afterward, it was sectioned into different longitudinal fragments to be used for biochemical determination and Western blotting.

Histopathological examinations

The paraffin sections were stained with hematoxylin and eosin (H&E) or saved for immunohistochemical staining. Pathologic index was graded according to criteria (13). Pathologic data and slides were blindly reviewed by two independent gastrointestinal specialists (Kim KJ and Hahm KB appeared as author). For periodic acid and Schiff's (PAS) staining, histochemical staining of glycoconjugates was carried out as per the method of Pandurangan (14), using 2% PAS reagent in dark for 20 min.

Immunohistochemistry

Immunohistochemistry was performed on replicate sections of mouse colon tissues. Sections fixed in 10% buffered formalin and embedded in paraffin were deparaffinized, rehydrated, and boiled three times in 100 mM Tris-buffered saline (pH 7.6) with 5% urea in an 850 W microwave oven for 5 min each. Sections were also incubated with F4/80 and COX-2 antibody in the presence of 1.0% bovine serum albumin and finally incubated for 16 h at 4ºC. The sections were counterstained with hematoxylin.

Western blot analysis

The colon tissues were homogenized with ice-cold cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After 20 min of incubation, samples were centrifuged at 12,000 g for 15 min. Supernatants were then collected. Total protein-equivalents for each sample were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes, which were incubated with which were incubated with appropriate antibodiesand then visualized using West-zol Plus (Intron Biotechnol, Seongnam, Korea).

Total malondialdehyde (MDA) assay

MDA-586 kit was purchased from Oxis International (Foster city, CA) and used according to the manufacturer's instructions.

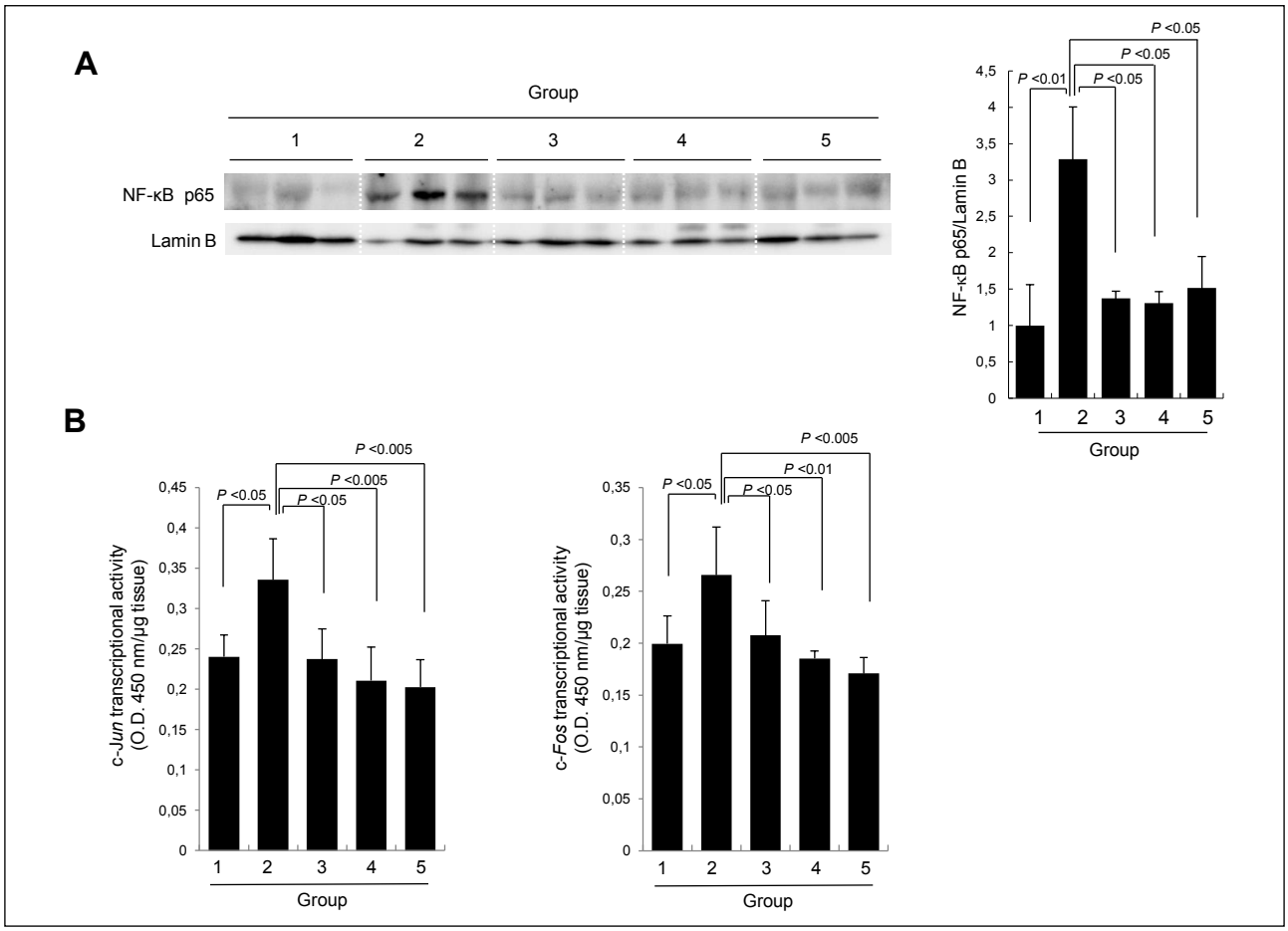


Fig. 3. (A) The nuclear translocation of NF-κB p65. NF-κB p65 is a ubiquitous, constitutive and inducible heterodimer. The administration of DSS increased the nuclear translocation of NF-κB p65, which was suppressed by pretreatment with oligonol. (B) Level of *c-Jun* and *c-Fos* activity reflecting acute phase response. *c-Fos* and *c-Jun* are the AP-1 components. Administration of DSS significantly increased *c-Jun* and *c-Fos* transcription activity ($P < 0.05$) whereas pretreatment of oligonol ameliorated *c-Jun* and *c-Fos* transcription activity which level similar to normal group.

To measure total level of malondialdehyde (MDA), isolated tissues were incubated with PBS (pH 2.0) for 80 min at 60ºC before carrying out this assay.

Total antioxidation capacity (TAC) measurement

Total antioxidant measurement of serum was done using the total antioxidant status assay kit (Calbiochem, San Diego, CA). The principal of the assay is dependent on antioxidants in the sample inhibiting the oxidation of ABTS™ (2,2'-Azino-di-[3-ethylbenz-thiazoline sulphonate]) substrate to ABTS™• + product by metmyoglobin (a peroxidase). The amount of ABTS™• + product can be monitored by reading the absorbance at 600 nm. Under the reaction conditions used, the antioxidants in the sample cause suppression of the absorbance at 600 nm to a degree that is proportional to their concentration.

