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Research article

American ginseng significantly reduced the progression of high-fat-diet-enhanced colon carcinogenesis in $Apc^{Min/+}$ mice

Chunhao Yu [1,2,3,4,☆], Xiao-Dong Wen [3,4,☆], Zhiyu Zhang [3,4], Chun-Feng Zhang [3,4], Xiaohui Wu [3,4], Xin He [3,4], Yang Liao [3,5], Ningning Wu [3,6], Chong-Zhi Wang [3,4], Wei Du [3,5], Tong-Chuan He [3,6], Chun-Su Yuan [3,4,7,*]

[1] School of Life Science and Chemical Engineering, Huaiyin Institute of Technology, Jiangsu, China
[2] Jiangsu Provincial Engineering Laboratory for Biomass Conversion and Process Integration, Huaiyin Institute of Technology, Jiangsu, China
[3] Tang Center for Herbal Medicine Research, University of Chicago, Chicago, IL, USA
[4] Department of Anesthesia and Critical Care, University of Chicago, Chicago, IL, USA
[5] Ben May Department for Cancer Research, University of Chicago, Chicago, IL, USA
[6] Molecular Oncology Laboratory, Department of Surgery, University of Chicago Medical Center, Chicago, IL, USA
[7] Committee on Clinical Pharmacology and Pharmacogenomics, University of Chicago, Chicago, IL, USA

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ABSTRACT

Background: Colorectal cancer (CRC) is a leading cause of death worldwide. Chronic gut inflammation is recognized as a risk factor for tumor development, including CRC. American ginseng is a very commonly used ginseng species in the West.

Methods: A genetically engineered $Apc^{Min/+}$ mouse model was used in this study. We analyzed the saponin composition of American ginseng used in this project, and evaluated its effects on the progression of high-fat-diet-enhanced CRC carcinogenesis.

Results: After oral ginseng administration (10–20 mg/kg/d for up to 32 wk), experimental data showed that, compared with the untreated mice, ginseng very significantly reduced tumor initiation and progression in both the small intestine (including the proximal end, middle end, and distal end) and the colon (all $p < 0.01$). This tumor number reduction was more obvious in those mice treated with a low dose of ginseng. The tumor multiplicity data were supported by body weight changes and gut tissue histology examinations. In addition, quantitative real-time polymerase chain reaction analysis showed that compared with the untreated group, ginseng very significantly reduced the gene expression of inflammatory cytokines, including interleukin-1α (IL-1α), IL-1β, IL-6, tumor necrosis factor-α, granulocyte-colony stimulating factor, and granulocyte-macrophage colony-stimulating factor in both the small intestine and the colon (all $p < 0.01$).

Conclusion: Further studies are needed to link our observed effects to the actions of the gut microbiome in converting the parent ginsenosides to bioactive ginseng metabolites. Our data suggest that American ginseng may have potential value in CRC chemoprevention.

1. Introduction

Colorectal cancer (CRC) is a leading cause of patients' morbidity and mortality. In 2014 alone, there were an estimated 136,830 new CRC cases and 50,310 CRC-related deaths in the United States [1,2]. A significant number of men and women worldwide are at risk of developing invasive CRC in their lifetime [3]. Because currently available therapies for advanced CRC have

* 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 contributed equally to this work.

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limited efficacy, increased attention has been focused on CRC prevention.

Using Asian ginseng, a case–control study on over a thousand participants in Korea showed that participants who consumed ginseng had a decreased risk of many different cancers compared with those who did not. In addition, ginseng has a nonorgan-specific cancer prevention effect [4,5]. In contrast to Asian ginseng [5–7], however, the effects of American ginseng on CRC therapeutics have not been evaluated.

Chronic inflammation is recognized as a risk factor for tumor development, including CRC [8–10]. There is growing evidence to support the efficacy of natural products possessing anti-inflammatory activities. Published studies suggested that ginseng not only has cancer prevention potential [4,5,11,12], but also has anti-inflammatory effects [13,14]. Various data suggest that ginseng reduces inflammation and suppresses colitis by restoring gut homeostasis [15–17], and this anti-inflammatory activity likely plays a critical role in cancer prevention and treatment [18–20].

American ginseng (*Panax quinquefolius* L.) is one of the most commonly used herbal medicines in the United States [21,22]. Similar to Asian ginseng, a significant number of ginsenosides have been identified in American ginseng. These ginsenosides fall into two major groups: the protopanaxadiol group and the protopanaxatriol group, which differ in the presence of the carboxyl group at the C-6 position [21,23]. However, the ginsenoside profile between American ginseng and Asian ginseng is different, and this difference may contribute to their different pharmacological effects. Interestingly, American ginseng has approximately over onefold higher ginsenoside content than Asian ginseng [21,24,25]. In addition to ginsenosides, ginseng also contains other bioactive compounds [21,24].

