



Research article

American ginseng attenuates azoxymethane/dextran sodium sulfate-induced colon carcinogenesis in mice



Chunhao Yu^{1,2,3,☆}, Xiao-Dong Wen^{1,2,☆}, Zhiyu Zhang^{1,2}, Chun-Feng Zhang^{1,2},
Xiao-Hui Wu^{1,2}, Adiba Martin^{1,2}, Wei Du⁴, Tong-Chuan He⁵, Chong-Zhi Wang^{1,2},
Chun-Su Yuan^{1,2,6,*}

¹ Tang Center for Herbal Medicine Research, University of Chicago, Chicago, IL, USA

² Department of Anesthesia and Critical Care, University of Chicago, Chicago, IL, USA

³ School of Life Science and Chemical Engineering, and Jiangsu Provincial Engineering Laboratory for Biomass Conversion and Process Integration, Huaiyin Institute of Technology, Huaian, China

⁴ Ben May Department for Cancer Research, University of Chicago, Chicago, IL, USA

⁵ Department of Surgery, University of Chicago, Chicago, IL, USA

⁶ Committee on Clinical Pharmacology and Pharmacogenomics, University of Chicago, Chicago, IL, USA

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ABSTRACT

Background: Colorectal cancer is a leading cause of cancer-related death, and inflammatory bowel disease is a risk factor for this malignancy. We previously reported colon cancer chemoprevention potential using American ginseng (AG) in a xenograft mice model. However, the nude mouse model is not a gut-specific colon carcinogenesis animal model.

Methods: In this study, an experimental colitis and colitis-associated colorectal carcinogenesis mouse model, chemically induced by azoxymethane/dextran sodium sulfate (DSS) was established and the effects of oral AG were evaluated. The contents of representative ginseng saponins in the extract were determined.

Results: AG significantly reduced experimental colitis measured by the disease activity index scores. This suppression of the experimental colitis was not only evident during DSS treatment, but also very obvious after the cessation of DSS, suggesting that the ginseng significantly promoted recovery from the colitis. Consistent with the anti-inflammation data, we showed that ginseng very significantly attenuated azoxymethane/DSS-induced colon carcinogenesis by reducing the colon tumor number and tumor load. The ginseng also effectively suppressed DSS-induced proinflammatory cytokines activation using an enzyme-linked immunosorbent assay array, in which 12 proinflammatory cytokine levels were assessed, and this effect was supported subsequently by real-time polymerase chain reaction data.

Conclusion: AG, as a candidate of botanical-based colon cancer chemoprevention, should be further investigated for its potential clinical utility.

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1. Introduction

Colorectal cancer is one of the most common malignancies worldwide [1,2], and the 5-year survival rate is < 10% in the advanced stages [3]. Numerous effective drugs, including those

currently used for cancer treatment, have been developed from botanical sources [4,5]. Thus, there still is a significant unexploited resource in herbal medicines.

In our previous studies, we assessed the colon cancer chemoprevention potential of American ginseng, a very commonly used

* Corresponding author. Tang Center for Herbal Medicine Research, The Pritzker School of Medicine, University of Chicago, 5841 South Maryland Avenue, MC 4028, Chicago, IL 60637, USA.

E-mail address: cyuan@dacc.uchicago.edu (C.-S. Yuan).

☆ These two authors are co-first authors in this work.

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herbal medicine in the USA. [6,7]. In an *in vivo* investigation, the tumor xenograft nude mice model was used and significant anti-tumor effects of ginseng compounds were observed [8]. However, the xenograft mice model was not a commonly appreciated model for colon cancer studies. In addition, the ginseng compound was administered via intraperitoneal injection, an experimental approach, compared to the real world in which the route of administration of herbal medicines in humans is nearly always oral.

Inflammatory bowel disease is a group of chronic dysregulated inflammatory conditions in the large and small intestine of humans, and it is well known that chronic inflammation in the colon can lead to cancer [9–11]. An experimental colitis and colitis-associated colorectal carcinogenesis mouse model, chemically induced by azoxymethane (AOM)/dextran sodium sulfate (DSS), has been used often for colorectal cancer research [12,13]. AOM is a genotoxic colonic carcinogen frequently used to induce colon tumors [14,15].

We previously evaluated the effects of American ginseng (AG) in colorectal cancer chemoprevention in the AOM/DSS mouse model using a high-fat diet (20% fat) to mimic Western food [16]. In the present study, this established animal colon carcinogenesis model was used in mice fed with regular mouse chow (5% fat) reflecting an oriental diet, with or without AG supplement. To ensure the quality of the study botanical, high-performance liquid chromatography (HPLC) analysis was performed on the herb, and the contents of a number of important ginseng saponins were quantified. To extend previous tumor-related protein regulator observations, in this study, selected enzyme-linked immunosorbent assay (ELISA) for inflammatory cytokines and quantitative real-time polymerase chain reaction (qRT-PCR) were performed to elucidate the IBD related mechanisms of action.

