Walnut phenolic extract inhibits nuclear factor kappaB signaling in intestinal epithelial cells, and ameliorates experimental colitis and colitis-associated colon cancer in mice

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Abstract

Purpose  Walnuts (Juglans regia) are known to have anti-cancer and immunomodulatory effects. However, little information is available on the effects of walnut phenolic extract (WPE) on intestinal inflammation and colitis-associated colon cancer.

Methods  COLO205 cells were pretreated with WPE and then stimulated with tumor necrosis factor (TNF)-α. In the acute colitis model, wild type mice (C57BL/6) were administered 4% dextran sulfate sodium (DSS) for 5 days. In the chronic colitis model, interleukin (IL)-10−/− mice were administered with either the vehicle or WPE (20 mg/kg) by oral gavage daily for 2 weeks. In an inflammation-associated tumor model, wild type mice were administered a single intraperitoneal injection of azoxymethane followed by three cycles of 2% DSS for 5 days and 2 weeks of free water consumption.

Results  WPE significantly inhibited IL-8 and IL-1α expression in COLO205 cells. WPE attenuated both the TNF-α-induced IκB phosphorylation/degradation and NF-κB DNA binding activity. The administration of oral WPE significantly reduced the severity of colitis in both acute and chronic colitis models, including the IL-10−/− mice. In immunohistochemical staining, WPE attenuated NF-κB signaling in the colons of both colitis models. Finally, WPE also significantly reduced tumor development in a murine model of colitis-associated colon cancer (CAC).

Conclusions  WPE ameliorates acute and chronic colitis and CAC in mice, suggesting that WPE may have potentials for the treatment of inflammatory bowel disease.

Keywords  Walnut · Inflammatory bowel disease · Nuclear factor kappaB · Colon cancer

Introduction

Inflammatory bowel disease (IBD) including Crohn’s disease (CD) and ulcerative colitis is characterized by chronic intestinal inflammation, resulting in chronic abdominal pain, diarrhea, and hematochezia [1]. Its incidence and prevalence have been rapidly increasing in both developed and developing countries [2, 3]. Patients with IBD have an increased risk of colitis-associated colon cancer (CAC), which has higher mortality than de novo colon cancer [4]. Although biological agents, such as anti-tumor necrosis (TNF)-α provides symptom improvement and mucosal healing in some patients with IBD and are expected to have preventive effects on CAC, drugs that can induce and maintain long-term remission have not yet been developed [5].

The mucosal surface of the intestine is exposed to microorganisms but the intestinal epithelial cells (IECs) have various defense mechanisms against harmful bacteria.
in the intestinal lumen [6]. The mechanical barrier provided by the IECs contributes to the isolating the host from the bacteria [7], and anti-microbial peptide secretion from IECs inhibits microbial growth, which in turn regulates innate immune responses [8]. IECs also regulate intestinal homeostasis by activating intracellular molecular cascades. For example, NF-κB signaling in IECs is a key transcription factor for the regulation of intestinal inflammation [9]. This NF-κB signaling regulates the production of cytokines such as TNF-α, interleukin (IL)-1, IL-6, and IL-8 [9]. In addition, NF-κB signaling contributes to the development of CAC by regulating apoptosis and angiogenesis [5].

Walnuts (Juglans regia) are round, single-seeded fruits that are commonly grown in southeast Europe, southwest and central Asia, southeast China, and the United States of America. It has been reported that walnuts provide health benefits, including anti-oxidative and anti-cancer effects [10–12]. Epidemiological studies have demonstrated that the intake of nuts and seeds associated with low colorectal cancer risk [13, 14], and walnut consumption inhibited tumor growth in colon cancer by regulating colon cancer stemness [15]. Additionally, our co-authors demonstrated that walnut phenolic extract (WPE) and its bioactive compounds suppress cell growth in colon cancer by regulating colon cancer stemness [16]. However, little information is available on the effect of walnut on intestinal inflammation and CAC. Therefore, the aim of this study was to evaluate the effect of walnut phenolic extract on intestinal inflammation and CAC.