Significant antitumor effects of American ginseng were observed in the CRC cell-xenografted nude mouse model [26,27]. However, the nude mouse is not a gut disease-specific animal model. Thus, it is desirable to use specific gut inflammatory and malignancy animal models. In this study, we used $Apc^{Min/+}$ mice, an animal model with mutations in the *Apc* gene. This multiple intestinal neoplasia (*Min*) mouse is characterized by early lethality, colon tumors, and development of a number of polyps in the small intestine [28–30]. Our data demonstrated that American ginseng significantly reduced the progression of high-fat-diet-enhanced carcinogenesis in this $Apc^{Min/+}$mouse model.

2. Materials and methods

2.1. Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade ethanol, *n*-butanol, acetonitrile, and dimethyl sulfoxide were purchased from Fisher Scientific (Pittsburgh, PA, USA). Milli-Q water was supplied by a water purification system (US Filter, Palm Desert, CA, USA). Standards of ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg2, 20R-Rg2, Rg3, Rh1, and Rh2 were obtained from INDOFINE Chemical Company (Somerville, NJ, USA) or Delta Information Center for Natural Organic Compounds (Xuancheng, Anhui, China). All standards were of biochemical-reagent grade and at least 95% pure. Other reagents were of biochemical-reagent grade.

2.2. Botanical material and preparation

The 4-year-old roots of American ginseng (*P. quinquefolius* L.) were purchased from Roland Ginseng, LLC (Wausau, WI, USA). The voucher samples were authenticated by C.-Z.W. and deposited at the Tang Center for Herbal Medicine Research at the University of Chicago (Chicago, IL, USA). The American ginseng extract was prepared with a slight modification from previous works [31–33]. The air-dried roots of American ginseng were pulverized into powder form and sieved through an 80-mesh screen. One kilogram of the powder was placed into a 12-L flask and 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 while hot. The gathered and combined filtrate was evaporated under vacuum with a Buchi rotary evaporator (Buchi Corporation, New Castle, DE, USA). The obtained extract was dissolved in water, and then extracted with water-saturated *n*-butanol. The *n*-butanol phase was evaporated at 65°C under vacuum and lyophilized.

2.3. Phytochemical analysis

The American ginseng extract was analyzed using HPLC [34,35]. The HPLC system used 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 i.d.; Phenomenex, Torrance, CA, USA) with a guard column (Prodigy, 3.0 mm × 4.0 mm i.d.; Phenomenex).

For the HPLC analysis, a 20-μL 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% Solvent A for 20 min, then to 26% Solvent A for 3 min and held for 19 min, maintained at 36% Solvent A for 13 min, at 50% Solvent A for 9 min, at 95% Solvent A for 2 min and held for 3 min. Finally, eluting solvents were changed to 17.5% Solvent 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-μm pore size). The content of the constituents was calculated using standard curves of the ginsenosides. The contents of American ginseng were measured in triplicate. Malonyl ginsenosides were not measured in this investigation.

2.4. Animals and experimental protocol

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago. Male C57BL/6J-Apc^{Min}/J and female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) for breeding. Mice were caged under controlled room temperature, humidity, and light (12/12-h light/dark cycle) and had free access to mouse chow and tap water. After weaning, genotyping was carried out by tail biopsy using PCR-based assays to identify $Apc^{Min/+}$mice [28].

The study protocol is shown in Fig. 1. There were six animal groups (n = 6–9 animals/group). Prior to 6 wk of age, all mice consumed standard mouse chow. The $Apc^{Min/+}$ mice were randomized at 6 wk of age and placed into the following experimental groups: (1) mice that received Western high-fat diet were categorized as the model group (M-HF); (2) mice that received standard diet as the control group (M-SD); (3) mice that received Western high-fat diet supplemented with 200 ppm of the American ginseng extract, equivalent to 20 mg/kg/d, as the high-dose ginseng group (M-GH); (4) mice that received Western high-fat diet supplemented with 100 ppm of the American ginseng extract, equivalent to 10 mg/kg/d, as the low-dose ginseng group (M-GL). As the control or negative control group, two wild-type mice groups were used: (1) wild-type mice fed with standard diet (wild-type standard diet control group, or W-SD); (2) wild-type mice fed with Western high-fat diet (wild-type high-fat-diet control group, or W-HF). The Western diet (Harlan Laboratories, Madison, WI, USA)