2. Materials and methods

2.1. Chemicals and reagents

Standards of ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, Rg2, 20(R)-Rg2, Rg3, and Rh1 were obtained from Indofine Chemical Company (Somerville, NJ, USA) and Delta Information Center for Natural Organic Compounds (Xuancheng, AH, China). All standards were of biochemical-reagent grade and at least 95% pure. AOM was obtained from the NCI Chemical Carcinogen Reference Standard Repository, Midwest Research (Kansas City, MO, USA). DSS (molecular weight of 36–50 kDa) was obtained from MP Biomedicals (Solon, OH, USA). HPLC grade ethanol, *n*-butanol, acetonitrile, and dimethylsulfoxide were obtained from Fisher Scientific (Pittsburgh, PA, USA). Milli Q water was supplied by a water purification system (US Filter, Palm Desert, CA, USA). Hemocult Sensa test strips were obtained from Beckman Coulter (Brea, CA, USA). Multi-Analyte ELISArray Kits for inflammatory cytokine analysis were obtained from Qiagen (Germantown, MD, USA).

2.2. Botanical materials and extract preparation

AG roots (4-year-old, *Panax quinquefolius* L.) were obtained from Roland Ginseng, LLC (Marathon, WI, USA). The voucher samples were authenticated by Dr Chong-Zhi Wang and deposited at the Tang Center for Herbal Medicine Research at the University of Chicago. AG extract was prepared with a slight modification from previous works [17–19]. The air-dried roots of AG were pulverized into powder and sieved through an 80 mesh screen. One kilogram of the powder placed into 12 L flask was extracted three times by heat-reflux with 8 L of 75% (v/v) ethanol at 95°C for 4 h each time. The extracting solution was filtered when hot. The gathered and combined filtrate was evaporated under vacuum with a Büchi

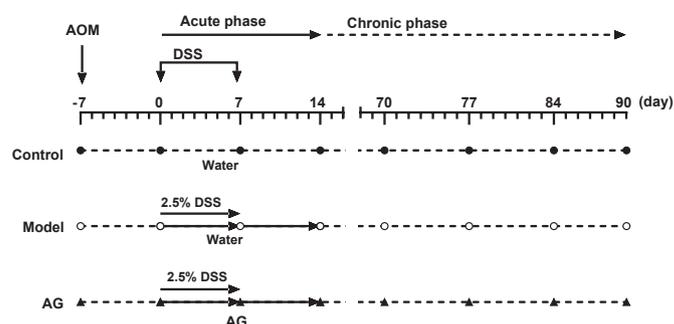


Fig. 1. Experimental protocol. Experimental A/J mice were divided into three groups, i.e., control (or negative control) group, model group, and American ginseng (AG) group. Animals in the model and AG groups initially received a single intraperitoneal injection of azoxymethane (AOM; 7.5 mg/kg). One week after the AOM administration (set as Day 1), mice in the model and AG groups received 2.5% dextran sodium sulfate (DSS) in drinking water for 8 consecutive days. Mice in the AG group also received oral AG extract 30 mg/kg/day for 90 consecutive days (Day 1–14 is the acute phase and then up to 90 days is the chronic phase).

Rotary Evaporator. The obtained extract was dissolved in 700 mL of water. The solution was extracted 3 times with 500 mL of water-saturated *n*-butanol. The mixed *n*-butanol phase was evaporated under vacuum and then lyophilized.

2.3. HPLC analysis of herbal extract

Prior to pharmacological evaluation, the AG extract was analyzed using HPLC [20,21]. The HPLC system was a Waters Alliance 2960 instrument (Milford, MA, USA) with a quaternary pump, an automatic injector, a photodiode array detector (Model 996), and Waters Millennium 32 software for peak identification and integration. The separation was carried out on a Prodigy ODS(2) column (250 mm × 3.2 mm inner diameter) with a guard column (3.0 mm × 4.0 mm inner diameter) (Phenomenex, Torrance, CA, USA). For HPLC analysis, a 20- μ L sample was injected into the column and eluted at room temperature with a constant flow rate of 1.0 mL/min. For the mobile phase, acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 17.5% solvent A and 82.5% solvent B. Elution solvents were then changed to 21% A for 20 min, then to 26% A for 3 min and held for 19 min, at 36% A for 13 min, at 50% A for 9 min, at 95% A for 2 min, and held for 3 min. Lastly, eluting solvents were changed to 17.5% A for 3 min and held for 8 min. The detection wavelength was set at 202 nm. All sample solutions were filtered through a membrane filter (0.2 μ m pore size). The content of the constituents were calculated using the standard curves of 13 ginsenosides. The measurement for the content analysis of the AG was performed in triplicate.