Measurement of serum levels of IL-1β, IL-6, and TNF-α and transcriptional activity of c-Jun, c-Fos and Nrf2

After sacrifice of the mice, blood and protein were collected for ELISA assay. IL-1β, IL-6 and TNF-α (R&D Systems, Mineapolis, MN) were measured using ELISA kits according to manufacturer's instruction. All samples were measured for their individual levels, and each sample was analyzed in triplicate manner, taking the mean of the three determinations. The level of *c-Jun*, *c-Fos* and Nrf2 were measured using transcriptional activity kits (R&D, Systems) according to manufacturer's instruction. All samples were measured for their individual levels, and each sample was analyzed in triplicate manner, taking the mean of the three determinations.

Statistical analysis

The data are presented as means ± standard deviations (SD). The data were analyzed by ONE-WAY ANOVA followed by Dunnet. Some statistical significance between two groups was determined by Student's t-test. Statistical significance was accepted when $P < 0.05$. The survival curves between the groups were compared using log-rank test.

RESULTS

Oligonol pretreatment significantly attenuated DSS-induced colitis

The administration of 3% DSS in drinking water for 7 days resulted in significant degree of colitis manifested with a significant reduction colon length ($P < 0.001$, *Fig. 1B*) and mean body weight ($P < 0.05$, *Fig. 1C*). However, in the groups pretreated with oligonol, there was significant reduction in either