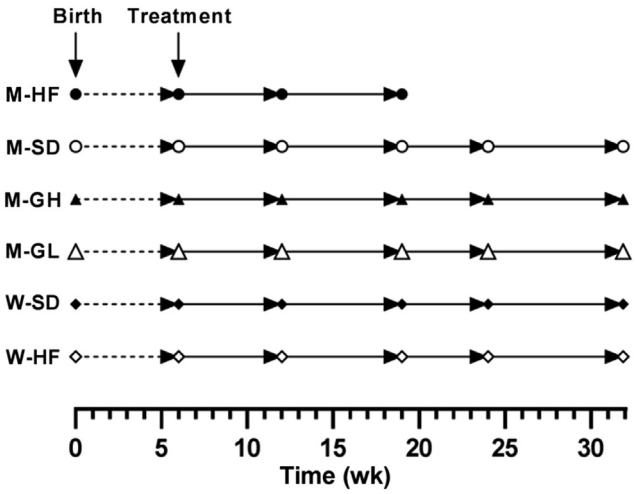


Fig. 1. Experimental groups and study time lines. Prior to the age of 6 wk, all mice consumed standard mouse chow. The six experimental groups (n = 6–9 animals/group) are as follows: (1) M-HF, mice fed with Western high-fat diet as the model group; (2) M-SD, mice fed with standard diet as the control group; (3) M-GH, mice fed with Western diet supplemented with high-dose American ginseng extract; (4) M-GL, mice fed with Western diet supplemented with low-dose American ginseng extract; (5) W-SD, wild-type mice fed with standard diet; and (6) W-HF, wild-type mice fed with Western high-fat diet. Study protocols are presented in the "Materials and methods" section.

contains 20% fat and includes beef tallow (35 g/kg), lard (30 g/kg), and corn oil (80 g/kg) [36]. Body weight of the animals was obtained at least once per wk. No significant adverse events were observed in the mice after the ginseng treatment.

At the end of each observation period, samples of small intestine and colon were harvested, flushed immediately with ice-cold phosphate-buffered saline, and slit open longitudinally. Under a dissection microscope, tumor numbers in the proximal, middle, and distal ends of the small intestine and the colon were counted by two independent investigators who were blinded with respect to the treatment group. Small intestinal and colon samples were fixed in 10% neutral-buffered formalin, embedded in paraffin blocks, and processed by routine histological staining. Some colon tissue was collected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis of interleukin-1α (IL-1α), IL-1β, IL-6, tumor necrosis factor (TNF-α), granulocyte-colony stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF).

2.5. Histological assessment

Paraffin-embedded tissue samples were serially sectioned, and some of these sections were stained with hematoxylin and eosin. The stained sections were subsequently examined for histopathological changes by a gastrointestinal pathologist.

2.6. RNA extraction and qRT-PCR

Total RNA was isolated from the mouse colonic tissues using the miRNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and was used as a template to synthesize complementary DNA for qRT-PCR. First-strand cDNA was synthesized using Thermo Scientific Maxima first-strand cDNA synthesis kit. RT-qPCR was performed on a real-time PCR system (7900HT; Applied Biosystems, Foster City, CA, USA). RT-qPCR with SYBR Green dye (Qiagen) was used to determine the expression of genes.

Table 1
Primers used for RT-qPCR analysis of inflammatory cytokines

Gene	Primer	Sequence
IL1α	Forward primer	5′-CGAAGACTACAGTTCTGCCATT-3′
	Reverse primer	5e-GACGTTTCAGAGGTTCTCAGAG-3G
IL1β	Forward primer	5o-GCAACTGTTCCTGAACTCAACT-3G
	Reverse primer	5e-ATCTTTTGGGGTCCGTCAACT-3A
IL6	Forward primer	5o-TAGTCCTTCCTACCCCAATTTCC-3T
	Reverse primer	5e-TTGGTCCTTAGCCACTCCTTC-3T
IFN-γ	Forward primer	5o-ATGAACGCTACACACTGCATC-3A
	Reverse primer	5e-CCATCCTTTTGCCAGTTCCTC-3C
G-CSF	Forward primer	5o-ATGGCTCAACTTTCTGCCCAG-3A
	Reverse primer	5e-CTGACAGTGACCAGGGGAAC-3C
GM-CSF	Forward primer	5o-GGCCTTGGAAGCATGTAGAGG-3G
	Reverse primer	5e-GGAGAACTCGTTAGAGACGACTT-3G
β-actin	Forward primer	5o-GGCTGTATTCCCCTCCATCG-3G
	Reverse primer	5e-CCAGTTGGTAACAATGCCATGT-3C

RT-qPCR, quantitative real-time polymerase chain reaction

Primers for RT-qPCR are presented in Table 1. β actin was used as the endogenous control. Each sample was run in triplicate.

2.7. Statistical analysis

Data were presented as mean ± standard deviation. Data were analyzed using analysis of variance for repeated measures and Student t test. All analyses were performed using SPSS version 14.0 (IBM Corporation, Somers, NY, USA). The level of statistical significance was set at $p < 0.05$.

