**Original Article**

**Gingko biloba extract (Ginaton) ameliorates dextran sulfate sodium (DSS)-induced acute experimental colitis in mice via reducing IL-6/STAT3 and IL-23/IL-17**

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**Abstract:** This study explored the underlying mechanism of Gingko biloba extract (Ginaton) on dextran sulfate sodium (DSS)-induced acute experimental colitis in mice. 40 male C57BL/6 mice were randomly divided into four groups: normal control group, Ginaton group, Ginaton treatment group, and DSS group. After 7 days administration, mice were sacrificed and colons were collected for H-E staining, immunohistochemistry, real-time PCR and Western blot. By observing clinical disease activity and histological damage, we assessed the effect of Ginaton on DSS-induced acute experimental colitis in mice and observed the effect of Ginaton on normal mice. We also explored the specific mechanism of Ginaton on DSS-induced acute experimental colitis in mice through examining the expression of inflammatory related mediators (gp130, STAT3, p-STAT3, ROR-γt) and cytokines (IL-6, IL-17, IL-23). Ginaton-treated DSS mice showed significant improvement over untreated DSS mice. Specifically, Ginaton improved clinical disease activity (DAI score, weight loss, colon shortening, and bloody stool) and histological damage, and reduced the expression of inflammatory-related mediators (p-STAT3, gp130, ROR-γt) and cytokines (IL-6, IL-17, IL-23). In addition, clinical disease activity, histological damage, the expression of inflammatory related mediators (STAT3, p-STAT3, gp130, ROR-γt) and cytokines (IL-6, IL-17, IL-23) in mice of Ginaton group were similar to normal control group. In conclusion, Ginaton ameliorates DSS-induced acute experimental colitis in mice by reducing IL-17 production, which is at least partly involved in inhibiting IL-6/STAT3 signaling pathway and IL-23/IL-17 axis. Moreover, Ginaton itself does not cause inflammatory change in normal mice. These results support that Ginaton can be as a potential clinical treatment for ulcerative colitis (UC).

**Keywords:** Ginaton, acute experimental colitis, IL6/STAT3, IL-23/IL-17

**Introduction**

Inflammatory bowel disease (IBD) is a complex set of non-specific intestinal inflammatory diseases with unknown etiology, including ulcerative colitis (UC) and Crohn’s disease (CD). In UC, lesions begin in the rectum, retrograde to the proximal segment, which involve the entire colon and terminal ileum. These lesions are localized in the mucosa and submucosal layers in an uninterrupted pattern [1]. In CD, damage is commonly found in the distal ileum and adjacent colon but can be observed in any part of the gastrointestinal tract from mouth to anus. These lesions involve the whole layer of the bowel wall and occur in a discontinuous pattern [2]. IBD is believed to result from the abnormal interaction among genetic, environmental, microbial, immunological, and infectious factors [3, 4]. Experimental evidences are mounting to support immune disorder as a leading factor in the pathogenesis of IBD [5].

Many studies found that IL-6-gp130-STAT3 signaling pathway plays a crucial role in the development of IBD [6-8]. One study conducted by Weaver et al. showed a novel role of the IL-6/STAT3 signaling pathway in Th17 reaction and IL-17 production [9]. Moreover, multiple studies have shown that ROR-γt, which induced by IL-6-gp130-STAT3 signaling pathway, is very important for differentiation of Th17 cells [10-12]. Meanwhile, some researchers have demonstrated that IL-23 participates in generation of
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Th17 cells and stimulates secretion of IL-17 [13].

Traditional medicine for IBD is divided into three main categories: 5-aminosalicylic acids, glucocorticoid steroids, and immunosuppressive agents. With improved understanding of the pathogenesis of IBD, many effective biological agents have been created, but these biological agents are quite expensive. Furthermore, studies have shown that these biological agents can increase the risks of infection and cancer [14, 15]. Thus, identifying new treatments for IBD is a priority.