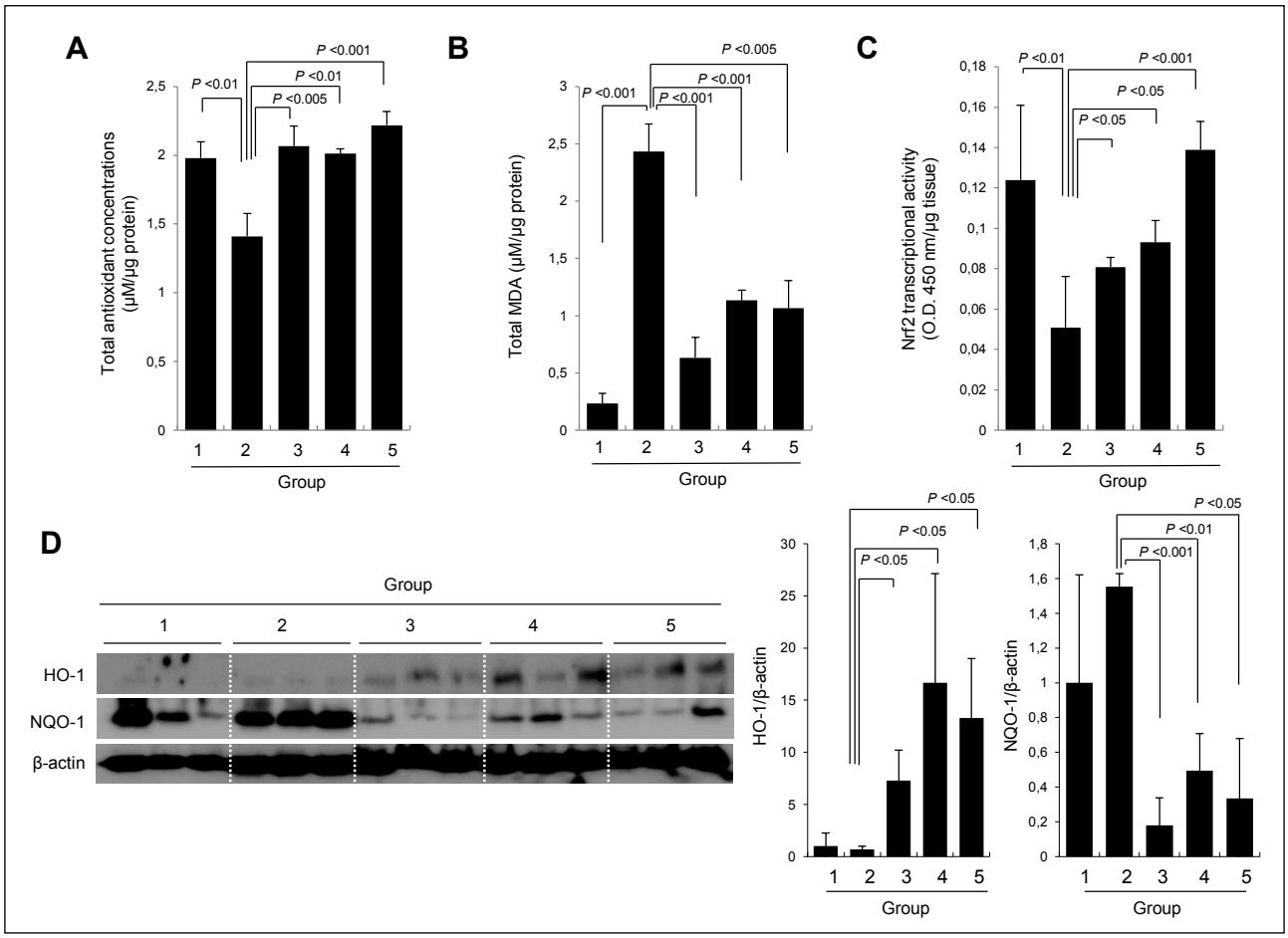


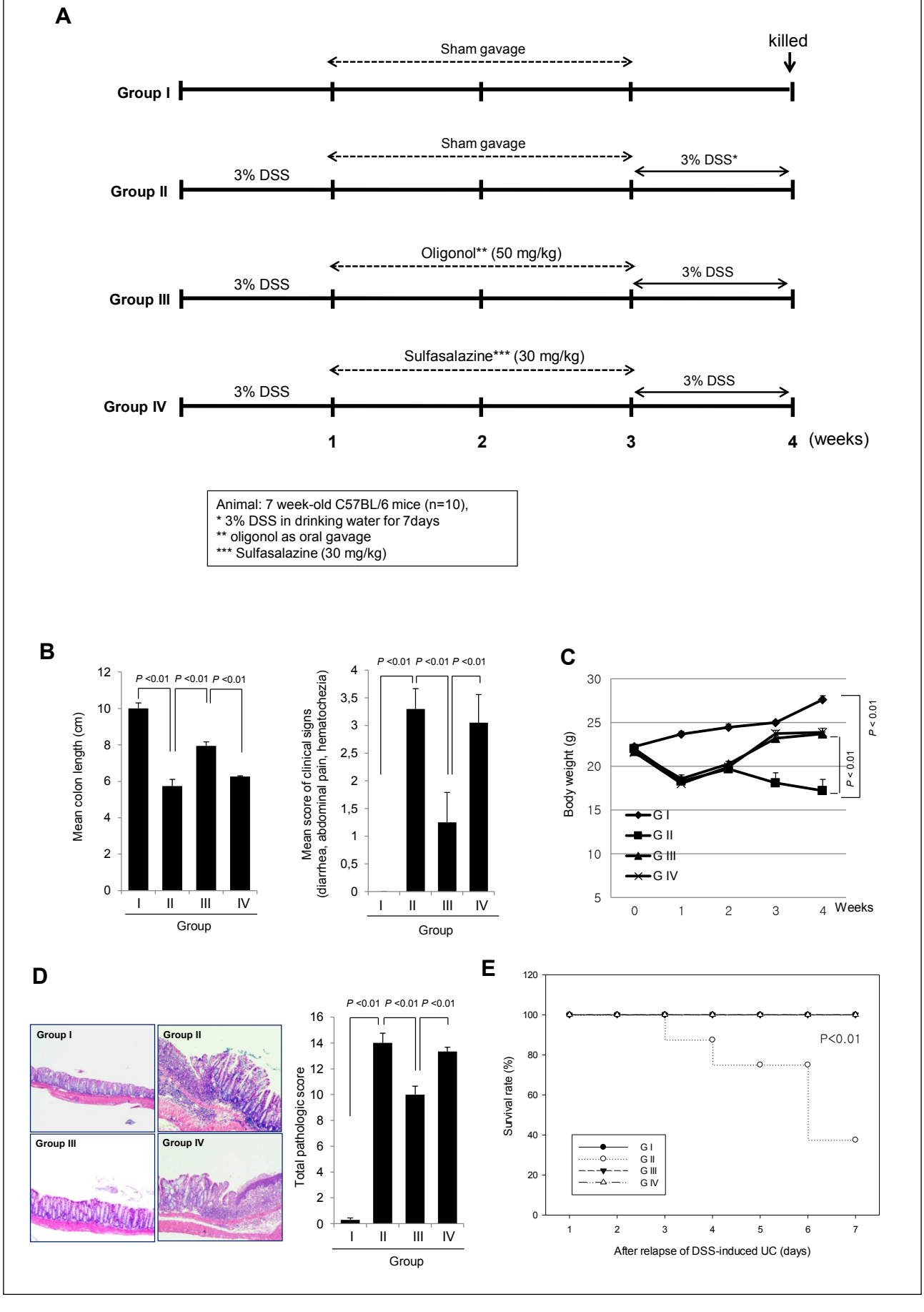
Fig. 4. Oligonol activated the antioxidant activity and Nrf2-mediated antioxidant signaling pathway. (A) Total antioxidant concentration (TAC). The induction of the total antioxidant activity was significantly decreased by administration of DSS whereas the total antioxidant activity significantly increased by pretreatment of oligonol. (B) MDA levels. Colon MDA levels were significantly increased after DSS administration compare with the normal group, but the colon MDA levels were significantly lower in all dose of oligonol compare with DSS administration. (C) Nrf2 transcription activity. Nrf2 transcription activity levels were significantly lower in DSS administration in mice than in normal controls, whereas pretreatment of oligonol showed markedly increased Nrf2 transcription activity levels compared to DSS administration. (D) Protein expression of HO-1 and NQO-1. All protein expression of HO-1 and NQO1 are the phase II enzymes downstream of Nrf2 were examined. The protein expression of HO-1 in oligonol pretreatment was significantly increased compared to DSS administration.

colon length or body weight ($P < 0.01$). Calculating clinical scores, as seen in *Fig. 1D*, the mean scores of anal bleedings or diarrhea were significantly decreased in group pretreated with oligonol in a dose-dependent manner (*Fig. 1D*, $P < 0.05$). On individual evaluation of inflammatory cell infiltrates, mucosal ulcers, and submucosal edema, as seen in *Fig. 1E*, there was significant decrease in pathological score in group pretreated with oligonol administration ($P < 0.05$).

Oligonol pretreatment significantly decreased inflammatory mediators such as IL-1β, IL-6, COX-2, iNOS, and TNF-α

To determine whether the anti-inflammatory activity of oligonol against DSS-induced colitis is executed through inhibiting inflammatory mediators, the serum levels of IL-1β (*Fig. 2A*), IL-6 (*Fig. 2B*) and TNF-α (*Fig. 2C*) were measured, respectively. As shown in *Fig. 2A*, *2B*, and *2C*, IL-1β, IL-6, and TNF-α in sera were significantly higher after DSS administration ($P < 0.01$), but oligonol pretreatment before DSS administration resulted in a significant decrease in the concentration of serum IL-1β, IL-6, and TNF-α ($P < 0.05$). DSS administration led to significant increases in the expressions of iNOS and COX-2 ($P < 0.05$), but oligonol pretreatment led to significant decreases in

Fig. 5. Oligonol prevented repeated DSS administration-induced relapse of colitis. (A) Experimental design for evaluating the protective effects of oligonol on relapse of DSS-associated colitis. The experimental details are described in Materials and Methods. (B) Survival of mice. Mice were examined for survival every day, up to 7 days after the DSS re-administration. (C) The changes of colon lenght hand; (D) Body weight; (E) Microscopic feature of colitis and total pathologic score. Microscopically, 3% DSS administration for 4 week provoked definite colitis such as distorted glandular formation and recruited inflammatory cells especially in submucosal layer, leading to mucosal destruction. Pathologic lesion index included scores of inflammation, extent of follicle aggregates, edema, erosion/ulceration, crypt loss, and infiltration of monomorphonuclear and polymorphonuclear cells. Total pathology score was all increased in DSS administration group whereas treatment of oligonol in all dose significantly attenuated these pathologic indices. Bar represents mean ± SD.