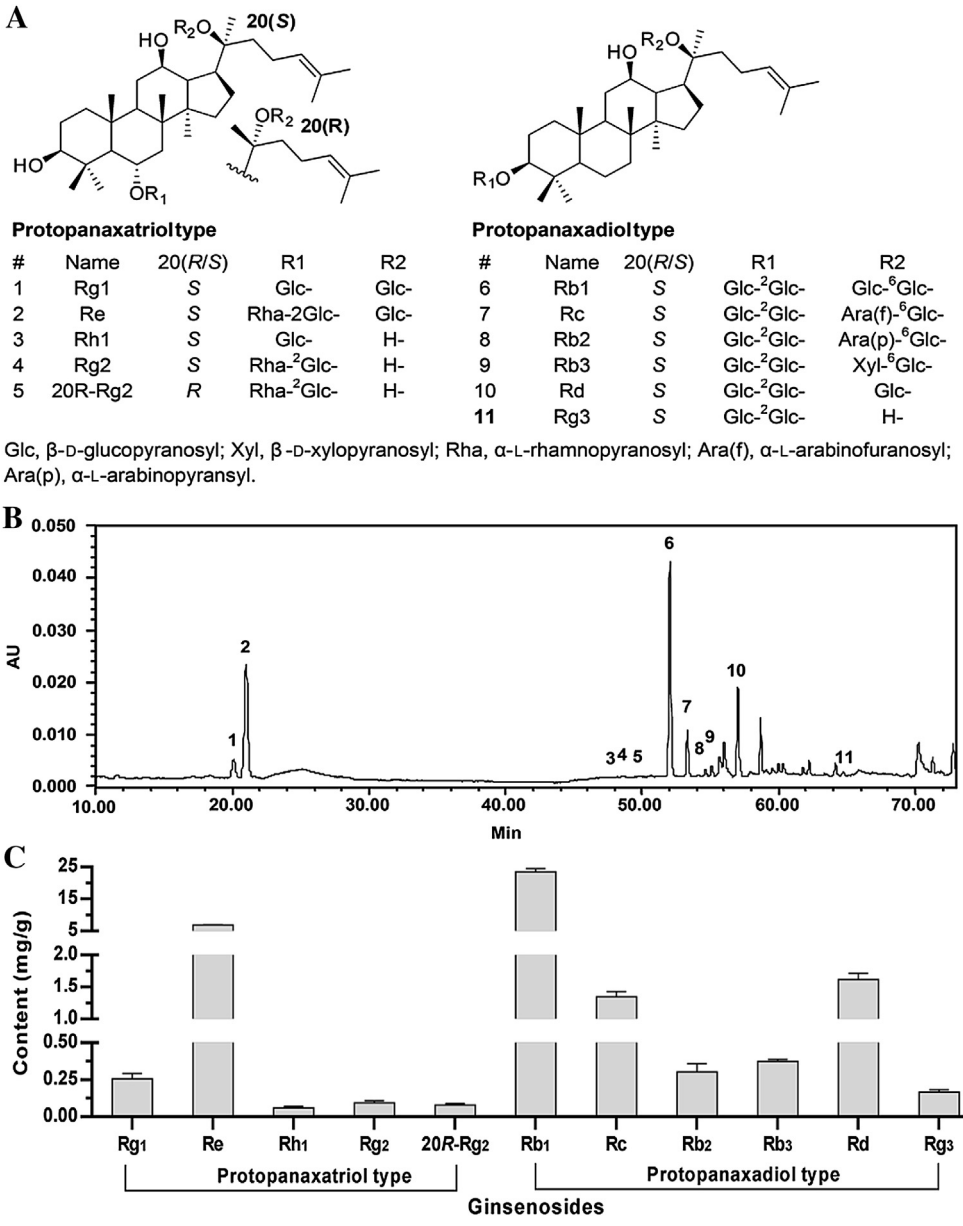
3. Results

3.1. Saponin composition in the study samples

Fig. 2A shows the chemical structures of ginsenosides in both the protopanaxadiol and protopanaxatriol groups in the American ginseng root. A typical HPLC chromatogram of the ginseng extract is shown in Fig. 2B. The levels of the measured 11 ginsenosides are presented in Fig. 2C. The major constituents of the protopanaxatriol group are Re, followed by Rg1. More than 70% of the ginsenosides are in the protopanaxadiol group, including Rb1, Rd, Rc, Rb3, and Rb2. Unlike in Asian ginseng, no ginsenoside Rf was detected in American ginseng.