2.4. Animals and treatment protocols

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago, Chicago, IL, USA. All experiments were carried out in male A/J mice, aged approximately 6 weeks, weighing 18–22 g, obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained under controlled room temperature, humidity, and light (12/12 h light/dark cycle) and allowed *ad libitum* access to standard mouse chow and tap water. The mice were allowed to acclimate to these conditions for at least 7 days prior to inclusion in the experiments.

As shown in Fig. 1, animals were separated into three groups ($n = 12$ per group): control (or negative control), model, and AG groups. All animals initially received a single intraperitoneal injection of AOM (7.5 mg/kg). One week after the AOM injection (set

Table 1
Primers used for quantitative real-time polymerase chain reaction analysis of inflammatory cytokines

Gene	Primer	Sequence
IL-1 α	Forward	5'-CGAAGACTACAGTTCTGCCAAT-3'
	Reverse	5'-GACGTTTCAGAGGTTCTCAGAG-3'
IL-1 β	Forward	5'-GCAACTGTCTCTGAACTCAACT-3'
	Reverse	5'-ATCTTTTGGGGTCCGTCAACT-3'
IL-6	Forward	5'-TAGTCCTTCTACCCCAATTTC-3'
	Reverse	5'-TTGGTCCTTAGCCACTCCTC-3'
IFN- γ	Forward	5'-ATGAACGCTACACACTGCATC-3'
	Reverse	5'-CCATCTTTTGCCAGTTCCTC-3'
G-CSF	Forward	5'-ATGGCTCAACTTTCTGCCAG-3'
	Reverse	5'-CTGACAGTGACCAGGGAAAC-3'
GM-CSF	Forward	5'-GGCCTTGAAGCATGTAGAGG-3'
	Reverse	5'-GGAGAACTCGTTAGAGACGACTT-3'
β -actin	Forward	5'-GGCTGTAATCCCTCCATCG-3'
	Reverse	5'-CCAGTTGGTAACAATGCCATGT3'

G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin, TNF, tumor necrosis factor

as Day 1), the animals began to receive 2.5% DSS in drinking water for 8 consecutive days. The animals in AG group also received AG extract 0.15 mg/mL in drinking water for up to 90 consecutive days. We calculated that the daily dose of American ginseng was approximately 30 mg/kg.

For the acute phase observation, six animals per group were sacrificed on Day 14. The remaining animals were kept in the chronic phase and were sacrificed on Day 90. The gut tissue samples of all animals were collected for further observations.

2.5. Disease activity index

AOM/DSS induced colitis was scored as the disease activity index (DAI) as described previously [22]. In brief, the DAI was the combined scores of weight loss (0, none; 1, 0–5%; 2, 5–10%; 3, 10–20%; and 4, >20%), stool consistency change (0, none; 2, loose stool; and 4, diarrhea), and bleeding (0, none; 1, trace; 2, mild hemocult; 3, obvious hemocult; and 4, gross bleeding), and then divided by three. The animals were scored for the DAI at the same time of each day, blind to the treatment. The minimal score was 0 and the maximal score was 4.

2.6. Histological assessment

Paraffin-embedded gut tissue samples were serially sectioned, and some sections were stained with hematoxylin and eosin (H&E). The stained sections were subsequently examined for histopathological changes by a gastrointestinal pathologist.

