Effects of ginsenoside compound K on alleviating colitis via modulating gut microbiota

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Research Article

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Abstract

Background: Ginsenoside compound K (GC-K) potentially alleviates ulcerative colitis involved in gut microbiota, which is significantly correlated with the occurrence and development of colitis. However, the effect and mechanism of GC-K on anti-colitis in relation with gut microbiota are not unambiguous. This study focused on the preventive effect and mechanism of GC-K on Dextran sulfate sodium (DSS)-induced colitis in mice involved in gut microbiota.

Methods: DSS was used to establish a chronic colitis mouse model. Body weight analysis, colon length measurement, HE staining and inflammatory factors levels were carried out in animal experiments. Flow cytometry was used to analyze Th17/Treg cells in the mouse spleen and blood. 16S rRNA sequencing was used to analyze gut microbiota. Fecal microbiota transplantation (FMT) experiment was employed to verify the anti-colitis efficacy of GC-K by reshaping gut microbiota.

Results: GC-K significantly relieved colitis-related symptoms by decreased disease activity index (DAI) scores, spleen weight, and increased colon length. Additionally, tight junction proteins were enhanced and pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-17 were decreased after GC-K treatment. Furthermore, Bacteroides spp. were significantly increased after modeling. Moreover, FMT experiments confirmed that GC-K-driven gut microbiota significantly relieved DSS-induced colitis.

Conclusion: GC-K alleviated colitis via the modulation of gut microbiota.

Introduction

Ulcerative colitis (UC), a chronic nonspecific intestinal inflammatory disease, clinically manifests as abdominal pain, bloody diarrhoea and even degrees of systemic symptoms, which is becoming a global disease in gastroenterology [1, 2]. Colitis triggers inflammatory cell infiltration followed by cytokine release syndrome and the systemic inflammation eventually. The pathological mechanism of colitis is not well-known, so that colitis is symptomatically treated in clinic along with side effects and easy recurrence. Therefore, it is particularly important to develop safe and effective drugs for colitis.

Although the underlying pathogenesis of UC is incompletely understood, numerous studies have demonstrated that gut microbiota plays an important role in the pathogenesis of UC. Intestinal inflammation disappears in a spontaneous model of colitis in germ-free IL-10(−/−) mice [3, 4]. Meanwhile, the gut microbiota in UC patients remains dysbiosis by comparing with healthy human. The α-diversity of gut microbiota and some beneficial gut microbials, such as Faecalibacterium prausnitzii and Akkermansia muciniphila, were reduced in UC patients. F. prausnitzii can destroy the activation of the NF-κB pathway and block the production of IL-8 by producing butyrate, while A. muciniphila ameliorates colitis by modulating immune responses and gut barrier repair [5-7]. On the contrary, the abundances of pathogenic bacteria were increasing, e.g., Bacteroides vulgatus, which exacerbates UC by secreting proteases [8]. Due to disordered gut microbiota as a pathogenic factor that leads to an impaired intestinal
barrier and promotes the onset of UC, targeting the gut microbiota could be an effective therapeutic approach for preventing and curing colitis.

Ginsenoside compound K (GC-K) is one of the main metabolites of ginseng saponins bio-converted by gut microbiota \textit{in vivo} [9-11], which possesses various pharmacological activities, such as anti-diabetic, anti-hepatic lipid accumulation, anti-inflammation and antitumor effects. In addition, GC-K ameliorates colitis and inhibits the inflammatory responses by suppressing NF-kB activation [12]. However, the absolute oral bioavailability of GC-K is only about 35% [13]. GC-K is catalyzed by β-glycosidase only secreted from gut microbiota to generate its metabolite protopanaxadiol \textit{in vivo}, which implies that GC-K interplays with gut microbiota after oral administration [14]. Furthermore, our previous study found that GC-K could suppress the tumor growth of AOM/DSS-induced colitis-associated CRC through the modulation of gut microbiota, partially by up-regulation of \textit{A. muciniphila} [9]. Accordingly, we hypothesized that GC-K could alleviate colitis via regulating gut microbiota.

In this study, the influence of GC-K on gut microbiota was explored in DSS-induced colitis to verify whether gut microbiota mediates the improvement in DSS-induced colitis. We observed that GC-K ameliorated experimental colitis and regulated gut microbial such as \textit{Bacteroides spp.}, which were enriched in colitis group. In addition, co-incubation experiments further demonstrated the regulatory effect of GC-K on \textit{Bacteroides spp.} Spearman correlation analysis showed that the improvement of UC phenotype was closely related to gut microbiota. To verify the beneficial effects of a treatment-naïve gut microbiota on colitis, fecal microbiota transplantation (FMT) confirmed that gut microbiota from GC-K-dosed mice improved intestinal barrier function and relieved experimental colitis. Collectively, our study demonstrated the anti-inflammatory effects of GC-K in a DSS-induced colitis model mainly via regulating gut microbiota.