3.2. Body weight changes

The body weight changes of the animals in different experimental groups are shown in Fig. 3. Wild-type mice with standard diet had an approximate weight increase of 12 g over the 10-wk observation period. As expected, those wild-type mice fed with Western high-fat diet had an even higher weight increase of approximately 17 g. However, the $Apc^{Min/+}$ mice in the model group did not respond to the high-fat diet well, and their weight only increased slightly. For the model group mice, the body weight started to decrease from Wk 10, and all mice died on or prior to Wk 18. By contrast, the $Apc^{Min/+}$ mice with standard diet had obvious weight gain (∼ 13 g), suggesting that the high-fat diet effectively caused some pathological changes in the $Apc^{Min/+}$ mice. High-dose American ginseng showed some small increase in body weight. Interestingly, low-dose American ginseng perceptibly increased body weight, suggesting the reduced pathological problems. The low-dose American ginseng data were consistent with the gut histology and tumor multiplicity data presented in the following section.



#	Name	20(*R*/*S*)	R1	R2
1	Rg1	*S*	Glc-	Glc-
2	Re	*S*	Rha-2Glc-	Glc-
3	Rh1	*S*	Glc-	H-
4	Rg2	*S*	Rha-^{2}Glc-	H-
5	20R-Rg2	*R*	Rha-^{2}Glc-	H-

#	Name	20(*R*/*S*)	R1	R2
6	Rb1	*S*	Glc-^{2}Glc-	Glc-^{6}Glc-
7	Rc	*S*	Glc-^{2}Glc-	Ara(f)-^{6}Glc-
8	Rb2	*S*	Glc-^{2}Glc-	Ara(p)-^{6}Glc-
9	Rb3	*S*	Glc-^{2}Glc-	Xyl-^{6}Glc-
10	Rd	*S*	Glc-^{2}Glc-	Glc-
11	Rg3	*S*	Glc-^{2}Glc-	H-

Glc, β-D-glucopyranosyl; Xyl, β-D-xylopyranosyl; Rha, α-L-rhamnopyranosyl; Ara(f), α-L-arabinofuranosyl; Ara(p), α-L-arabinopyransyl.

Fig. 2. Major ginsenosides in American ginseng (*Panax quinquefolius*). (A) Chemical structures of ginsenosides in both the protopanaxadiol and protopanaxatriol groups. (B) A representative high-performance liquid chromatography chromatogram of the American ginseng extract. (C) Levels of the measured 11 ginsenosides.

3.3. Gut histopathology changes

Fig. 4 shows the representative hematoxylin and eosin staining histological sections of experimental animals with different treatments. Histological analysis of the tissues from the model group showed prominent adenomatous change along with inflammatory lesions, such as some neutrophil infiltration. Focal adenomatous change can also be seen in the $Apc^{Min/+}$ mice with standard diet. However, in the ginseng-treated groups, especially the low-dose ginseng group, the dysplastic changes were greatly reduced in both small intestine and colon slides.

3.4. Tumor multiplicity changes

Compared with the high-fat diet, $APC^{min/+}$mice that received standard diet had reduced tumor numbers. After treatment with American ginseng, the tumor numbers were very significantly reduced in both the small intestine (including the proximal, middle, and distal ends) and the colon (all $p < 0.01$). In addition, tumor number reduction was more obvious in those mice treated with low-dose American ginseng (Fig. 5).

3.5. qRT-PCR analysis

Fig. 6 shows the qRT-PCR analysis of IL-1α, IL-1β, IL-6, TNF-α, G-CSF, and GM-CSF levels in the tissue obtained from the model group and the American ginseng groups. The results of the analysis indicated that low-dose American ginseng had better activities in the small intestine. Only low-dose ginseng was used in colon samples because this dose was more effective in the small intestine (Figs. 6A, 6B).

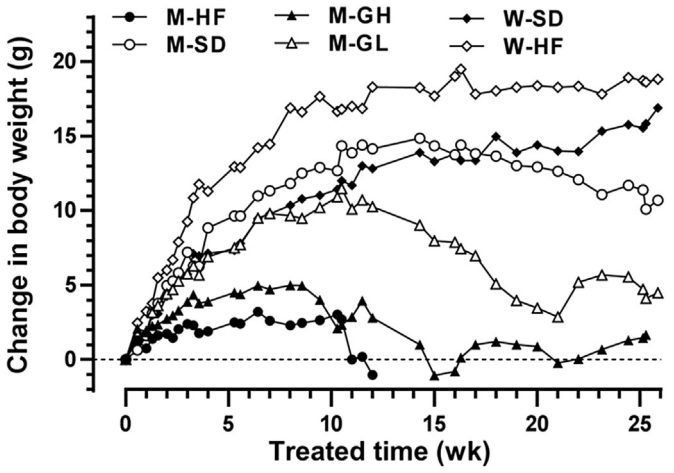


Fig. 3. Changes in animal body weight. As discussed in the text, the $Apc^{Min/+}$ mice received low-dose American ginseng in this study, which appeared to have evident therapeutic effects. The six experimental groups ($n = 6–9$ animals/group) are as follows: (1) M-HF, mice fed with Western high-fat diet as the model group; (2) M-SD, mice fed with standard diet as the control group; (3) M-GH, mice fed with Western diet supplemented with high-dose American ginseng extract; (4) M-GL, mice fed with Western diet supplemented with low-dose American ginseng extract; (5) W-SD, wild-type mice fed with standard diet; and (6) W-HF, wild-type mice fed with Western high-fat diet. Study protocols are presented in the "Materials and methods" section.