Methods and materials

Preparation of WPE and its bioactive compounds

WPE was kindly donated by professor Yuri Kim. WPE preparation was performed according to the methanolic extraction method as previously described [16]. Briefly, shelled kernels of frozen walnuts were finely ground and immersed in a solution of 75% acetone containing sodium metabisulphite. The solution was incubated with N2 at 4 °C, and after 24 h, centrifuged at 8000 g for 10 min. The resulting supernatant was filtered, the acetone was removed under reduced pressure, and 50% methanol was added. After three consecutive hexane extractions, the extracts were lyophilized to a dry powder after removing the methanol to prevent oxidation. All of the prepared samples were stored at −80 °C until needed. The major components of WPE were analyzed by high-performance liquid chromatography. In 100 g of walnut, 10.7 mg of gallic acid, 137.5 mg (+)-catechin, 13.6 mg of chlorogenic acid, and 12.6 mg of ellagic acid were included.

Cell line and treatment of walnut extract

The human colonic epithelial cell line, COLO205 (American Type Culture Collection [ATCC], CCL-222, Rockville, MD, USA), obtained from the Korea cell line bank were used between passages 15 and 30. Cells were pretreated with WPE, dissolved in phosphate-buffered saline (PBS) for 24 h, and then treated with TNF-α for varying durations.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time RT-PCRs were conducted as described previously [17]. Whole cellular RNA was extracted using TRIzol® (GIBCO, Gaithersburg, MD). Of the extracted RNA, 1 µg of RNA was reverse-transcribed and amplified using the LightCycler® 480 DNA SYBR Green I Master system (Roche Applied science, Penzberg, Germany) and the LightCycler® 480 II system (Roche Diagnostics Ltd. Rotkreuz, Switzerland) with specific primers for human interleukin (IL)-8, IL-1α, and β-actin. The primer sequences were as follows: IL-8 (S): 5′-AACCAACCAAGGAACCAT-3′, (AS) 5′-CTCTACACAGGCTGGAA-3′; IL-1α (S) 5′-TCAGGCTGTCGATCTACAT-3′, (AS) 5′-GCCGTTAGTGAGGACGGAAGAAGA-3′; TNF-α (S): 5′-CATCTCTCTCTAAACGAGCAGCAGAA-3′; β-actin (S): 5′-GTTGCTATCGATGGAGAGACGCAAAG-3′; (AS) 5′-CTGGAAGTGTGCATGAGCTATCAGAGTACCA-3′; IL-1β (S): 5′-GTTGCTATCGATGGAGAGACGCAAAG-3′; (AS) 5′-CTGGAAGTGTGCATGAGCTATCAGAGTACCA-3′; β-actin (S): 5′-GTTGCTATCGATGGAGAGACGCAAAG-3′; (AS) 5′-CTGGAAGTGTGCATGAGCTATCAGAGTACCA-3′; β-actin (AS): 5′-GTTGCTATCGATGGAGAGACGCAAAG-3′.

Electrophoretic mobility shift assays (EMSAs)

COLO205 cells were harvested and nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL). Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA) and EMSAs were performed using a commercially available kit (Promega, Madison, WI) [18].

Immunoblot assays

Immunoblot assays were performed as described previously [18]. COLO205 cells were washed with ice-cold PBS, and cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). After determining protein concentration using the Bradford Assay, 50 µg of protein from each lane was size-fractionated on a 12% polyacrylamide minigel and transferred to
a nitrocellulose membrane with 0.45-µm pore size. Anti-IκBα (Cell Signaling, Beverly, MA), phospho-IκBα (Cell Signaling), and anti-β-actin (Cell Signaling) were used as primary antibodies. Protein detection was performed using a Luminescent Image Analyzer, LAS 4000 (Fuji Film, Tokyo, Japan).