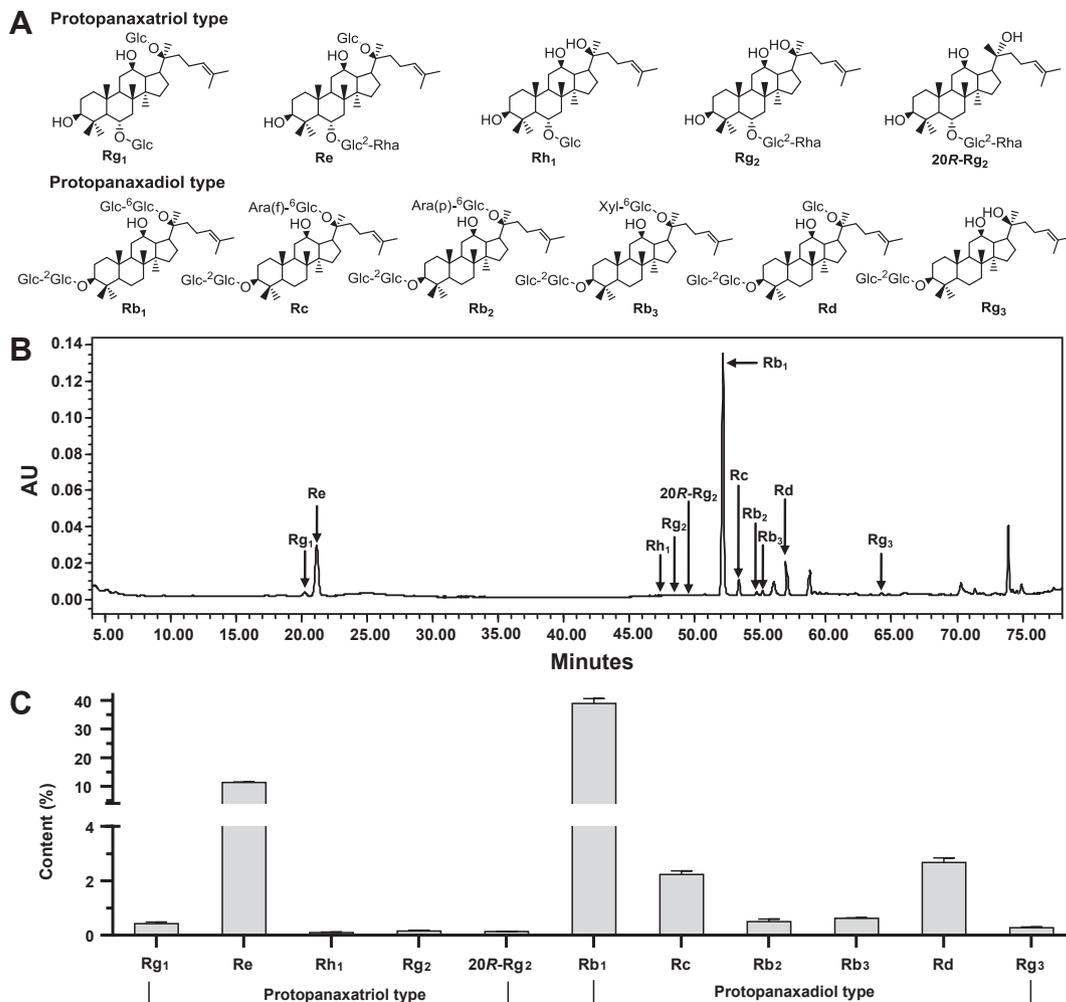


Fig. 2. Major ginsenosides in American ginseng (AG) extract. (A) Chemical structures of 11 ginsenosides. (B) Typical chromatograph of AG extract. (C) Contents of the major ginsenosides in the AG extract. Glc, β -D-glucopyranosyl; Xyl, β -D-xylopyranosyl; Rha, α -L-rhamnopyranosyl; Ara(f), α -L-arabinofuranosyl; Ara(p), α -L-arabinopyranosyl.

2.7. ELISA analysis

Proteins of the mouse colonic tissue that was collected on Day 14 were extracted with radio-immunoprecipitation assay lysis buffer (Thermo Scientific, Hanover Park, IL, USA) adding 10 $\mu\text{L}/\text{mL}$ proteinase inhibitor cocktail and phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA). ELISA was performed with Multi-Analyte ELISA Array Kit containing 12 mouse inflammatory cytokines [interleukin (IL)1 α , IL1 β , IL2, IL4, IL6, IL10, IL12, IL17A, interferon (IFN)- γ , tumor necrosis factor- α (TNF- α), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF)] according to the manufacturer's instructions.

2.8. RNA extraction and quantitative RT-PCR

Total RNA was isolated from the mouse colonic tissues using the miRNeasy kit (QIAGEN, Valencia, CA, USA) based on the manufacturer's instructions and was used as a template to synthesize cDNA for qRT-PCR. First strand cDNA was synthesized using Thermo Scientific Maxima First Strand cDNA Synthesis Kit. qRT-PCR was performed on a 7900HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). qRT-PCR with SYBR Green dye (QIAGEN) was used to determine the gene expression. Primers for qRT-PCR are listed in Table 1. β -actin was used as an endogenous control. Each sample was run in triplicate.

2.9. Statistical analysis

Data are presented as mean \pm standard deviation. Data were analyzed using analysis of variance (ANOVA) for repeated measures and Student *t* test. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. HPLC analysis of AG saponins

The chemical structures of 11 major ginsenosides, in the protopanaxadiol or protopanaxatriol groups, are shown in Fig. 2A. The chromatograph of AG extract is shown in Fig. 2B. As shown in Fig. 2C, the contents of protopanaxatriol type ginsenosides Rg1, Re, Rh1, Rg2, and 20R-Rg2 in AG extract were 0.43%, 11.33%, 0.10%, 0.15%, and 0.13%, respectively, whereas the contents of protopanaxadiol type ginsenosides Rb1, Rc, Rb2, Rb3, Rd, and Rg3 were 38.89%, 2.24%, 0.50%, 0.62%, 2.68%, and 0.28%, respectively. The total ginsenoside content was 57.4%.