4. Discussion

Inflammatory bowel disease is a group of inflammatory conditions in the large and small intestine in humans. Inflammation can initiate and promote stimuli and mediators, generating a tumor-prone microenvironment. Chronic inflammation is recognized as a risk factor for tumor development, including CRC [8–10]. Targeting inflammatory pathways has been shown to be effective in preventing the formation of colon tumors and their malignant progression in both animal and human studies [37]. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce CRC tumorigenesis, but concerns and long-term risks of NSAIDs make this form of prevention unsuitable as a general recommendation [38,39]. Given the limitations of today's clinical practice, there is a strong motivation for exploring alternative strategies, including using herbal medicines, in the management of malignancies in the gastrointestinal system [40–43].

To evaluate the pharmacological effects on gut inflammation and tumorigenesis, two gut-specific animal models have been used in this study, namely, the AOM/DSS and $Apc^{Min/+}$ mice. AOM/DSS is a chemical-compound-induced model, characterized by early inflammation followed by tumor development [44,45]. This chemically induced murine model has been used for inflammation-related colon carcinogenesis in the last decade. To further characterize the activities of American ginseng, we used a genetically engineered mouse model that has a mutation in the *Apc* gene resulting in the growth of small intestine polyps and colon tumors associated with inflammation [46,47]. The association of inflammation with tumorigenesis in the $Apc^{Min/+}$ mouse model has been reported [46]. For example, celecoxib, a cyclooxygenase-2 inhibitor that belongs to the NSAID family, has been reported to possess potent preventive and therapeutic effects in $Apc^{Min/+}$ mice [47].

In our study, we demonstrated that low oral dose of American ginseng significantly reduced the progression of high-fat-diet-enhanced carcinogenesis in $Apc^{Min/+}$ mice. qRT-PCR analysis data showed that compared with the model group, ginseng, especially at low doses, very significantly reduced the gene expression inflammation cytokines, such as IL-1α, IL-1β, IL-6, TNF-α, G-CSF, and GM-CSF in the tissue from both the small intestine and the colon. The qRT-PCR results were consistent with survival, tumor growth inhibition, and histology data.

As with many other herbal medicines, American ginseng is almost always taken orally. Unlike parenteral administration that has been used in many animal studies, the enteric microbiome likely plays an important role after the oral intake of ginseng [29,36,37,48]. When ingested orally, the bioavailability of ginseng is low due to incomplete absorption of parent compound and the