**Materials And Methods**

**Materials**

Fluorescein isothiocyanate (FITC)-labeled dextran was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies of ZO-1 and Occludin were purchased from Proteintech Group, Inc. (Wuhan, Hubei, China).

**Animals and experimental design**

Dextran sulfate sodium (DSS; molecular weight, 36–50 kDa) and Sulfasalazine (SASP) were purchased from MP Biomedicals (San Jose, CA, USA) and Aladdin (Shanghai, China), respectively. Female C57BL/6 mice (6 weeks; 17-20 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). The Animal Ethics Committee of Central South University (No. 2020sydw1037) permitted the animal protocol. Mice after acclimation were divided into groups as control, DSS, DSS+LCK (20 mg/kg/day, low dose), DSS+HCK (60 mg/kg/day, high dose), and SASP (200 mg/kg/day, positive control) (Fig. 1A). Mice were fed with 2% DSS in drinking water for 5-7 days, replaced by autoclaved water for another 10-14 days for 3 cycles, and sacrificed on day 42.
Disease activity index (DAI) and histologic assessment

The DAI was calculated every two days to evaluate the severity of DSS-induced colitis as described previously [15]. The distal colorectal segments were immersed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E) to perform histopathology assay.

Quantitative Real-Time PCR Analysis

According to previous studies [16], total RNA of colon tissue was extract by Trizol reagent. The concentration and purity of total RNA were determined by Nanodrop. SYBR Green Premix Ex TaqII was used for qPCR amplification. The relative regulation in target genes after normalization to β-actin between two groups was calculated using the $2^{-\Delta\Delta Ct}$ method. The qPCR primers of IL-17a, TNF-α, IL-β, IL-10, IL-6, Foxp3, ZO-1, Occludin, E-cadherin and Mucin-2 were synthesized by Sanggon Biotech (Shanghai, China) (Table S1).

Flow cytometry analysis

Spleens were collected, ground and filtered using 70 μm cell strainers to obtain single cell suspension. For Th17 cells measurement, cells were counted and stimulated by Leukocyte Activation Cocktail for 4 h. Then, Fixable Viability Dye eFluor™ 780 (eBioscience) was used to identify the Viability of cells. After lysis of the red blood cells, cells were subsequently stained with antibodies as following, (a) Anti-mouse CD45 Briliant Violet510, Anti-mouse CD3 FITC, Anti-mouse CD4 Briliant Violet421, (b) Anti-mouse IL-17A PE. For Treg cells measurement, cells were accordantly stained with antibodies as following, (a) Anti-mouse CD45 Briliant Violet510, Anti-mouse CD3 FITC, Anti-mouse CD4 Briliant Violet421, Anti-mouse CD25 PE/Cy™ (b) Anti-mouse Foxp3 APC. All the anti-bodies were purchased from BD Biosciences (San Jose, CA, USA).

Western blotting

Proteins were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer and then centrifuged at 12 000 rpm and 4°C for 15 min. 200 μL of supernatant were subsequently added and mixed well with 40 μL of 5 × SDS-PAGE(100°C, 10 min). Protein quantification was performed according to the BCA kit procedure. The polyvinylidene difluoride membranes were incubated with the specific primary antibodies and then with the appropriate secondary antibodies for 1.5 h at room temperature. The blot was visually detected with an enhanced chemiluminescence (ECL) substrate and a Bio-Rad imaging system.

Measurement of FITC-Dextran leakage

Mice were fasted overnight and gavaged with FITC-Dextran (Sigma) at a dosage of 500 mg/kg. After administration of FITC-Dextran for 4 h, serum was collected to measure the fluorescence intensity.

16S ribosomal RNA gene sequencing and data analysis
After sequencing, data processing and quality control were performed using fastq-join (Version: 1.3.1) and pear (v0.9.11) to generate Raw Tags. Cutadapt (version 1.18) was used to get the Clean Tags from the original sequences. Usearch (Version11.0.667) software was used to operate OTU (Operational Taxonomic Units) clustering. Silva (Release132) database was used to pair OTUs sequences for taxonomic analysis. The α-diversity and β-diversity of the samples were analyzed by Mothur software and R language tools. LEfSe analysis was performed on LEfSe software to assess the effective size (LED > 2 or 4) of each distinct taxa or OTU. Correlation analysis was conducted on Wekemo Bioincloud (https://www.bioincloud.tech/).