3.2. AG reduced experimental colitis

Starting from Day 4 after DSS treatment, animals in the model group showed apparent diarrhea and rectal bleeding. As DSS

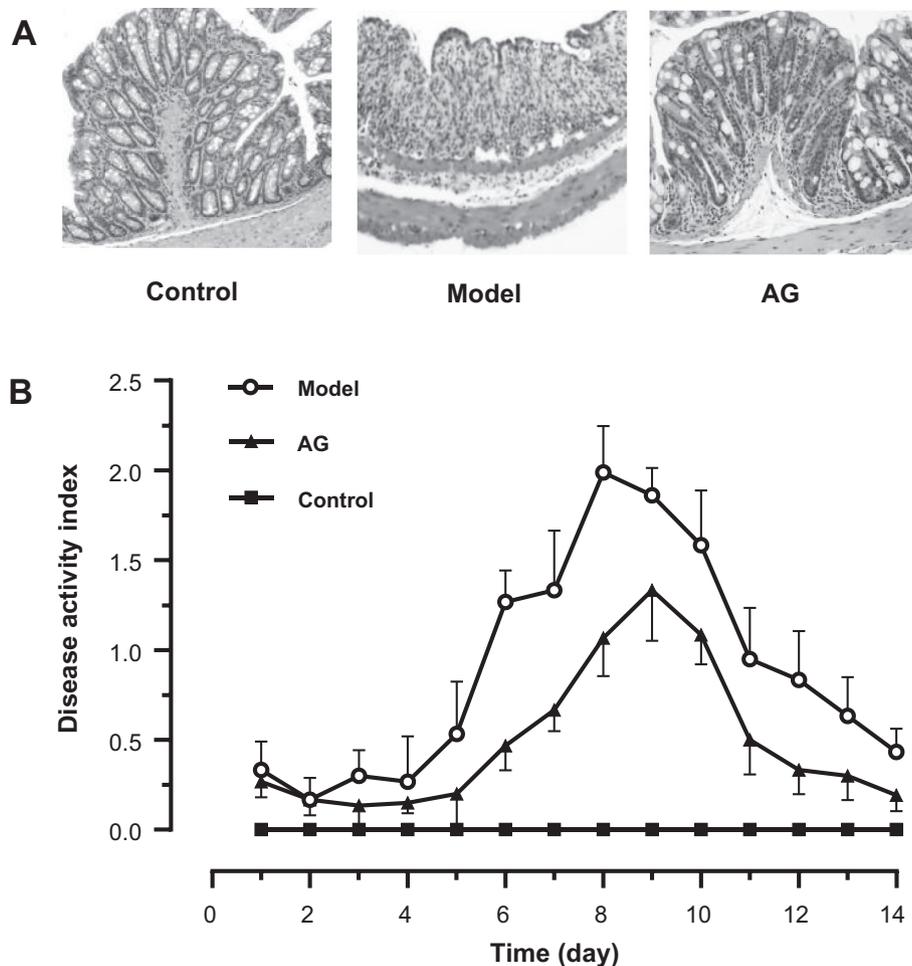


Fig. 3. Effects of American ginseng (AG) on azoxymethane/dextran sodium sulfate-induced change in colitis ($n = 6$ per group). (A) Representative hematoxylin and eosin stained histological sections of control (or negative control), model, and AG groups. (B) AG ameliorates the colitis, expressed as disease activity index. Data from the control (or negative control) group are all zeros from Day 1 to Day 14, shown as a thick horizontal line.

administration continued, the presence and development of inflammation manifested clearly. During the acute phase (Day 14), H&E staining colon tissue from model animals showed: increasingly severe inflammatory lesions extensively throughout the colon; significant and complete loss of crypts; surface erosion with exuberant inflammatory exudates; patchy re-epithelization; lamina propria fibrosis with acute and chronic inflammatory infiltrate; submucosal edema; and mixed inflammatory cell infiltration. In the AG group, mucosa had tightly packed glands with a normal amount of goblet cells (Fig. 3A). The disease severity, scored by the DAI, reached its highest level on Day 8. Fig. 3B shows significant effects of AG on the reduction of the DAI score ($p < 0.05$). This suppression of the experimental colitis by the herb was not only evident during DSS treatment, but also very obvious after the cessation of DSS administration (i.e., Day 8), suggesting that AG significantly promoted recovery from the colitis.