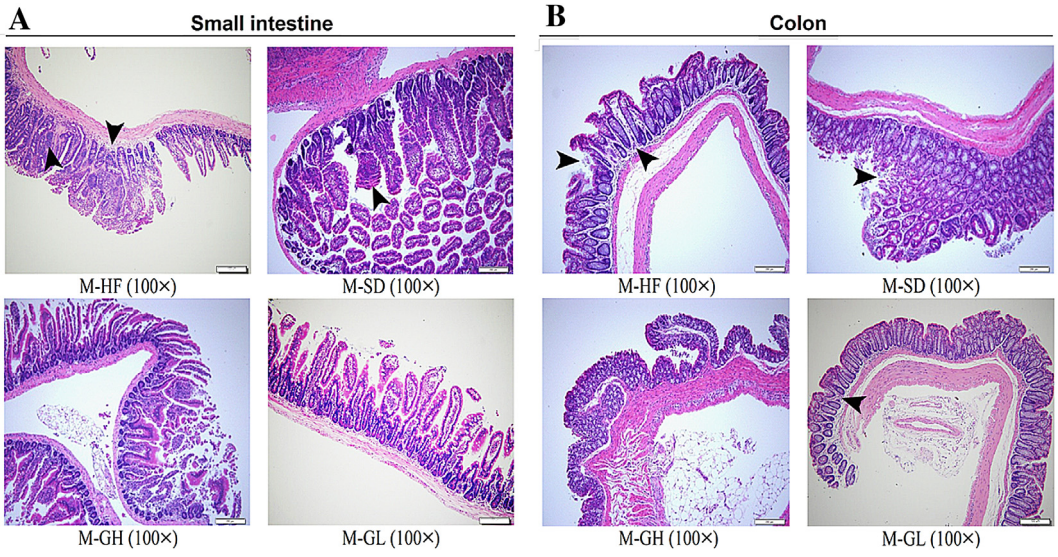


Fig. 4. Effects of American ginseng on the histological characterization in $Apc^{Min/+}$ mice. Representative hematoxylin and eosin staining histological sections showing inflammatory changes in (A) the small intestine and (B) the colon with different treatments. The scale in the low right figure is 200μM. Arrowheads indicate inflammation lesions in the gut tissue. The six experimental groups ($n = 6–9$ animals/group) are as follows: (1) M-HF, mice fed with Western high-fat diet as the model group; (2) M-SD, mice fed with standard diet as the control group; (3) M-GH, mice fed with Western diet supplemented with high-dose American ginseng extract; (4) M-GL, mice fed with Western diet supplemented with low-dose American ginseng extract; (5) W-SD, wild-type mice fed with standard diet; and (6) W-HF, wild-type mice fed with Western high-fat diet. Study protocols are presented in the "Materials and methods" section.