**Bacterial strains and growth curve**

All the bacterial strains were stored in 30% glycerol at -80°C until use. The bacteria were divided into the administration group (500 mg/mL or 20 μmol/mL of GC-K) and the control group (DMSO). Their growth dynamics were detected in an anaerobic chamber with mixed anaerobic gas (5% carbon dioxide, 5% hydrogen, 90% nitrogen).

**Fecal microbiota transplantation**

FMT was performed according to an established protocol with an appropriate modification [17]. Stools from mice treated with HCK or DSS were collected and stored at -80°C. 100 mg of stools from donor mice were re-suspended in 1 mL of sterile saline. The solution centrifuged at 600 × g for 5 min to obtain the supernatant, which was then centrifuged at 12 000 × g for 10 min. The pellet was re-suspended and used as transplant bacteria. Mice were fed with antibiotic water (0.5 g/L of ampicillin, 0.5 g/L of metronidazole, 0.5 g/L of neomycin and 0.25 g/L of vancomycin) for 5 days. Transplantation was performed by oral gavage of 200 μL of bacterial fluid 3 times a week.

**Statistical analysis**

The data were expressed as the mean ± SEM and analyzed by GraphPad Prism 8.0 and SPSS software (Version 23). Significant differences between the two groups were evaluated by the two-tailed unpaired Student’s t-test, one-way ANOVA or Kruskal-Wallis with non-normal or non-parametric distribution. The level of significance was set at \( p < 0.05 \) (\( * p < 0.05, ** p < 0.01, *** p < 0.001 \), and **** \( p < 0.0001 \)).

**Results**

**GC-K alleviated DSS-induced chronic colitis in mice**

Chronic DSS treatment caused a reduction in body weight and an increase of DAI, which was reversed after treated with GC-K (Fig. 1A). The body weight showed a slight increase with GC-K treatment (Fig. 1B). GC-K alleviated colon shortening, reduced spleen weight and exhibited less immune cell infiltration and tissue damage by comparing with DSS Group (Fig. 1C-F).
DSS-induced upregulations of pro-inflammatory cytokines such as IL-1β, IL-17a, TNF-α and IL-6 were significantly downregulated by GC-K (Fig. 2A−D). In addition, the expression of anti-inflammatory cytokine IL-10 was significantly reduced by DSS (Fig. 2E), but neither GC-K nor SASP had any effect on the production of IL-10. GC-K down-regulated the expression of Foxp3 increased by DSS without significance (Fig. 2F). Th17 cells in the blood significantly increased after modeling, while GC-K effectively reduced the quantities of Th17 cells and restored the Th17/Treg ratio (Fig. 2G-L). Meanwhile, the quantities of Treg cells in the blood and spleen were recovered with GC-K treatment.

The concentration of FITC-dextran was increased in model group, while SASP and GC-K significantly reduced the concentration of dextrose in the plasma (Fig. 3A). Furthermore, GC-K reversed the expressions of Occludin, ZO-1, E-cadherin and MUC2, which implied that mucus layer thickness was restored (Fig. 3B-F).

**Alterations of gut microbiome by GC-K treatment**

The α-diversity showed no significant difference among all groups (Fig. 4A and B). The β-diversity revealed distinct clustering of gut bacterial for each group (Fig. 4C and 4D). The results indicated that the profiles of gut microbiota were significantly different among each group.

Taxonomic histograms of gut bacterial on phylum and genus levels were shown in Fig. 4E-F. The phylum analysis revealed that GC-K increased the relative abundances of Verrucomicrobia and Patescibacteria and decreased that of Proteobacteria in DSS-induced colitis mice (Fig. 4F and Fig.S2), while GC-K significantly increased the relative abundances of Akkermansia, Candidatus_Saccharimonas and Ruminococcaceae_UCG-014, and decreased Bacteroides and Rumboutsia in genus levels (Fig. S3). The biomarkers for the Model group cluster were Bacteroides and Rumboutsia, while the biomarkers of LCK group cluster were Akkermansia, Ruminococcaceae_UCG-014 and Turicibacter (Fig. 4G and H). The results showed that GC-K ameliorated gut microbiota dysbiosis in DSS-induced mice with significantly decreasing the abundance of Bacteroides.