A
Sham gavage
killed
Group I
Sham gavage
3% DSS*
3% DSS
Group II
Oligonol** (50 mg/kg)
3% DSS
3% DSS
Group III
Sulfasalazine*** (30 mg/kg)
3% DSS
3% DSS
Group IV
1
2
3
4
(weeks)
Animal: 7 week-old C57BL/6 mice (n=10),
* 3% DSS in drinking water for 7days
** oligonol as oral gavage
*** Sulfasalazine (30 mg/kg)
B
Mean colon length (cm)
P <0.01 P <0.01 P <0.01
I II III IV
Group
Mean score of clinical signs (diarrhea, abdominal pain, hematochezia)
P <0.01 P <0.01 P <0.01
I II III IV
Group
C
Body weight (g)
P < 0.01
P < 0.01
G I
G II
G III
G IV
Weeks
D
Group I
Group II
Group III
Group IV
Total pathologic score
P <0.01 P <0.01 P <0.01
I II III IV
Group
E
Survival rate (%)
P<0.01
G I
G II
G III
G IV
After relapse of DSS-induced UC (days)

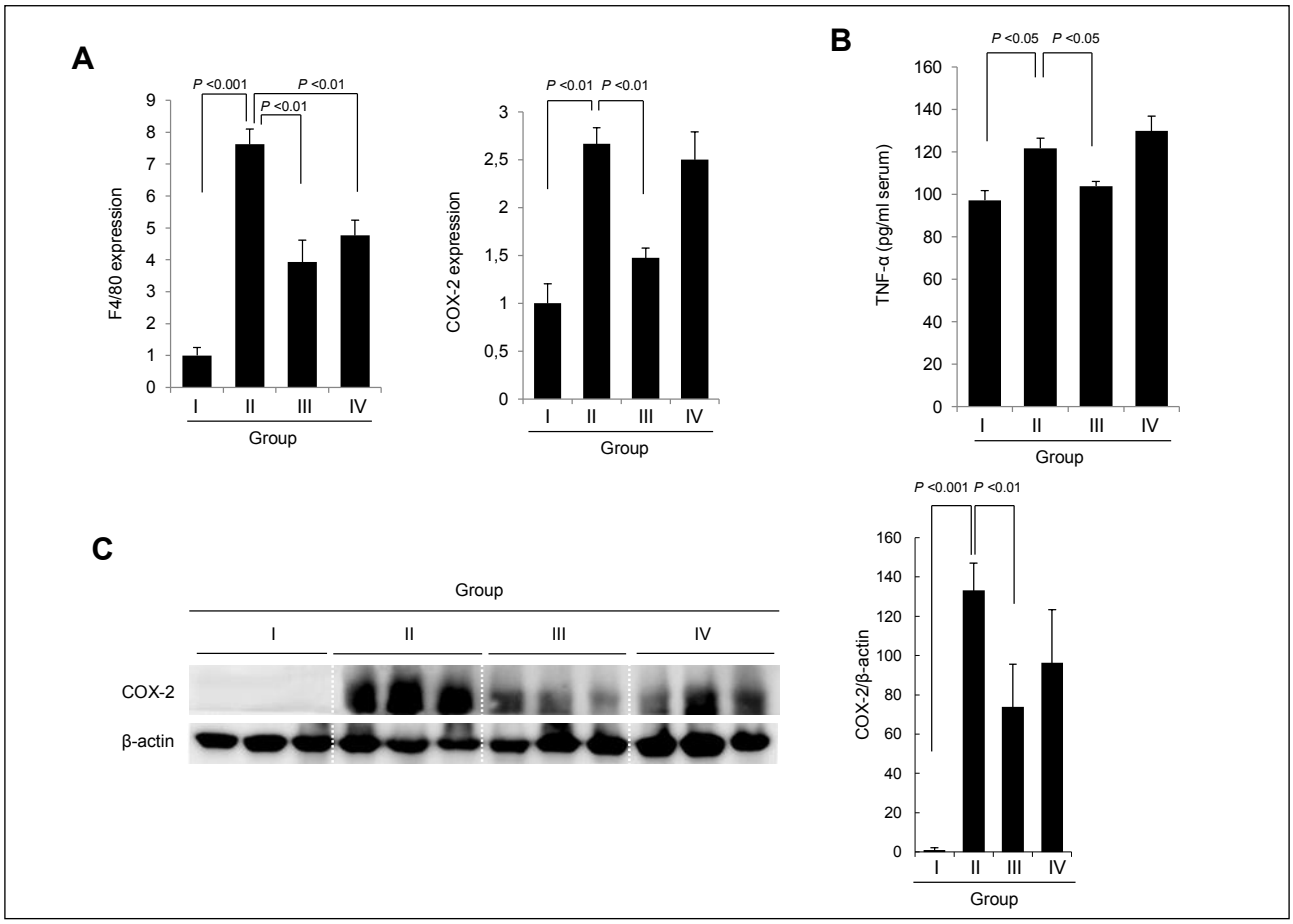


Fig. 6. (A) F4/80 macrophage infiltrations. Macrophage infiltrations were measured with F4/80 immunistaining. (B) TNF-α levels, serum level of TNF-α were all significantly increased after DSS administration, but oligonol were significantly attenuated them. (C) COX-2 immunohistochemical staining and its expression, Immunohistochemical detection of COX-2 in mouse colon according to group. (D) The protein expression of COX-2. The protein expression of proinflammatory signaling pathway was detected by Western blot. Administration of DSS significantly increased the expressions of COX-2 compared with normal group but significantly decreased in pretreatment of oligonol.

either iNOS or COX-2 expression ($P < 0.05$, *Fig. 2D*). Next, we have compared the mean nuclear expression of NF-κB p65 according to group and the expressions of NF-κB were significantly inhibited in group pretreated with oligonol ($P < 0.05$, *Fig. 3A*). Looking at other transcription factor implicated in acute inflammation, as seen in *Fig. 3B*, *c-Jun* and *c-Fos* was significantly increased with DSS administration, but their expressions were significantly decreased in group pretreated with oligonol ($P < 0.05$). All of these results consistently showed oligonol pretreatment significantly repressed inflammation-associated transcriptional activations, NF-κB and AP-1.

Significant antioxidative and phase 2 antioxidant enzyme response was responsible for preventive action of oligonol

To compare the difference in antioxidant condition between groups, the mean levels of total antioxidant concentration (TAC) was measured. As seen in *Fig. 4A*, DSS administration led to significant reduction in TAC ($P < 0.01$) significantly. However, oligonol pretreatment preserved significant levels of TAC compared with Group 2 ($P < 0.01$). These decreases in TAC in DSS administration were significantly associated with significant increments in lipid peroxidation. The mean levels of malondialdehyde (MDA), reflecting the extent of oxygen derived free radical induced-lipid peroxidation, were significantly increased in Group 2 ($P < 0.001$), but significantly decreased in oligonol pretreated group ($P < 0.005$, *Fig. 4B*). To demonstrate whether these ameliorating efficacies of oligonol on DSS-induced colitis are mediated by antioxidative phase 2 enzyme reaction, we measured not only Nrf2 transcription activity, but also the expression of Nrf2 downstream enzymes, HO-1 and NQO-1, in the colon tissues. As shown in *Fig. 4C*, Nrf2 transcription activity levels were significantly lowered after DSS administration compared to that before administration ($P < 0.01$). However, oligonol pretreatment significantly preserved Nrf2 activity even after DSS administration ($P < 0.01$, *Fig. 4C*). The expression of HO-1 among the phase 2 antioxidative and anti-mutagenic enzymes down-stream of Nrf2 was increasingly observed in group pretreated with oligonol ($P < 0.05$, *Fig. 4D*). However, NQO-1 was not influenced by oligonol pretreatment.