Animals

All procedures using mice were approved by the Institutional Animal Care and Use Committee of Seoul National University Boramae Hospital. Six to seven-week-old specific pathogen-free (SPF) wild type mice (C57BL/6NCrljBgi) were purchased from Orient (Seongnam, Korea). IL-10 knock-out (IL-10−/−) mice on a C57BL/6 strain background were obtained from the Center for Animal Resource and Development (Seoul, Korea). These mice were acclimated for a week and we used 7–8-week old mice (20–22 g) for these studies. The mice were housed in sterile plastic cages under specific pathogen-free conditions with standard humidity and temperature. Mice were supplied with standard chow and free sterile water.

Induction of dextran sulfate sodium (DSS)-induced colitis

Seven mice were randomly assigned to each group. Mice in the negative control group received free water alone. Mice assigned to the vehicle-treated group were administered PBS (100 µL), and mice assigned to the walnut-treated group were administered with low-dose (10 mg/kg), or high-dose (20 mg/kg) WPE once daily by oral gavage 2 days before DSS administration. Mice were then fed 4% DSS (MP Biological, Irvine, CA) dissolved in free water for 5 days. To investigate the effect of WPE on normal intestinal mucosa, WPE (20 mg/kg) was administered without DSS exposure in a group of mice. Body weight, stool consistency, and occurrence of bloody stool were checked by a blinded researcher daily to assess the disease activity index (DAI).

Induction of colitis in IL-10 knockout mice

IL-10−/− mice were used to evaluate the effect of WPE on chronic colitis. To induce rapid colitis, chow containing piroxicam (200 ppm) was fed to the IL-10−/− mice for 10 days as previously described [19]. After inducing colitis, 5 (vehicle) or 7 (WPE 20 mg/kg/day) mice were randomly assigned according to body weight. Mice were administered either vehicle or WPE (20 mg/kg) once daily for the next 2 weeks using oral gavage.

Gross and histological assessment

After extracting the colons, data on colon length were obtained. For hematoxylin and eosin (H&E) staining, the removed tissues were fixed using 10% formalin and embedded in paraffin. The severity of DSS-induced colitis was evaluated based on the severity and extent of inflammation, the degree of crypt damage, and the area of involvement [20]. Briefly, the sum of the score regarding inflammation, extent, and crypt damage was multiplied by the score of the area of involvement. The severity of chronic colitis in IL-10−/− mice was graded from 0 to 4 as previously described [19]. These evaluations were performed by a pathologist in a blinded manner.

Primary IEC isolation

Primary IECs were isolated as previously described [21]. The extracted colons were cut longitudinally and washed three times in Hank’s Balanced Salt Solution (HBSS; Hyclone, Logan, UT, USA) containing 2% fetal bovine serum (FBS). Each colon was cut into 5 mm pieces and incubated, with shaking, for 30 min at 37 °C in HBSS solution containing 2% FBS and 2 mM EDTA. The supernatants was filtered through nylon mesh (100 µm pore-size; BF bioscience, San Jose, CA) and then centrifuged at 2000 rpm for 5 min. The cell pellets were washed and resuspended in RPMI-1640 with 10% FBS. Cellular RNA was extracted using TRizol® (GIBCO) and analyzed using real-time RT-PCR.

Induction of CAC in a murine model

Male, 7-week-old specific pathogen-free C57BL/6 mice were used to induce CAC as previously described [22]. Mice were randomly divided between the vehicle-administered (n = 10) and walnut-treated (n = 10) groups. Azoxymethane (AOM, 12.5 mg/kg/mouse; Sigma) was injected into the intraperitoneal cavity of each mouse on day 0. The first cycle of 2% DSS was administered via drinking water from day 8 for 5 days, followed by 16 days of DSS-free water. This cycle was repeated three times. Mice were administered either vehicle or WPE (20 mg/kg) daily by oral gavage from day 8. Body weight was checked by a blinded researcher. Mice were killed 10 days after the final cycle of DSS administration. Postmortem, the colon was extracted, cut longitudinally, and photographed. The number of tumors and their dimensions were assessed using ImageJ (National Institute of Health, Bethesda, MD, USA). The extracted colons were fixed and embedded in paraffin. After H&E staining was performed, the severity
of intestinal inflammation was evaluated by a blinded pathologist as described earlier [20].