3.3. AG attenuated on AOM/DSS-induced colon carcinogenesis

Fig. 4A is a representative macroscopic morphology for the control group, model group, and AG group. Obvious tumorigenesis was observed in the model group. However, in the AG treatment group, the tumor number and size were significantly less and relative small. Fig. 4B shows representative H&E staining histological sections of the three groups. In the colon tissue from the model animals, multifocal adenomatous lesion was observed, and there was no invasion into submucosa; there was mild

inflammation with cryptitis, mild degree loss of goblet cells, fibrosis, and apoptotic changes. For the AG treatment group, mucosa shows tightly packed glands with a normal amount of goblet cells while crypt architecture remained normal. Compared to the model, the histological sections of the AG treatment group are more similar to those of the control group.

Fig. 4C shows colon carcinogenesis data. Our results showed that compared to the model group, AG treatment very significantly reduced the total number of colon tumors and load of tumors ($p < 0.01$ and $p < 0.001$, respectively). Tumor distribution data reflected this reduction, in which the number of large tumors (1–2 mm and > 2 mm) decreased while the number of small tumors (< 1 mm) increased.

3.4. AG suppressed DSS-induced proinflammatory cytokines activation

Previous studies have shown that blockade of inflammatory cytokines significantly decrease the severity of colitis. To explore mechanisms of inhibition of AOM/DSS induced colitis and tumorigenesis by AG treatment, using an ELISA array, we determined proinflammatory cytokine levels in the colon tissues collected on Day 14. Colonic levels of the proinflammatory cytokines IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN- γ , tumor necrosis factor- α , G-CSF, and GM-CSF were markedly elevated in the DSS model group. Treatment with AG significantly inhibited the levels of those 12 cytokines by 44%, 35%, 42%, 39%, 46%, 34%, 37%, 44%, 51%, 40%,

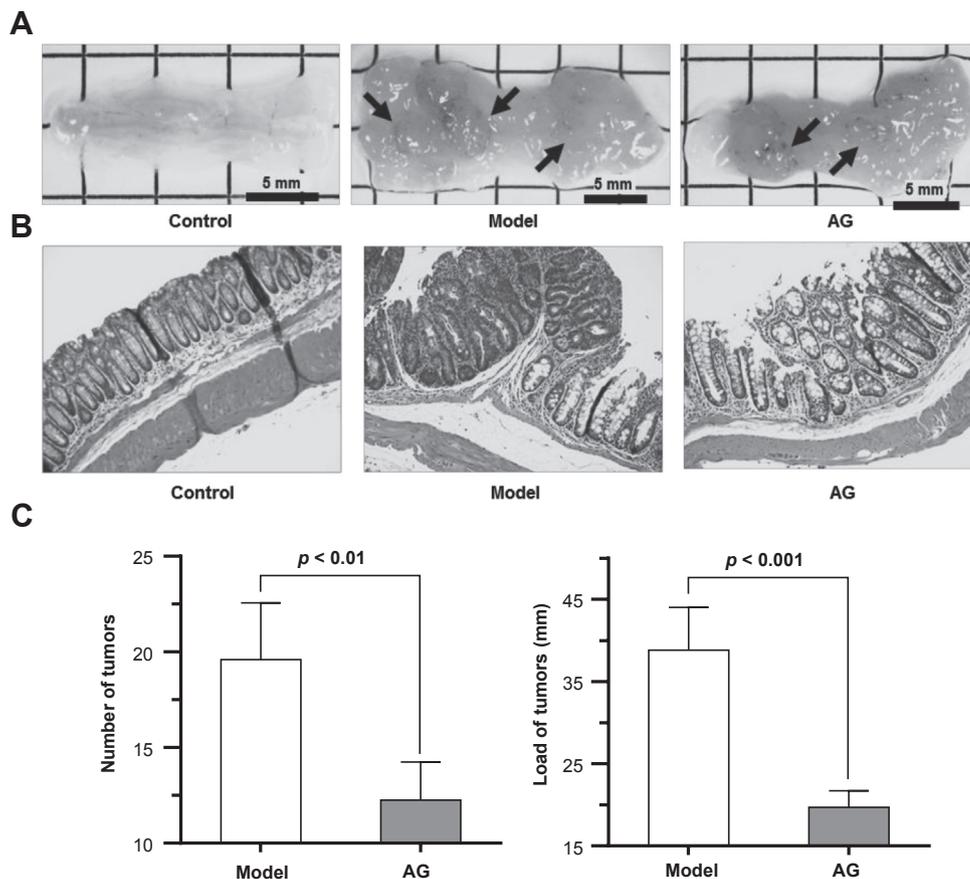


Fig. 4. Effects of American ginseng extract (AG) on azoxymethane/dextran sodium sulfate-induced colon carcinogenesis ($n = 6$ per group). (A) Representative macroscopic morphology of colon tumors in the control (or negative control), model, and AG groups. Arrows indicate the azoxymethane/dextran sodium sulfate-induced tumors. (B) Representative hematoxylin and eosin stained histological sections of control, model, and AG groups. (C) Number of colon tumor and load reduced very significantly in the AG group compared to the model group ($p < 0.01$ and $p < 0.001$, respectively).