Oligonol prevented relapse of colitis better than sulfasalazine

In order to represent relapse model of IBD, we have designed model as shown in *Fig. 5A* that DSS was re-administered after two weeks pause of the first DSS administration. Our model of colitis relapse was significantly led to mortality of mice as seen in *Fig. 5E*, leading to aggravated colitis through relapse. Evaluating colon length, clinical signs, body weight, and pathological assessment, repeated bout of 3% DSS after 2 weeks pause led to significant

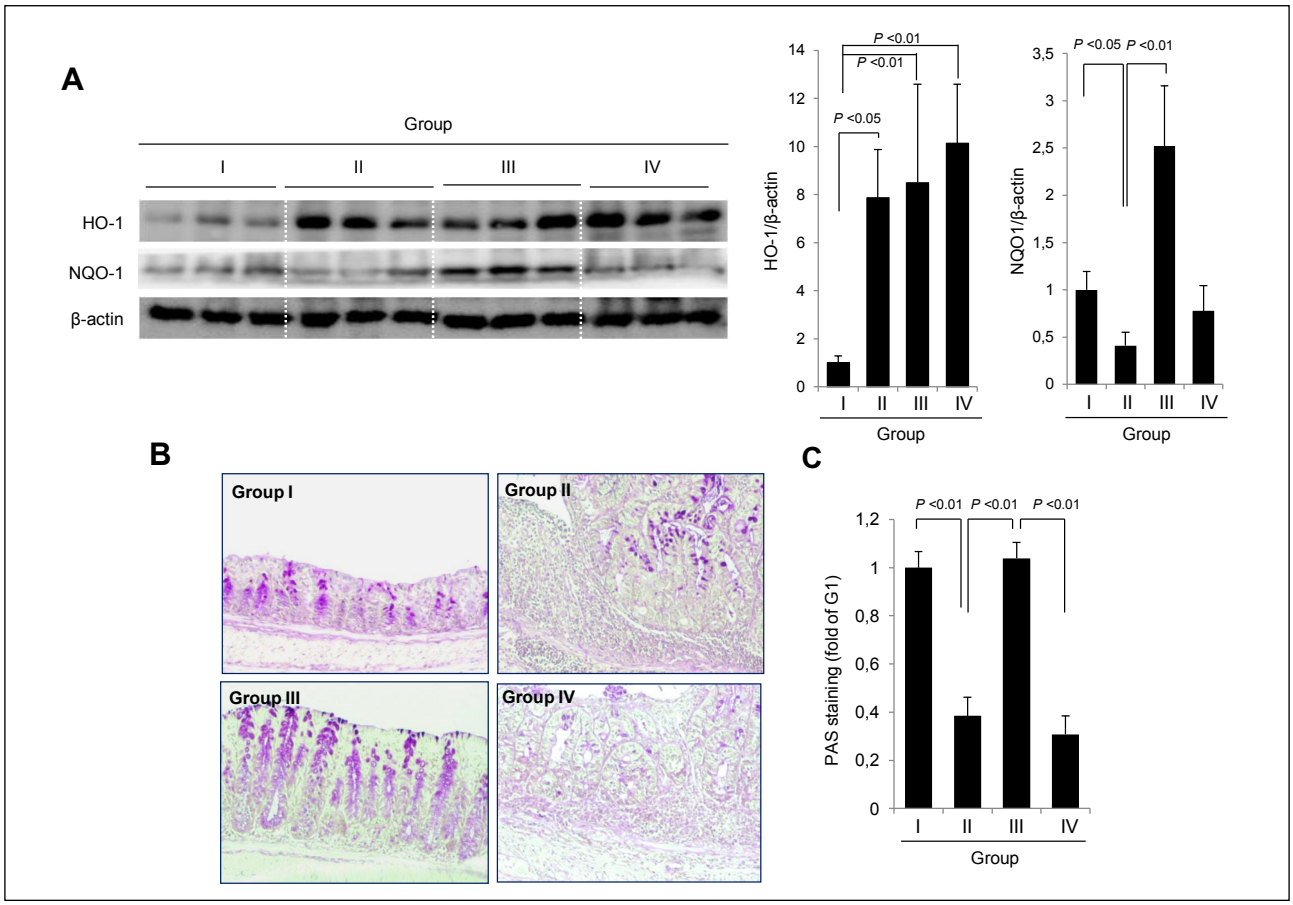


Fig. 7. Oligonol protected the colonic mucosa and induced the expression of antioxidant enzymes in mouse colon. (A) Protein expression of HO-1 and NQO-1 which phase 2 enzymes downstream of Nrf2 were detected by Western blot. All protein expressions of NQO-1 and HO-1 are the phase 2 enzymes downstream of Nrf2 were examined. The protein expressions of NQO-1 in oligonol treatment were significantly increased compared to DSS administration. (B) PAS staining for intestinal mucin immunohistochemical detection of the mucus production in mouse colon. (C) Quantitative analysis of PAS staining concluding oligonol might benefit colitis in preventing relapse.

shortening in colon length ($P < 0.01$, *Fig. 5B*), significant increases in clinical signs ($P < 0.01$, *Fig. 5B*), significant decreases in mean body weight $P < 0.01$, *Fig. 5C*), and significant increases in pathological scores ($P < 0.01$, *Fig. 5D*). However, oligonol treatment for 2 weeks before repeated DSS administration led to significantly increases in colon length ($P < 0.01$), significantly ameliorated clinical signs ($P < 0.01$, *Fig. 5B*), increment in mean body weights ($P < 0.001$, *Fig. 5C*), and significant reduction in mean pathological score ($P < 0.01$) (*Fig. 5D*). On pathological evaluation, repeated administration of 3% DSS led to significant increases in colitis and ulcerations, but these pathological changes were significantly ameliorated (*Fig. 5D*). As shown in *Fig. 5E*, relapse model of DSS-induced colitis was associated with significant mortality as time passes in control, but these increased mortality *via* repeated DSS administration were significantly prevented in Group III and Group IV, signifying the importance of maintenance therapy in ulcerative colitis ($P < 0.01$).