Figure 1. Effect of Ginaton on histological damage in DSS-induced acute experimental colitis. H-E staining of mice colons (× 10) from normal control group (A, saline), DSS mice treated with Ginaton 300 mg/kg (B), 200 mg/kg (C), and 100 mg/kg (D); and DSS group (E, DSS + saline). (F) Histological scores are presented as means ± SEM. *P < 0.01 vs. normal control group, †P < 0.05 vs. DSS group, ‡P < 0.01 vs. 100 mg/kg Ginaton group, §P < 0.01 vs. 200 mg/kg Ginaton group.
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Ginkgo Biloba extract (EGb) is derived from the leaves of Ginkgo biloba, and the main active constituents are flavonoid glycosides. EGb can remove oxygen free radicals, inhibit lipid peroxidation, inflammation and allergic reaction, modulate immune responses, and promote cell proliferation and apoptosis [16-21]. EGb761 was shown to inhibit the spread of colon cancer cells by up-regulation of P53 and down-regulation of bcl-2 genes [22]. Zhou et al. [23] found EGb to reduce expression of inflammatory factors (e.g. IL-6 and TNF-α) in TNBS-induced colitis. In addition, a study by Kotakadi et al. [24] suggested that EGb could ameliorate DSS-induced acute experimental colitis by promoting apoptosis. But until now, EGb relieves DSS-induced acute experimental colitis whether involves in IL-6/STAT3 signaling pathway and IL-23/IL-17 axis is not elucidated.

In this study, an animal model of acute experimental colitis was induced by freely drinking 3% Dextran Sodium sulfate (DSS) solution for 7 days. Meanwhile, the effect of Ginaton on IL-6/STAT3 signaling pathway and IL-23/IL-17 axis in acute experimental colitis was explored.

Materials and methods

Animal model of acute experimental colitis

Male C57BL/6 mice, aged 6-8 weeks and weighing 22-24 g, were purchased from the experimental animal center of Shengjing Hospital of China Medical University [license number: SCXK (Liao) 2003-0009]. Mice were fed in the SPF laboratory animal room on a 12:12-h light-dark cycles with room temperature 22±2°C and relative humidity 50%-60%. All animal experiments were performed in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the China Medical University Animals Committee.

To induce acute experimental colitis, mice were given ad libitum access to drinking 3% DSS solution [3 g DSS powder (MP Biomedicals, USA, MW 36,000-50,000) in 100 ml drinking water] for 7 days.

Table 1. DAI score chart

<table>
<thead>
<tr>
<th>Score</th>
<th>Body loss (%)</th>
<th>Stool-consistency</th>
<th>Occult/gross bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>1-5</td>
<td>Loose</td>
<td>Occult bleeding</td>
</tr>
<tr>
<td>2</td>
<td>5-10</td>
<td>Looseness</td>
<td>Occult bleeding</td>
</tr>
<tr>
<td>3</td>
<td>10-15</td>
<td>Loose</td>
<td>Occult bleeding</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 15</td>
<td>Diarrhea</td>
<td>Gross bleeding</td>
</tr>
</tbody>
</table>

The disease activity index = (combined score of weight loss, stool consistency and bleeding)/3. Normal stool, shaped stool; loose Pasty, uniform stools which do not adhered to the anus; Diarrhea stool, Watery stools which adhered to the anus.

In a preliminary dose-response experiment, mice were randomly assigned to three groups (n = 8) by different dose of Ginaton (100 mg/kg, 200 mg/kg, 300 mg/kg) administrated, then sacrificed for histological assessment. We found that 300 mg/kg Ginaton has the best therapeutic effect (Figure 1). The dose of 300 mg/kg Ginaton was chose to be used in subsequent experiment.

A total of 40 male C57BL/6 mice were randomly divided into four groups (n = 8): Normal control group, 0.8 mL saline was fed everyday by intragastric administration. Ginaton group, 300 mg/kg.d Ginaton (dissolved in 0.8 mL saline) was administrated in the same way. Ginaton treatment group, acute experimental colitis was induced by freely drinking 3% DSS solution for 7 days, while 300 mg/kg.d Ginaton was given by intragastric administration. DSS group, acute experimental colitis was induced as described above, while 0.8 mL saline was fed every day. After Ginaton or saline was administered for 7 days, mice were sacrificed by cervical dislocation and colons were collected. Colons were washed 2-3 times with pre-cooling saline, then 0.5-1.0 cm segments which is 2 cm away from the anus were collected and fixed in 10% neutral buffered formalin for H-E staining and immunohistochemistry. The remaining colon tissue of each mouse was divided into two sections and stored at 80°C for real-time PCR and Western blot.