46%, and 37%, respectively ($p < 0.05$; Fig. 5). This result suggested that within the acute phase (Day 14), AG significantly reduced the levels of proinflammatory cytokines in colon tissue, which was induced by DSS treatment.

3.5. AG downregulated inflammatory cytokine gene expression

To validate inflammatory cytokine data observed in the ELISA analysis, we examined the effect of AG on the expression of inflammatory cytokine genes in both the acute (Day 14) and chronic (Day 90) phases. We used RT-PCR to test the effects of AG on the target genes in colon tissues, which were collected on Day 14 and Day 90. As shown in Fig. 6A, in the acute phase (Day 14), the expression of six inflammatory cytokines (IL-1 α , IL-1 β , IL-6, IFN- γ , G-CSF, and GM-CSF) in the model group is much higher than in the control group (all $p < 0.001$). Compared to the model, ginseng treatment significantly downregulated the expression of the tested inflammatory cytokines (all $p < 0.01$). In the chronic phase (Day 90), similar effects were also observed, and AG treatment more significantly inhibited inflammatory cytokine expression (all $p < 0.001$ vs. model; Fig. 6B). This result indicate that the oral administration of AG transcriptionally repressed inflammatory cytokines in the gut tissue.

4. Discussion

Colorectal cancer is the second leading cause of cancer-related death in the West [2,23]. This cancer also remains a foremost cause of morbidity and mortality, a significant contributor to the burden of disease of global public health. Inflammatory bowel disease, including ulcerative colitis and Crohn's disease, is a risk factor for colon cancer initiation and development [10,11]. Nonsteroidal anti-inflammatory drugs can reduce colon cancer tumorigenesis. For example, celecoxib has potent preventive and therapeutic effects on the cancer [24]. Concerns about the risks of long-term use of such drugs, however, make this form of chemoprevention unsuitable as a general recommendation [25,26]. Epidemiological, experimental, and clinical studies provide evidence that inflammatory phytochemicals possess unique modes of action against cancer development and growth [27–29]. In the present study, the effects of AG were investigated, as one of the efforts to search for the botanical sources against this significant medical problem.

Experimentally, AOM (a mutagenic agent) and/or DSS (a proinflammatory reagent) have often been used in colorectal cancer chemoprevention animal studies [15,30–32]. In this study we

used the AOM/DSS mouse model to mimic the inflamed colon and carcinogenesis conditions in humans [15,33].

There were two observation phases in this study. The acute phase (Day 1–14) reflected the manifestation of inflammatory colitis, measured by DAI (Fig. 3). The chronic phase (up to 90 days) revealed the colon carcinogenesis (Fig. 4), measured by colon tumor number and tumor load. Compared with the model group, we observed that AG treatment significantly attenuated the experimental colitis. Our data also suggest that the AG not only could be used to treat DSS-induced colitis during the induction by the chemical, but also to promote the recovery from the colitis after the cessation of DSS administration. In the chronic phase, our data show that ginseng treatment very significantly reduced colon tumor number and load. The H&E staining histological observations support these pharmacological observations.

We used HPLC analysis to determine the major ginsenosides in the AG used in this study. Previously, we evaluated the effects of another herb in the ginseng family, notoginseng, on experimental colitis for up to 14 days. We reported that notoginseng attenuated the acute colitis [34] comparable to what was observed using AG in this study. Although the ginseng saponin profiles are different between AG and notoginseng, the two botanicals also share a number of common ginsenosides. It would be interesting to identify which is/are the key ginsenoside(s) responsible for the observed effects reported in these two studies.

AG and Asian ginseng are two major ginseng species. These two ginsengs, especially Asian ginseng, are the most studied natural products in the world [35,36]. It is generally accepted that the main bioactive constituents of both ginsengs are ginsenosides [37,38]. Over 80 ginsenosides have been identified, and nearly all these ginsenosides can be found in the two species. However, the ginsenoside profile between the two ginseng species is different, and this difference may contribute to their different pharmacological effects [18,35]. Of note, AG has approximately two times higher total ginsenoside content than Asian ginseng, largely due to its obvious high levels of Rb1, Re, and Rd [35].

Using the extract of AG, Cui et al [39] showed that the extract suppressed colon cancer associated with colitis in the AOM/DSS model. In particular, these authors investigated the molecular mechanisms of ginseng's anticancer effects using antibody array observations on colon cells isolated at a precancerous stage. Our study also used oral ginseng administration, and it is likely that enteric microbiome plays a role in ginseng metabolism and bioavailability.