Anti-inflammatory actions of oligonol contributed to decreased relapse of colitis

In order to explain why oligonol treatment significantly decreased relapse of repeated DSS administration, first, counting macrophage infiltrations, F4/80 staining for macrophages was done in all groups and as seen in *Fig. 6A*, repeated DSS administration (Group II) led to significantly increased expressions of F4/80 ($P < 0.001$). However, either oligonol or sulfasalazine treatment for 2 weeks before repeated DSS administration significantly decreased macrophage infiltration ($P < 0.01$). Mucosal levels of COX-2 (*Fig. 6A*) and TNF-α (*Fig. 6B*) were also significantly increased with second DSS administration, but significantly decreased only in oligonol treatment, not with sulfasalazine ($P < 0.05$). Investigation of COX-2 expressions significantly showed that repeated DSS led to significant increments in COX-2 expressions ($P < 0.01$) as seen in Western blot for COX-2 *Fig. 6C*, but only oligonol treatment led to significantly decreased expressions of COX-2 ($P < 0.01$), consistently signifying that oligonol treatment significantly prevented DSS-induced relapse *via* significant anti-inflammatory actions, while sulfasalazine was inferior to oligonol in this matter.

NQO-1 induction as significant phase 2 antioxidative host response with oligonol treatment led to prevention of colitis relapse

Different with HO-1 and NQO-1 expressions as seen in acute colitis, repeated induction of colitis through repeated DSS administration led to significant decrements in NQO-1, while HO-1 significantly increased ($P < 0.05$, *Fig. 7A*). Focusing on NQO-1, repeated bout of DSS colitis led to significant decreases in NQO-1, while oligonol treatment significantly increased NQO-1 expressions ($P < 0.05$, *Fig. 7A*). Lastly, as biological response implicated in relapse prevention, we have measured the

distribution of PAS-positive mucosal cells according to group. As seen in *Fig. 7B*, significantly decrements in PAS positive cells were noted with repeated DSS administration, but oligonol treatment significantly preserved PAS (+) cells in colon mucosa ($P < 0.01$).

DISCUSSION

Translating our study from point of clinical aspect, oligonol treatment can be applied to prevent IBD relapse, better than current standard maintenance therapeutics to

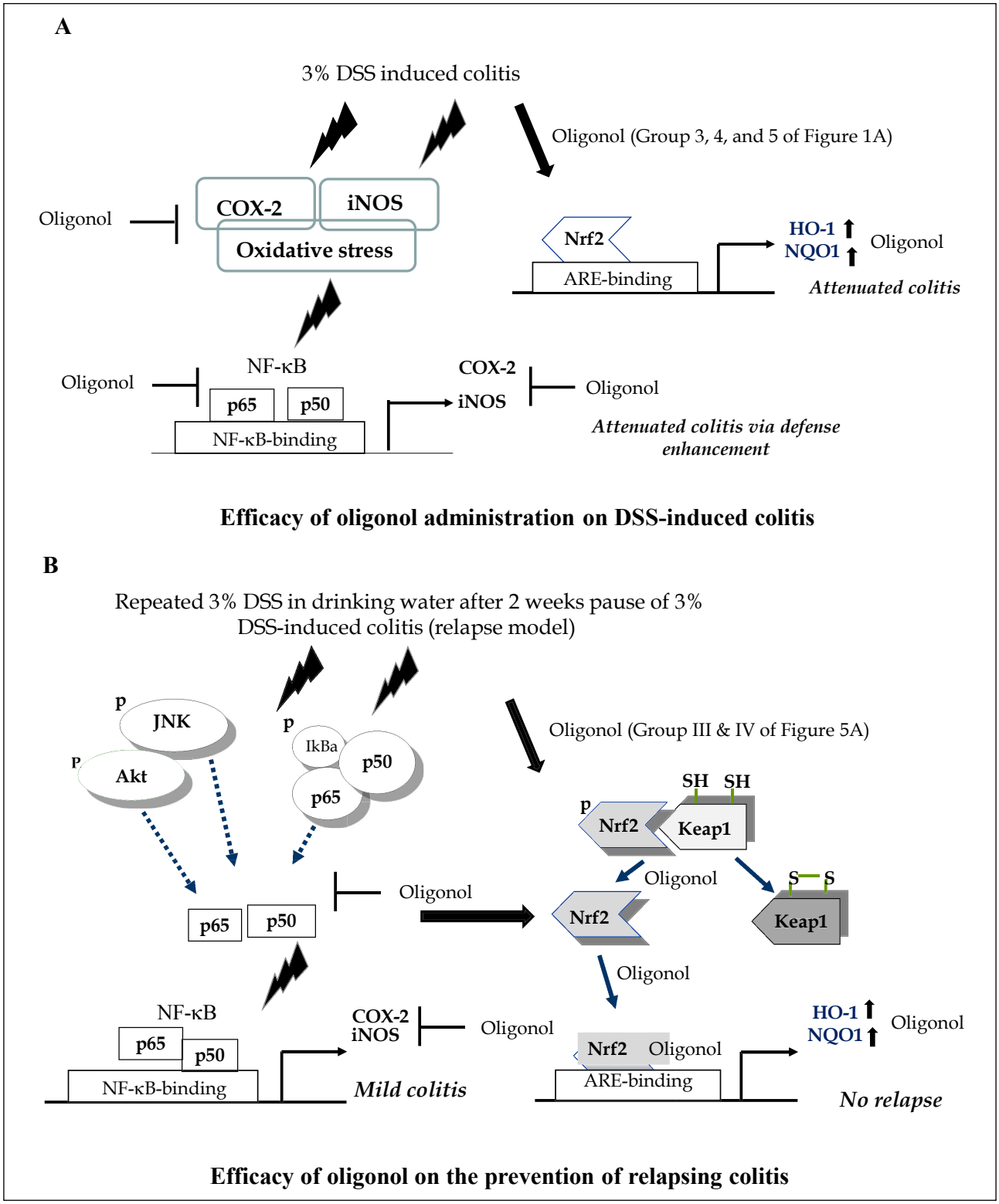


Fig. 8. Schematic figure showing either preventive or relapse preventive effects of oligonol against DSS-induced colitis. (A) Oligonol pretreatment significantly ameliorated DSS-induced colitis *via* anti-inflammatory, antioxidative, and cytoprotective action. (B) On colitis relapse model provoked with repeated DSS administration, oligonol significantly mitigated inflammatory and oxidative stress, resulting in significant attenuation of colitis relapse.

prevent relapse, sulfasalazine. Currently, gold standard for maintenance therapy is *sine qua non* 5-ASA compounds such as sulfasalazine or mesalazine, but after careful translation of our study, oligonol alone or combination with 5-ASA affords faithful gate-keeper for IBD maintenance therapy. Oligonol pretreatment significantly either ameliorated DSS-induced colitis or mitigated relapse through repeated DSS-induced colitis. As summarized in *Fig. 8*, oligonol treatment significantly induced phase 2 antioxidative response in DSS-induced colitis in addition to inactivation of redox-sensitive NF-κB and other inflammatory signaling.