Evaluation of acute experimental colitis

Acute experimental colitis was assessed by disease activity index (DAI) score [25-27] (Table 1), weight change, colon length, histological damage, and histological score [28] (Table 2). Histological score of colons was compared.
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Table 2. Histological score chart

<table>
<thead>
<tr>
<th>Integral</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>Depth of the lesion</td>
<td>None</td>
<td>Mucous layer</td>
<td>Submucosa</td>
<td>Muscularis and serosa</td>
<td></td>
</tr>
<tr>
<td>Crypt damage</td>
<td>None</td>
<td>1/3</td>
<td>2/3</td>
<td>100%</td>
<td>100% with epithelium loss</td>
</tr>
<tr>
<td>Pathological change range</td>
<td>None</td>
<td>0%-25%</td>
<td>26%-50%</td>
<td>51%-75%</td>
<td>76%-100%</td>
</tr>
</tbody>
</table>

Figure 2. Effect of Ginaton on clinical disease activity in DSS-induced acute experimental colitis, assessed by DAI score (A), weight change (B), and colon length (C). (D) Representative colon of each group (Line 1, normal control group; Line 2, Ginaton group; Line 3, Ginaton treatment group; Line 4, DSS group).

Completed independently by two pathologists, and the average score in each group was calculated.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from colon tissue with Trizol. Next, 500 ng RNA was reverse transcribed using 200 U M-MLV (Promega Corporation, Madison, WI, USA), and PCR was performed using a real-time PCR system (Applied Biosystems, Forster, CA, USA). All PCR reactions were done in triplicate, using the gene GAPDH as an endogenous control. The primer sequences used for cDNA amplification were as follows: GAPDH, 5'-ACTCCACTCACGGCAAATT-3' and 5'-TCTCCATGTTGGAAGACA-3'; IL-6, 5'-AGAAATCTGCAGCTCCCACC-3' and 5'-CTGTGCTAGCGAGCTGTT-3'; gp130, 5'-TAACTCCGTATTCGCCACG-3' and 5'-TTTGTCCGAACAGTCGGTCC-3'; STAT3, 5'-CCCGTACCTGAAGACCATG-3' and 5'-TCCATGTCAAACGTGAGCGA-3'; ROR-γt, 5'-GGAGCTCTGCCAGAATGACC-3' and 5'-CAAGGCTCGAAACAGCTCCAC-3'; IL-23, 5'-ACCTGCTGGACTCGGACAT-3' and 5'-GGCGAGGCATCTGTTGAT-3'; and IL-17A, 5'-TCCACGCTTTCCTCGCG-3'.
Western blot analysis

The protein levels of p-STAT3 and STAT3 in colon tissue were quantified by Western blot. Colon tissues of mice were added into RIPA lysis buffer. Then, these tissue samples were homogenized at 4°C, centrifuged at 12,000 × g for 30 min, and supernatant was retained. After degeneration, 50 μg samples were separated by SDS-PAGE and transferred to Polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk (5 g non-fat dry milk powder in 100 ml of TBST) at room temperature for 2 hours, then incubated with specific anti-phospho-STAT3 antibody (1:1000) and anti-STAT3 antibody (1:1000) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Finally, membranes were incubated with peroxidase-conjugated secondary antibody (1:5000) at room temperature for 1 hour. Protein levels were quantified by gray values, assessed by professional software (Software Total Lab Dynamics Ltd, Phoretix, Newcastle, UK).