**Immunohistochemistry and in situ terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) analysis**

Immunohistochemical analysis was performed as previously described [22]. The slides were stained using anti-NF-κB p65 antibody (1:250, Cell Signaling), anti-phospho-IκB kinase (IKK)-α/β antibody (1:100, Cell Signaling) and anti-β-catenin antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactivity was assessed by determining the percentage of positive cells using a visual scoring system and was classified as 0 (no staining), 1 + (< 10%), 2 + (10–30%), 3 + (31–60%) and 4 + (61–100%) as previously described [22]. The immunoreactivity index was calculated in each of three nonadjacent fields (magnification: 400×), which were randomly selected. Apoptosis was measured using in situ TUNEL assays. In situ TUNEL assays were performed using an apoptosis detection kit (Chemicon, Temecula, CA). The apoptotic index was calculated based on the ratio of TUNEL reactive cells to the total number of cells in each of three nonadjacent fields (magnification: 100×) which were randomly selected.

**Statistical analysis**

Data are expressed as mean ± SD. Differences between groups were analyzed using an analysis of variance with Bonferroni correction or a Man Whitney U test. P values less than 0.05 were considered statistically significant.

**Results**

**WPE inhibits proinflammatory cytokine mRNA expression by inhibiting NF-κB signaling in COLO205 cells**

We conducted real-time RT-PCRs for IL-8 and IL-1α to evaluate the effect of WPE on proinflammatory cytokine production regulated by the NF-κB pathway. IL-8 expression was extensively increased after stimulation with TNF-α. Pretreatment with the WPE significantly reduced the expression of IL-8. In addition, IL-1α mRNA expression was decreased by the WPE treatment (Fig. 1a, b). We performed EMSAs to evaluate the effect of the WPE on NF-κB DNA binding activity. NF-κB DNA binding activity in IECs markedly increased after stimulation with TNF-α. However, pretreatment with the WPE reduced NF-κB DNA binding activity in IECs (Fig. 1c). As NF-κB is translocated into the nucleus by IκB phosphorylation, we measured IκB phosphorylation and degradation. Pretreatment with the WPE strongly inhibited IκB phosphorylation and degradation in IECs (Fig. 1d).

**WPE attenuates the severity of DSS-induced acute murine colitis**

WPE significantly attenuated the DSS-induced body weight reduction and DAI (Fig. 2a, b). In addition, colon shortening was improved by the WPE treatment (Fig. 2c). In the histological evaluation, acute and severe colitis were induced by the administration of DSS for 5 days in mouse treated with vehicle. These mice showed marked wall thickening, inflammatory cell infiltration, crypt damage, and ulceration. However, WPE administration attenuated the severity of intestinal damage, resulting in statistically significant differences in the distal colons, but not in the proximal colon (Fig. 2d, e). WPE (20 mg/kg) alone did not induce DAI or histological damage to the intestinal mucosa (data not shown). We isolated primary IECs to evaluate the expression of genes regulated by NF-κB. WPE significantly inhibited the expression of genes such as TNF-α, IL-β, and IL6 in primary IECs (Fig. 2f).

**WPE inhibits NF-κB signaling in mouse colon of DSS colitis**

To elucidate the effect of WPE on NF-κB signaling in vivo, we assessed NF-κB and IKK activity in the distal colon. DSS upregulated NF-κB expression in both IECs and lamina propria mononuclear cells. However, the increased NF-κB activation was significantly attenuated in mice treated with WPE (20 mg/kg/day) (Fig. 3a). Similarly, exposure to DSS markedly induced IKK activity, but WPE treatment significantly reduced it (Fig. 3b). These results suggested that anti-inflammatory effects of WPE occur via suppression of NF-κB signaling in IECs.