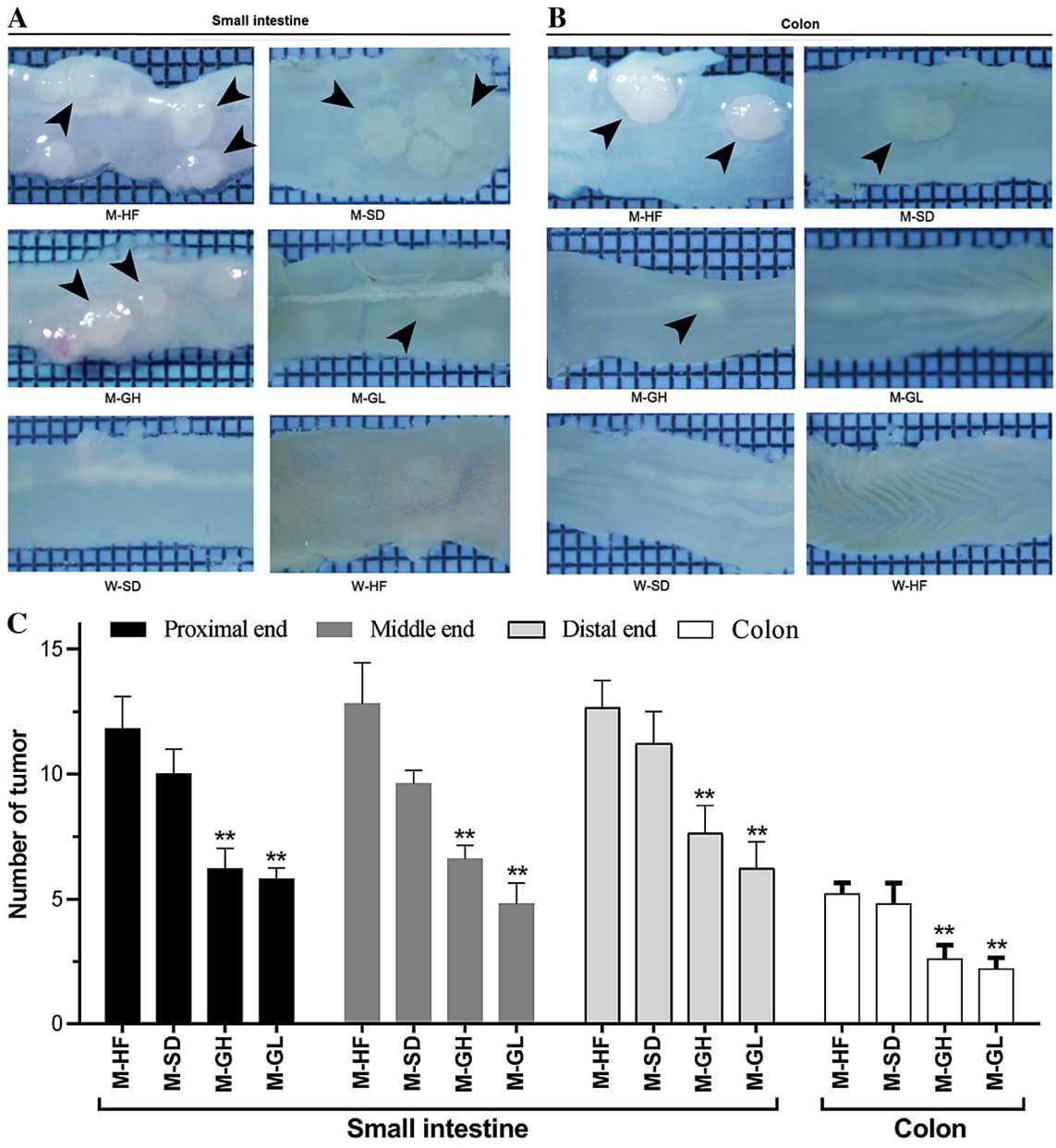


Fig. 5. Effects of American ginseng on gut carcinogenesis. (A) Macroscopic view of representative gut samples from the small intestine and the colon. (B) American ginseng significantly reduces the tumor numbers in the different gut segments. Tumors in the gut tissue are indicated by arrowheads (**$p < 0.01$ compared with the model group). The six experimental groups ($n = 6$–9 animals/group) are as follows: (1) M-HF, mice fed with Western high-fat diet as the model group; (2) M-SD, mice fed with standard diet as the control group; (3) M-GH, mice fed with Western diet supplemented with high-dose American ginseng extract; (4) M-GL, mice fed with Western diet supplemented with low-dose American ginseng extract; (5) W-SD, wild-type mice fed with standard diet; and (6) W-HF, wild-type mice fed with Western high-fat diet. Study protocols are presented in the "Materials and methods" section.

conversion of parent ginsenosides to bioactive ginseng metabolites by the enteric microbiome that involves a stepwise cleavage of sugar moieties [33,49,50]. After ginseng ingestion, compound K and protopanaxadiol are the major metabolites that reach systemic circulation, and these metabolites likely possess significant anti-inflammatory and anticancer properties [26,51]. At the same time, ginseng and its metabolites may alter the structure of the enteric microbiome. The conversion of ginseng saponins by human enteric microbiome was recently demonstrated, with > 20 metabolites detected [33]. In our ongoing investigations, ginseng metabolites in various biological samples (plasma, bile, urine, and stool) are being analyzed using ultraperformance liquid chromatography with quadruple time-of-flight mass spectrometry. In addition to compound K and protopanaxadiol, other ginseng metabolites should be searched, which may have even stronger bioactivities [26,52]. Further, other compounds identified in ginseng, such as polyacetylenes [24], are likely to have pharmacological activities, and they should also be evaluated in future studies.

In summary, using a gut-specific inflammation and carcinogenesis $Apc^{Min/+}$ mouse model, we reported that a low oral dose of American ginseng significantly reduced gut inflammation and tumor initiation and progression. The observed effects were supported by the body weight change, gut tissue histology, and gut inflammation cytokine data. Further studies are needed to link our observed effects to the actions of the gut microbiome in converting the parent ginsenosides to bioactive ginseng metabolites. Our data

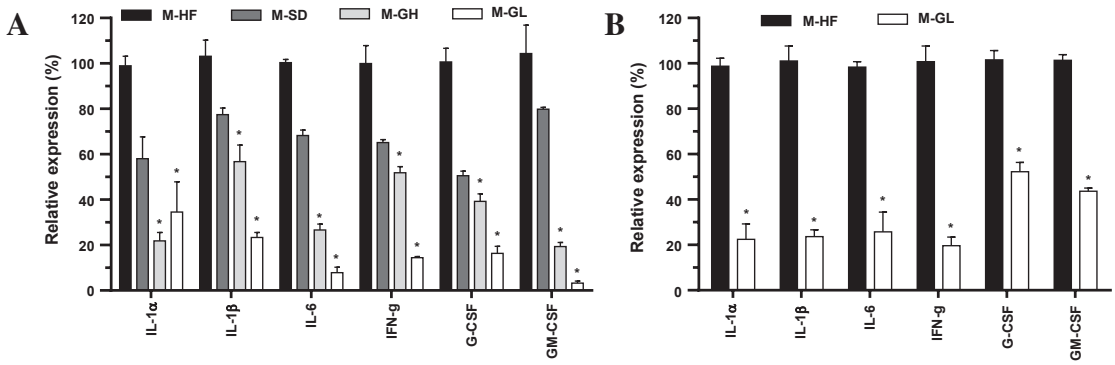


Fig. 6. Quantitative real-time polymerase chain reaction analysis of IL-1α, IL-1β, IL-6, IFN-g, G-CSF, and GM-CSF in the gut tissue from the model group and the American ginseng groups. (A) Small intestine. (B) Colon. Only low-dose ginseng is used in colon samples because this dose is more effective in the small intestine. $^{*}p < 0.01$ compared with the model group. The six experimental groups ($n = 6–9$ animals/group) are as follows: (1) M-HF, mice fed with Western high-fat diet as the model group; (2) M-SD, mice fed with standard diet as the control group; (3) M-GH, mice fed with Western diet supplemented with high-dose American ginseng extract; (4) M-GL, mice fed with Western diet supplemented with low-dose American ginseng extract; (5) W-SD, wild-type mice fed with standard diet; and (6) W-HF, wild-type mice fed with Western high-fat diet. Study protocols are presented in the "Materials and methods" section. G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin.

suggest that American ginseng may have a therapeutic role in CRC chemoprevention.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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