Immunohistochemical assay

Expression of IL-6, IL-17, and IL-23 in mouse colon tissue was determined by immunohistochemistry. Colons were fixed in 10% neutral buffered formalin for one week, then paraffin-embedded, sliced, dehydrated, and retrieved of antigen. Sections were treated with 3% hydrogen peroxide at room temperature for 5 min, then incubated with antibody IL-17 (1:500), IL-23 (1:500), and IL-6 (1:500) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) respectively. After washing with PBS, the sections were incubated with secondary antibody and DAB substrate. The color reaction was

Figure 3. Effect of Ginaton on bloody stool in DSS-induced acute experimental colitis. Normal control group (A, saline), Ginaton group (B, Ginaton), Ginaton treatment group (C, DSS + 300 mg/kg Ginaton) and DSS group (D, DSS + saline). Mice of normal control group and Ginaton group had no bloody around anus. There were lots of bloody stool adhering to the anus of mice in DSS group, but little bloody stool was found around the anus of mice in Ginaton treatment group. These pictures showed that Ginaton could improve the degree of bloody stool in DSS-induced acute experimental colitis.
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**Figure 4.** Effect of Ginaton on histological damage in DSS-induced acute experimental colitis. (H-E) staining of mice colons (× 10) (A-D) and (× 20) (E-H) from normal control group (A and E), Ginaton group (B and F), Ginaton treatment group (C and G), and DSS group (D and H). (I) Histological scores are presented as means ± SEM. *P < 0.01 vs. normal control group; *P < 0.05 vs. DSS group.
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stopped with distilled water, and sections were
counterstained with Hematoxylin. Sections in-
cubated with PBS instead of primary antibody
served as negative controls. For immunohisto-
chemistry analysis, data were expressed as
optical density.

Figure 5. Effect of Ginaton on relative mRNA expressions of IL-6 (A), gp130 (B), STAT3 (C), ROR-γt (D), IL-17 (E) and IL-23 (F) in DSS-induced acute experimental colitis. Treatment with Ginaton could effectively reduced mRNA expres-
sions of IL-6, gp130, STAT3, ROR-γt, IL-17 and IL-23. #P < 0.01 vs normal control group; *P < 0.05 vs. DSS group; §P
> 0.05 vs. Ginaton group.

Statistical analysis

All analyses were carried out using SPSS 18.0
(SPSS Inc, Chicago, IL, USA). Data are expressed
as mean ± SEM. Statistical analysis for signifi-
cant differences was conducted by use of one-
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way ANOVA (equal variances assumed) or Tamhane’s T2 tests (equal variances not assumed). P-values less than 0.05 were considered to be statistically significant.

Results

Clinical disease activity

Ginaton exhibited striking improvements in DSS-induced acute experimental colitis, as shown by reducing DAI score, inhibiting body loss and colon shortening (Figure 2). The DAI scores of normal control group and Ginaton group were 0. Compared with the DSS group, mice of Ginaton treatment group showed a reduced DAI score beginning at day 4 (P < 0.05). Body weight of mice in normal control group and Ginaton group increased gradually during the experiment. Compared with the DSS group, mice of Ginaton treatment group had slower weight loss starting at day 4 (P < 0.05). Similarly, mice of Ginaton treatment group showed significantly longer colons than DSS group (P < 0.01). Bloody stool around the anus was observed on day 7 (Figure 3).

Histology

Colons in normal control group and Ginaton group had intact membrane structure. In contrast, the membrane structures of colons in DSS group were disarranged. Specifically, glands had disappeared, and inflammatory cells had infiltrated into mucosa and submucosa. Colons of Ginaton treatment group showed damage in only part of membrane structure and reduced inflammatory cells infiltration. Histological score was significantly reduced in Ginaton treatment group compared with DSS group (P < 0.05) (Figure 4).

The mRNA expression of IL-6, gp130, STAT3, ROR-γt, IL-17, and IL-23

In contrast to normal control group, mice of DSS group exhibited elevated mRNA expression of IL-6, gp130, STAT3, ROR-γt, IL-17, and IL-23 mRNA (P < 0.05). Compared with DSS group, mRNA expression of these factors significantly reduced in Ginaton treatment group (P < 0.05). IL-6, gp130, STAT3, ROR-γt, IL-17 and IL-23 mRNA expressions in Ginaton group
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## Protein Expression

The protein expression of p-STAT3 and STAT3

Protein expression levels of p-STAT3 and STAT3 were quantified by Western blot (Figure 6). Compared to normal control group, mice of DSS group showed increased p-STAT3 protein expression in the colon (P < 0.05). p-STAT3 protein expressions in colons of Ginaton treatment group were significantly reduced in comparison with the DSS group (P < 0.05). The protein expression of STAT3 in colons of each group had no statistical difference (P > 0.05).