Among many experimental models for inducing colitis, the DSS induced colitis remains one of the best surrogate models that closely resembles the human ulcerative colitis in many facets (15-16), of course, easy to handle. DSS exhibits toxic effects toward colonic epithelium and destroys the mucosal barrier, allowing bacteria to contact lamina propria cells (17). The uncontrolled intestinal immune response against bacterial antigens leads to the production of abundant cytokines and chemokines by activated leukocytes and epithelial cells accompanied with excess oxidative stress in IBD and colitis-associated cancer (18). Excess generation of ROS caused by the gut microenvironment breaks intestinal antioxidant systems, thereby contributing to intestinal oxidative injury and initiating pro-inflammatory signaling such as COX-2, iNOS as well as diverse cytokines. In addition, inhibition of an antioxidant enzyme like HO-1 further aggravated DSS-induced colitis.

At first, we utilized the role of preventive action of oligonol in DSS-induced colitis model that mimics human IBD. Our study clearly demonstrated that oligonol administration was able to inhibit TNF-α and COX-2 both at protein and mRNA levels, suggesting that oligonol could be useful in the suppression of colon inflammation. On the other hand, TNF-α has been described as a key molecule in UC pathogenesis, and a monoclonal antibody against this molecule, biologics such as infliximab or adalimumab, has proven to be effective in the treatment of moderate to severe UC. Our study demonstrated that there was a significant amelioration of the colon length after 2 weeks of oligonol treatment and a significant reduction in mean scores of clinical symptoms as well as survival. To investigate a mechanism of preventive action of oligonol against colitis, we investigated whether oligonol can provide potency of remission maintenance in experimental models of both acute colitis and cyclic colitis representing relapse model. As results, we found potent effects of oligonol on the survival of the mice given 3% DSS in a cyclic protocol for up to 28 days (*Fig. 5A*). In the absence of oligonol, a protocol characteristically leads toalmost two-thirds over a period of 7 days. We found that treatment with oligonol nearly eliminated deaths during this period in relation to the dose given (*Fig. 5B*).

Another important role of oligonol in this study was the regenerating and rejuvenating action as documented with PAS staining and phase 2 host defense enzyme expression in affected colon tissues. That means oligonol may have potential to heal the mucosa. Currently, remission is defined as complete resolution of symptoms and endoscopic mucosal healing (19). In the cyclic protocol, DSS (3%) was given for repeated 7-day periods interrupted by recovery periods on water, oligonol or sulfasalazine. Oligonol or sulfasalazine was given for 2 weeks, and the disease activity was evaluated at the end of the second DSS-cycle. In these models, we compared the efficacy of oligonol (50 mg/kg) with that of sulfasalazine with the dose of 30 mg/kg. In human ulcerative colitis with mild to moderate activity, the standard therapy is sulfasalazine, given as oral therapy in doses of 2 g (30 mg/kg for an average 60 kg weighted person) a day for maintenance of remission. Though sulfasalazine, a drug composed of 5-ASA and sulfapyridine linked by an azo bond (20), can relieve inflammatory activities as well as some radical scavenging action, in spite that sulfasalazine has a double edged sword effect by several side effects, such as generating oxidative stress, hepatotoxicity, and severe blood disorders (21). From the current study, we found that oligonol was significantly better than sulfasalazine in preventing UC relapse and achieving mucosal healing, which was possible through innate anti-inflammatory actions as well as Nrf2-mediated cytoprotection.

Nrf2 plays a central role in cytoprotection by detoxifying and eliminating reactive oxygen species (ROS), xenobiotics and electrophilic carcinogens (22). Nrf2 also mediates induction of several other classes of antioxidant proteins such as thioredox in peroxiredoxin, sulfiredoxin, ferritin, metallothionein, and HO-1 and mediates the induction of phase II drug-metabolizing enzymes such as aldo-ketoreductases (AKRs), glutathione S-transferases (GSTs), and NAD(P)H: quinoneoxidoreductase 1 (NQO1). In this study, we demonstrated that oligonol increased Nrf2 transcription activity and it's downstream such as HO-1 and NQO1 accompanied with significantly decreased of nuclear translocation of NF-κB p65. During an acute phase response following inflammatory stimuli, AP-1 is a regulator of major physiological processes such as cell proliferation, differentiation, organogenesis, apoptosis, and response to stress. *c-Fos* and *c-Jun* are the best-studied AP-1 components (23).

Recent data also reveal that Nrf2 signaling plays an important role in reducing the inflammatory response. Nrf2 also represses multiple pro-inflammatory genes, including TNF-α, IL-1 and IL-6, which is thought to be primarily through its ability to antagonize redox sensitive transcription factor, NF-κB (24, 25). The HO-1 enzyme has prominent anti-inflammatory activity and it is up-regulated by Nrf2. This is likely to modulate innate immunity, inflammation. There is also considerable cross-talk between the Nrf2 pathway and inflammatory signaling. NF-κB has been reported to directly repress Nrf2 signaling at the transcriptional level (26). Despite of advancement of IBD treatment, many IBD patients also remain reluctant to the currently established medications largely due to potential adverse events. From the results of this study, oligonol may constitute a new pharmacologic treatment in IBD because it is able to attenuate the inflammation in acute and cyclic colitis model, probably because of its ability to re-enforce adaptive responses as well as cytoprotective actions.

Conclusively, oligomerized small molecular polyphenol, oligonol, showed not only the anti-inflammatory effects which are presumably secondary to its regulation of the release of some endogenous inflammatory endocoids namely, TNF-α and NO, but also the modulation of oxidant and anti-oxidant balance by increasing cytoprotective protein expression which together can prevent possible oxidative stress-induced apoptosis of colonic epithelial cells in colonic tissue. Two mechanisms possibly concerted the protective role of oligonolin DSS-induced colitis or repeated DSS-induced fatal relapsed colitis. In conclusion, our studies provide some clue that oligonol may have a potential in either suppressing colon inflammation or affording antioxidative defense system in the diseased bowel (*Fig. 7c*). However, a clinical trial is needed to confirm these findings in human patients with IBD.

Abbreviations: AP-1, activator of protein-1; CD, Crohn's disease; COX-2, cyclooxygenase 2; DSS, dextran sulfate sodium; HO-1, heme oxygenase-1; IBD, inflammatory bowel

disease; IL-1, interleukin-1; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; NF-κB, nuclear factor-κ B; NQO-1, NAD(P)H dehydrogenase (quinone 1); PAS, periodic acid and schiff; TAC, total antioxidation capacity; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis;

Author' contributions: K.J. Kim, J.M. Park, J.S. Lee, Y.S. Kim, N. Kangwan, Y.M. Han, E.A. Kang, and J.M. An initiated the project, performed most of the experiments, and K.J. Kim and K.B. Hahm wrote the manuscript. All authors performed a significant part of experiments. Among authors, K.J. Kim, J.M. Park, and K.B. Hahm were involved in project inception, design, supervision, and manuscript writing.