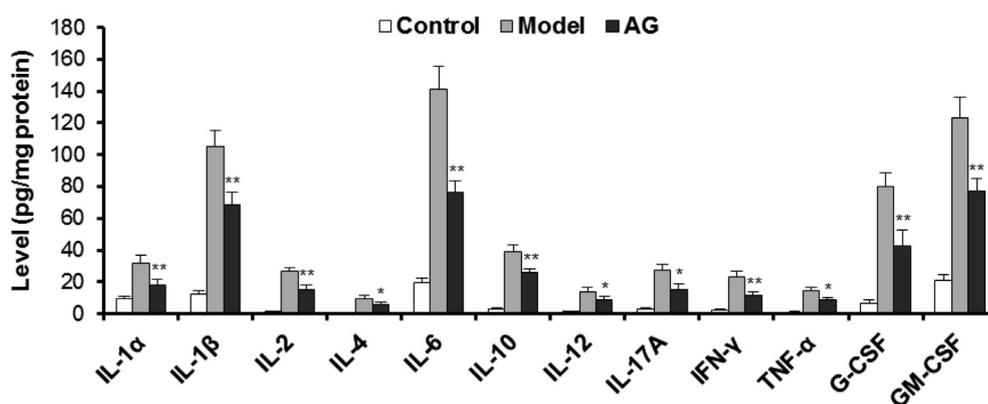


Fig. 5. Acute inflammatory responses in azoxymethane/dextran sodium sulfate model and American ginseng (AG) treatment groups. Protein lysis was prepared using colonic tissue from six mice, which was collected on Day 14. Cytokine expressions were analyzed using the mouse cytokine Multi-Analyte ELISAarray. * $p < 0.05$, compared to the model group. ** $p < 0.01$, compared to the model group. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin, TNF, tumor necrosis factor.

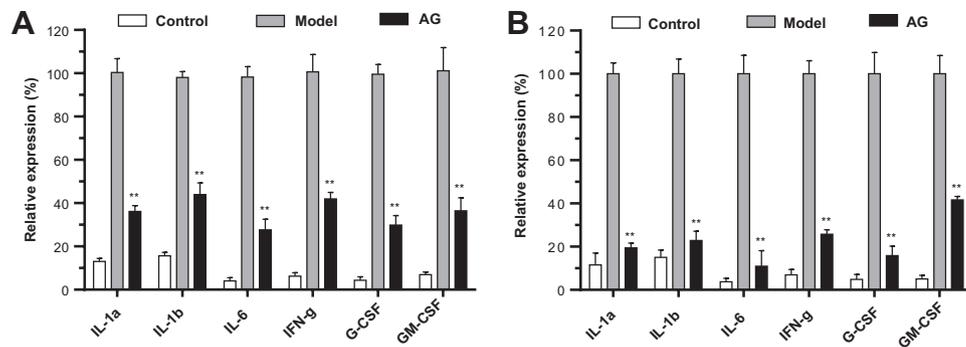


Fig. 6. Expression of inflammatory cytokine genes in colon tissues in the acute phase at Day 14 (A) and chronic phase at Day 90 (B). Colonic tissues from six mice were collected and used for total RNA isolation. Real-time polymerase chain reaction was used to determine cytokine gene expression. * $p < 0.01$, compared to the model group. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin, TNF, tumor necrosis factor.

After AG is ingested orally, the bioavailability of its saponins is low. This is due to incomplete absorption of the parent compounds and their conversion into metabolites by the enteric microbiome, mainly via step-wise cleavage of sugar moieties [35,40]. The ginseng metabolites may possess more significant pharmacological benefits than their parent compounds such as Rb1 [41], including the effects observed in this study. Because the diarrhea induced by DSS is likely to affect the activity and/or profile of enteric microbiome, AOM/DSS-induced, colitis-associated colorectal carcinogenesis may not be an ideal *in vivo* model to study the botanical chemoprevention of colorectal cancer in relation to the enteric microbiome. Future study should be extended to other colon cancer animal models, especially the APC mutant *Min* (multiple intestinal neoplasia) mice with detailed mechanisms of action [42,43].

Traditional medicine has been practiced for thousands of years based on clinical experience. Modern research suggested that herbal medicines could be used as adjuvants for cancer symptom management and cancer therapeutics [44,45]. To explore the potential role of AG in colorectal cancer chemoprevention, it is necessary to integrate existing traditional knowledge of diseases with modern biomedical technologies [46]. Data reported in this study suggested that AG, as a candidate of botanical-based colon cancer chemoprevention, should be further investigated for its potential clinical utility.

Conflicts of interest

The authors have no potential conflicts of interest.

Acknowledgments

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