The protein expression of IL-6, IL-17 and IL-23

The protein expressions of IL-6, IL-17 and IL-23 in mice were examined by immunohistochemistry.

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**Figure 7.** Effect of Ginaton on expressions of IL-6 (A-D), IL-17 (E-H), and IL-23 (I-L) in normal control group (A, E, I), Ginaton group (B, F, J), Ginaton treatment group (C, G, K), and DSS group (D, H, L). IL-6, IL-17 and IL-23 were mainly distributed in the mucosa and submucosa layer of the colon. A large number of brown granules were seen in DSS group in contrast to normal control group. Compared with DSS group, less brown granules were found in Ginaton treatment group.
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By observing immunohistochemistry films, we found that IL-6, IL-17 and IL-23 were mainly distributed in the mucosa and submucosa layer of colons (Figure 7). As shown in Figure 8, protein expressions of IL-6, IL-17 and IL-23 in DSS group were significantly increased compared with normal control group (P < 0.05). Conversely, protein expressions of these factors were significantly reduced in Ginaton treatment group (P < 0.05). IL-6, IL-17 and IL-23 protein expressions in Ginaton group were similar to normal control group (P > 0.05).

Discussion

Our study suggested that Ginaton can improve clinical disease activity (DAI score, weight loss, colon shortening, and bloody stool), reduce histological injury, and decrease expressions of inflammatory related mediators (gp130, p-STAT3, ROR-γt) and cytokines (IL-6, IL-17, IL-23) in colon tissues of mice with DSS-induced acute experimental colitis.

The underlying mechanisms of DSS-induced acute experimental colitis are unclear. Some researchers found that the mechanism involves the activation of intestinal macrophages [30, 32, 33]. However, recent studies found that the inflammatory process caused by DSS is related to immune disorders [34, 35]. In our study, mice were provided with 3% DSS instead of drinking water for one week to induce acute experimental colitis. Mice exposed to DSS were found with gross bloody stool, weight loss, colon shorten and histological injury compared with the normal control group.

TNBS, DSS, and acetic acid are used to build animal models of colitis. But now, oral administration of DSS is the most common method to induce experimental colitis. DSS-induced experimental colitis has similar pathological and clinical manifestations with UC [26, 29-31]. IL-6 produced by hyper-activation immune cells (e.g., monocytes and macrophages) involved in the pathogenesis of UC [36, 37]. Accumulating evidences suggest that IL-6 plays a crucial role in IBD [38]. A study by Feng et al. [39] demonstrated that serum IL-6 level was consistently elevated in patients with UC, indicating the pathogenesis of UC is closely related to IL-6. In another study, anti-IL-6R monoclonal antibody effectively reduced T-cell expansion and decreased adhesion molecules and inflammatory response in the CD45RB<sup>hi</sup>-SCID (sever combined immuno-deficient) adaptive transfer model of colitis [8, 40]. It proved that IL-6 couples with its receptor (sIL-6R) to form a complex which activates gp130-positive cells, inducing...
In conclusion, Ginaton ameliorates DSS-induced acute experimental colitis in mice by reducing IL-17 production, which is at least partly involved in inhibiting IL-6/STAT3 signaling pathway and IL-23/IL-17 axis. Meanwhile, Ginaton itself does not cause inflammatory change in colons of normal mice. These results support that Ginaton can be as a potential clinical treatment for ulcerative colitis. In addition, future research should be conducted to investigate other inflammatory pathways which may be involved in reducing the production of IL-17 in Ginaton-treated DSS mice.

Disclosure of conflict of interest

None.

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References


[29] Bjorck S, Jennische E, Dahlstrom A, Ahlman H. Influence of topical rectal application of drugs...