**WPE ameliorates chronic colitis in IL-10−/− mice**

As IL-10−/− mice exhibits chronic colitis slowly and the severity varies, we induced colitis using chow containing piroxicam. After 10 days of consuming the chow, mice exhibited severe colitis. We next administered either vehicle or WPE daily for 2 weeks. Mice treated with vehicle showed severe colitis such as epithelial hyperplasia, crypt abscesses, transmural inflammation, and ulceration. However, WPE administration significantly ameliorated chronic colitis in IL-10−/− mice, resulting in significant improvement in histological grading (Fig. 4a). Similar to the results observed for the acute colitis model, NF-κB activity increased after piroxicam administration in the colonic epithelium of
IL-10−/− mice, but it was significantly inhibited by WPE (20 mg/kg/day) treatment (Fig. 4b).

**WPE inhibits colitis-associated colon tumorigenesis in a murine model**

Two mice treated with PBS showed rapid body weight reduction after the first cycle of DSS administration and finally died 7 at week 5. In the WPE group, a mouse was euthanized because of severe weight reduction. We obtained eight samples from the PBS-treated group and nine samples from WPE-treated mice. Overall, mice treated with WPE showed improvement in body weight, compared to mice treated with vehicle (Fig. 5a). In the macroscopic evaluation, it was observed that the AOM injection and three cycles of DSS administration induced multiple colitis-associated colon tumorigenesis. Most of the tumors developed in the middle or distal colon. However, WPE administration significantly reduced tumor development (Fig. 5b, c). In addition, tumor size was significantly reduced by the WPE treatment (Fig. 5d). In the histological evaluation, WPE attenuated the histological grading of intestinal inflammation in the distal colon (Fig. 5e, f).

**WPE attenuates tumor growth by inhibiting IKK activity and β-catenin signaling independent of inducing apoptosis in a CAC model**

Because our in vitro and in vivo data showed that WPE inhibited NF-κB signaling in IECs, we performed immunohistochemical analysis in the distal colon. Exposure to AOM/DSS strongly induced IKK immunoreactivity and WPE significantly attenuated IKK activity in IECs (Fig. 6a). We evaluated whether treatment with WPE affect apoptotic cell death. Exposure to AOM-DSS induced extensive epithelial apoptosis but WPE did not significantly affect the apoptotic index (Fig. 6b). We next evaluated β-catenin translocation into the nucleus, a key signal of colon tumorigenesis.
Vehicle-treated mice showed marked β-catenin translocation into nucleus, whereas WPE significantly inhibited its translocation (Fig. 6c).

**Discussion**

Walnut, a member of the Juglandaceae family, has been known to have various beneficial effects on health. Recently, some studies have shown that WPE not only acts as antioxidants, but also acts as possible anti-inflammatory agents [23, 24]. A recent study reported that WPE has anti-inflammatory effects on human aorta endothelial cells [24]. However, little information is available on the effects of walnut intestinal inflammation and CAC. In the present study, we investigated the effect of WPE in intestinal inflammation and inflammation-associated tumorigenesis. WPE inhibited pro-inflammatory cytokine production by blocking NF-κB signaling. Oral administration of WPE attenuated DSS-induced acute colitis and chronic colitis in IL-10−/− mice. Finally, long-term administration of WPE significantly prevented the development of colitis-associated colon tumorigenesis in a murine model. To our knowledge, this is the first study to examine the beneficial effects of WPE in the intestinal inflammation.