To examine whether GC-K directly suppressed the growth of Bacteroides in vitro, the growth curve of 7 strains of Bacteroides co-incubated with GC-K was monitored (Fig. S4). GC-K directly suppressed the growth of B. vulgatus in vitro, but had no significant effect on other strains of Bacteroides.

**The transplant of GC-K-induced microbiota relieved colitis**

As shown in Fig. 5, a correlation map was constructed to discriminate the specific bacteria correlated with colitis disease indicators. Higher correlation scores indicated that the GC-K-driven gut bacteria potentially alleviated colitis disease indicators.

FMT significantly alleviated DSS-induced colitis and decreased immune cell infiltration in colon tissue and inflammatory factors expression of IL-1β, TNF-α, IL-17a (Fig. 6). Similar to donor mice, the quantities of Treg and Th17 cells in the spleen decreased significantly with DSS administration and increased after treated with GC-K (Fig. S5). Furthermore, FITC-dextran leakage experiment confirmed that FMT protected...
gut leakage. In addition, the expressions of Occludin, ZO-1, MUC2 and E-cadherin were increased, which indicated intestinal barrier function was restored (Fig. 7).

Discussion

Gut microbiota plays an important role in maintaining body health [18]. In addition, intestinal inflammation disappears in IL-10(-/-) mice when the intestinal tract is sterile [19], which indicates that the gut microbiota is closely correlated to the occurrence and development of IBD. Due to the gut microbiota dysbiosis, the abundance and diversity of the gut microbiota of UC patients are decreased [20, 21]. Therefore, Regulation of gut microbiota has become new therapeutic strategy of colitis.

GC-K is the main metabolite of the protopanaxadiol type of ginsenosides bio-converted by gut microbiota [10, 11, 22]. Studies have shown that GC-K can play a beneficial role in colitis by inhibiting IRAK-1 and NF-kB activation [12]. However, the absolute oral bioavailability of GC-K is only about 35% [13], which implied it could interplay with gut microbiota after oral administration [14]. Recently, natural products have been discovered to interplay with gut microbiota in vivo [23, 24]. Moreover, our previous work has confirmed GC-K suppressed the tumor growth of AOM/DSS-induced colitis-associated CRC via regulating gut microbiota, partially by up-regulation of A. muciniphila [9]. Thus, the purpose of this work was to determine the role of gut microbiota in the treatment of UC with GC-K.

GC-K relieved the symptoms of weight loss and colon length shortening in DSS-induced mice. Compared with SASP, HC-K has advantages in weight and DAI scores. In terms of colonic histopathology, GC-K was observed to effectively reduce histlogic inflammation. In addition, GC-K significantly decreased the mRNA expression of inflammatory cytokines (TNF-α, IL-6, IL-1β and IL-17a) in colon tissue. Intestinal mucosal barrier could isolate the intestinal lumen from the environment to prevent the invasion of bacteria and toxic substances [25], but an increase in proinflammatory cytokines further damages intestinal mucosal barrier function to make colitis worse [26, 27]. Occludin, ZO-1 and E-cadherin are crucial for connecting individual epithelial cells and maintaining the integrity of the epithelium [25]. The intestinal mucus layer is a protective gel-like substance covering the surface of the intestinal mucosa, which is the first barrier in the intestinal lumen [28]. Our experiment showed that DSS-induced colitis decreased the mRNA expressions of Occludin, ZO-1, E-cadherin and MUC2 in colon tissue, which could be reversed by GC-K treatment.

According to 16S rRNA sequencing analysis, lower abundances of Lachnospiraceae_NK4A136_group and Candidatus_Saccharimonas and higher abundances of Bacteroides, Romboutsia and Turicibacter were observed in the DSS group than in the control group, which were consistent with previous reports [29, 30]. GC-K reversed the intestinal dysbacteriosis caused by DSS. In addition, the abundances of Bacteroides and Romboutsia in HC-K was significantly decreased. Spearman correlation analysis showed that Bacteroides was mostly correlated with the disease indicators of UC. In vitro co-incubation experiments showed that GC-K significantly inhibited the growth of B. vulgatus. Studies have shown that the abundance of B. vulgatus in UC patients is significantly increased and the proteases from B. vulgatus...
could be involved in UC pathogenesis [8]. The above results indicate that GC-K regulated the structure and abundance of gut bacteria and reduced the abundance of *Bacteroides*. However, the detailed mechanisms of GC-K reshaped *Bacteroides spp.* remain unclear and need further investigation.