K.J. Kim and J.M. Park contributed equally in the current study.

Acknowledgements: This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. NRF-2017R1C1B2009057 to Y.M. Han) and supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA, 116015-03-1-CG000).

We appreciate help from CHA Organoid Research Center.

Conflict of interests: None declared.

REFERENCES

1. Simmonds NJ, Allen RE, Stevens TR, Van Someren RN, Blake DR, Rampton DS. Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. *Gastroenterology* 1992; 103: 186-196.
2. Blackburn AC, Doe WF, Buffinton GD. Colonic antioxidant status in dextran sulfate-induced colitis in mice. *Inflamm Bowel Dis* 1997; 3: 198-203.
3. Robinson A, Hankins M, Wiseman G, Jones M. Maintaining stable symptom control in inflammatory bowel disease: a retrospective analysis of adherence, medication switches and the risk of relapse. *Aliment Pharmacol Ther* 2013; 38: 531-538.
4. Feagan BG, MacDonald JK. Once daily oral mesalamine compared to conventional dosing for induction and maintenance of remission in ulcerative colitis: a systematic review and meta-analysis. *Inflamm Bowel Dis* 2012: 18: 1785-1794.
5. Biasi F, Astegiano M, Maina M, Leonarduzzi G, Poli G. Polyphenol supplementation as a complementary medicinal approach to treating inflammatory bowel disease. *Curr Med Chem* 2011: 18: 4851-4865.
6. Quezada SM, Briscoe J, Cross RK. Complementary and alternative medicine. *Inflamm Bowel Dis* 2016; 22: 1523-1530.
7. D'Argenio G, Mazzone G, Tuccillo C, *et al.* Apple polyphenols extract (APE) improves colon damage in a rat model of colitis. *Dig Liver Dis* 2012; 44: 555-562.
8. Park CH, Lee JY, Kim MY, *et al.* Oligonol, a low-molecular-weight polyphenol derived from lychee fruit, protects the pancreas from apoptosis and proliferation via oxidative stress in streptozotocin-induced diabetic rats. *Food Funct* 2016; 7: 3056-3063.
9. Kundu JK, Hwang DM, Lee JC, *et al.* Inhibitory effects of oligonol on phorbol ester-induced tumor promotion and COX-2 expression in mouse skin: NF-kappaB and C/EBP as potential targets. *Cancer Lett* 2009; 273: 86-97.
10. Yum HW, Zhong X, Park J, *et al.* Oligonol inhibits dextran sulfate sodium-induced colitis and colonic adenoma formation in mice. *Antioxid Redox Signal* 2013; 19: 102-114.
11. Aruoma OI, Sun B, Fujii H, *et al.* Low molecular proanthocyanidin dietary biofactor oligonol: its modulation of oxidative stress, bioefficacy, neuroprotection, food application and chemoprevention potentials. *Biofactors* 2006; 27: 245-265.
12. Lee N, Shin MS, Kang Y, *et al.* Oligonol, a lychee fruit-derived low-molecular form of polyphenol mixture, suppresses inflammatory cytokine production from human monocytes. *Hum Immunol* 2016; 77: 512-515.
13. Cheon JH, Kim JS, Kim JM, Kim N, Jung HC, Song IS. Plant sterol guggulsterone inhibits nuclear factor-kappaB signaling in intestinal epithelial cells by blocking IkappaB kinase and ameliorates acute murine colitis. *Inflamm Bowel Dis* 2006; 12: 1152-1161.
14. Pandurangan AK, Dharmalingam P, Ananda Sadagopan SK, Ganapasam S. Effect of luteolin on the levels of glycoproteins during azoxymethane-induced colon carcinogenesis in mice. *Asian Pac J Cancer Prev* 2012; 13: 1569-1573.
15. Perse M, Cerar A. Dextran sodium sulphate colitis mouse model: traps and tricks. *J Biomed Biotechnol* 2012; 2012: 718617. doi: 10.1155/2012/718617
16. Salmenkan H, Holappa M, Forsqard RA, Korpela R, Vapaatalo H. Orally administered angiotensin-converting enzyme-inhibitors captopril and isoleucine-proline-proline have distinct effects on local renin-angiotensin system and corticosterone synthesis in dextran sulfate sodium-induced colitis in mice. *J Physiol Pharmacol* 2017; 68: 355-362.
17. Moura FA, de Andrade KQ, dos Santos JC, Araujo OR, Goulart MO. Antioxidant therapy for treatment of inflammatory bowel disease: Does it work? *Redox Biol* 2015; 6: 617-639.
18. Kowalczyk P, Jaworek J, Kot M, *et al.* Inflammation increases oxidative DNA damage repair and stimulates preneoplastic changes in colons of newborn rats. *J Physiol Pharmacol* 2016; 67: 277-286.
19. Dignass A, Lindsay JO, Sturm A, *et al.* Second European evidence-based consensus on the diagnosis and management of ulcerative colitis Part 2: Current management. *J Crohn's Colitis* 2012; 6: 991-1030.
20. Oz HS, Ebersole JL. Application of prodrugs to inflammatory diseases of the gut. *Molecules* 2008; 13: 452-474.
21. Uko V, Thangada S, Radhakrishnan K. Liver disorders in inflammatory bowel disease. *Gastroenterol Res Pract* 2012; 2012: 642923. doi: 10.1155/2012/642923
22. Mazur-Bialy AI, Kozlowska K, Pochec E, Bilski J, Brzozowski T. Myokine irisin-induced protection against oxidative stress in vitro. Involvement of heme oxygenase-1 and antioxidazing enzymes superoxide dismutase-2 and glutathione peroxidase. *J Physiol Pharmacol* 2018; 69: 117-125.
23. Bossis G, Malnou CE, Farras R, *et al.* Down-regulation of c-Fos/c-Jun AP-1 dimer activity by sumoylation. *Mol Cell Biol* 2005; 25: 6964-6979.
24. Ahmed SM, Luo L, Namani A, Wang XJ, Tang X. Nrf2 signaling pathway: pivotal roles in inflammation. *Biochim Biophys Acta* 2016; 1863: 585-597.
25. Li Y, Shen L, Luo H. Luteolin ameliorates dextran sulfate sodium-induced colitis in mice possibly through activation of the Nrf2 signaling pathway. *Int Immunopharmacol* 2016; 40: 24-31.

26. Lu MC, Ji JA, Jiang YL, *et al.* An inhibitor of the Keap1-Nrf2 protein-protein interaction protects NCM460 colonic cells and alleviates experimental colitis. *Sci Rep* 2016; 6 26585. doi: 10.1038/srep26585

Received: June 1, 2018
Accepted: June 30, 2018

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