In the present study, WPE inhibited NF-κB signaling in IECs and this was confirmed in DSS-induced murine colitis. Therefore, we believe that the anti-inflammatory mechanism of WPE involves blocking NF-κB signaling. However, the DSS model has serious limitations because it is a chemically induced and self-resolved colitis model. Therefore, we tried to confirm the anti-inflammatory effect of WPE on chronic colitis in IL-10−/− mice. WPE ameliorated the severity of chronic colitis in IL-10−/− mice by inhibiting NF-κB signaling, suggesting that WPE has anti-inflammatory effects on intestinal inflammation. The anti-inflammatory effects of similar phenolic extracts such as those of mango, pomegranate, and Terminalia catappa containing gallic acid, ellagic
Acid or chlorogenic acid have been observed in murine models of colitis [25, 26]. Therefore, we believe that WPE has the potential to control intestinal inflammation in patients with IBD. CAC development includes three phases: initiation, promotion, and progression [27]. In the tumor initiation phase, tumor cells activate oncogenes and inactivate tumor suppressor genes such as KRAS and p53 [5]. In the tumor promotion phase, the mutated cells clonally expand because of increased cellular proliferation and reduced cell death. Proinflammatory cytokines such as TNF-α, IL-1, and IL-6 seem to play a key role in promoting tumor growth [27]. Our study demonstrated that WPE inhibited the production of various cytokine such as TNF-α and IL-1α. The severity of chronic colitis was reduced by the WPE treatment in two different animal models. Because NF-κB signaling regulates the expression of apoptosis-related genes and angiogenesis, and to elucidate the anti-tumor mechanism of WPE on CAC, we studied whether WPE induced apoptosis. However, there was no significant difference in the apoptotic index between the two groups. Therefore, we believe that the anti-tumor effect of WPE is achieved by reducing the severity of inflammation rather than by inducing apoptosis. From our study results, and considering that WPE inhibits colon cancer cell growth by regulating colon cancer stemness [16], we believe that WPE has the potential for the prevention of CAC.
compounds in WPE. A previous study showed that gallic acid ameliorates intestinal inflammation in a murine model [28]. An ellagic acid-enriched pomegranate extract has also been found to attenuate trinitrobenzene sulfonic acid-induced chronic colitis in rats [29]. Therefore, a combination of gallic acid and ellagic acid in WPE may contribute to the reduced intestinal inflammation. However, we could not conduct studies using each bioactive compound. Therefore, further studies are required to elucidate whether these compounds have synergic effects when compared to individual bioactive compounds.

Diet is an important factor in controlling disease activity in IBD. Epidemiological studies have shown that a higher incidence of IBD has been reported in South Asians migrating to Canada and the United Kingdom, suggesting that a Western lifestyle and diet have a major role in the pathogenesis of IBD [30, 31]. A previous study demonstrated that diet can rapidly alter the human gut microbiota, which may contribute to the development of IBD [32]. Those will help to conclude that WPE has the potential for clinical applications in the treatment of IBD.

In the present study, we attempted to demonstrate that the usual dietary intake of walnut exerted anti-inflammatory effects on intestinal inflammation. Therefore, we tested a lower dose of WPE and the results demonstrated WPE exerted anti-inflammatory effects in murine colitis. It is important to know the quantity of walnut that patients should consume to achieve similar effects on intestinal inflammation. Based on human equivalent dose calculation [33], we believe that a dietary intake of 5 g/day/60 kg person of walnut may be sufficient to exert anti-inflammatory effects on intestinal inflammation. However, the result should be interpreted with caution because there is a lack of information on the bioavailability differences between walnut and WPE.

In conclusion, we found that WPE inhibits the TNF-α-induced NF-κB signaling in IECs and attenuates acute and chronic colitis as well as CAC in mice. These results suggest that WPE has the potentials to reduce intestinal inflammation and prevent CAC in patients with IBD.
**Fig. 5** The effect of WPE on a colitis-associated colon cancer mouse model. 

a. Body weight change in both groups (mean ± SD). WPE significantly attenuated body weight reduction compared to that of mice treated with vehicle. 

b. Representative colon samples treated with or without WPE in CAC model. Administration of WPE significantly reduced the number of tumors which developed in the AOM/DSS model. *P < 0.05 compared to AOM/DSS. 

c. The number of tumors in the two groups. WPE significantly reduced tumorigenesis compared to that in mice treated with vehicle. 

d. Tumor size was measured using ImageJ as described in Materials and Methods. WPE significantly inhibited tumor growth. 

e, f Histologic evaluations of (e) proximal and (f) distal colons in the DSS-induced acute murine colitis. DSS dextran sulfate sodium, PBS phosphate buffered saline, WPE walnut phenolic extract (20 mg/kg/day). *P < 0.05 compared to vehicle.
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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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