Finally, in order to further explore whether GC-K relieved colitis by modulating gut microbiota, we used FMT to verify the anti-colitis of GC-K in the DSS model. Prior to FMT, the pseudo-sterile mice were constructed with antibiotics, since gut microbiota may lead to colonization resistance. FMT relieved colonic inflammation and decreased intestinal permeability, which transmitted the anti-colitis effects of GC-K. Collectively, our data supported GC-K ameliorated colitis via regulating gut microbiota.

**Conclusion**

Our study evidenced that the anti-colitis effects of GC-K was related to gut microbiota, which was modulated by GC-K. Fecal microbiota transplantation experiments demonstrated that GC-K-reshaped gut microbiota significantly relieved DSS-induced colitis. In conclusion, GC-K showed anti-colitis effects via regulating gut microbiota, but the pertinent mechanism needs further study.

**Abbreviations**

16S rRNA, 16S ribosomal RNA; **GC-K**, ginsenoside compound K; linear discriminative analysis; **LEfSe**, the linear discriminative analysis effect size; **PCoA**, principal component analysis; **PC**, principal component; **RT-PCR**, Real-time polymerase chain reaction; **QIIME**, Quantitative Insights Into Microbial Ecology.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The research data generated from this study are included within the article and supplementary files.

**Competing interests**

The authors declare that they have no competing interests.

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Authors’ contributions

WH designed the experiments; LW and LW participated in the experiments and analyzed the 16S rRNA sequencing data; MC, LW, LS, WZ and FBT provided the technical support and advices for the study; LW and WH wrote the manuscript. All authors approved the final version of manuscript.

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Not applicable.

References


Figures
Figure 1

GC-K alleviated DSS-induced chronic colitis. (A) Disease Activity Index during mouse model development. (B) Relative bodyweights during mouse model development. (C) Colon length in each group. (D) Spleen weight in each group. (E) Representative colon and spleen pictures from each group. (F) Representative H&E staining of colon tissue sections from each group.
Figure 2

GC-K decreased inflammatory cytokines production during DSS-induced colitis. Inflammatory factors IL-1β (A), IL-6 (B), TNF-α (C), IL-17a (D), IL-10 (E) and transcription factor Foxp3 (F) in mouse colon measured by RT-PCR. Flow cytometry analysis of Treg cells (CD3+CD4+CD25+Foxp3+) in blood (G) and spleen (J). Flow cytometry analysis of Th17 cells (CD3+CD4+IL-17a+) in blood (H) and spleen (K). Flow cytometry analysis of Th17/Treg cells in blood (I) and spleen (L).
Figure 3

GC-K improves intestinal barrier during DSS-induced colitis. Intestinal leakage measured by FITC-Dextran concentration in serum (A). Mucin MUC2 (B) and intestinal tight junction proteins E-cadherin (C), Occludin (D) and ZO-1 (E) in each group measured by RT-PCR. ZO-1 and Occludin in colon of different groups measured by Western blotting (F).
Figure 4

16S rDNA sequencing revealed altered microbiota composition after GC-K treatment. The α diversity of each group measured by Shannon (A) and Simpson (B) index. PCA score plot analysis based on the relative abundance of OTUs (C) and bray curtis-based PCoA analysis (D) were used to evaluate the β-diversity of each group. Relative abundance of microbial taxa was determined at the phylum (E) and genus (F) level. Taxonomic cladogram obtained (G) and linear discriminant analysis score (H) from linear
discriminant analysis effect size analysis of 16S rRNA sequences. Relative abundance of *Bacteroides* (I) and *Rombsia* (J) in each group.

**Figure 5**

Spearman correlation analysis between top 15 Taxonomic of gut microbiota and evaluation indexes of inflammatory bowel disease.
Figure 6

FMT alleviated DSS-induced chronic colitis. (A) Disease Activity Index during mouse model development. (B) Relative bodyweights during mouse model development. (C) Colon length in each group. (D) Spleen weight in each group. (E) Representative colon and spleen pictures from each group. (F) Representative H&E staining of colon tissue sections from each group. Inflammatory factors IL-1β (G), TNF-α (H) and IL-17a (I) in mouse colon measured by RT-PCR.
Figure 7

FMT improves intestinal barrier during DSS-induced colitis. Intestinal leakage measured by FITC-Dextran concentration in serum (A). ZO-1 and occludin in colon of different groups measured by Western blotting (B) Mucin MUC2 (C) and intestinal tight junction proteins ZO-1 (D), Occludin (F) and E-cadherin (G) in each group measured by RT-PCR.
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