

Original Article

Ascorbic acid ameliorates oxidative stress and inflammation in dextran sulfate sodium-induced ulcerative colitis in mice

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Abstract: Ascorbic acid (AA) has been shown to exert beneficial effects, including mitigating oxidative stress and inhibiting inflammation. However, the preventative effect of vitamin C in chronic inflammatory diseases such as inflammatory bowel disease (IBD) remains unclear. In our study, we investigated the anti-inflammatory effects of AA and possible mechanism involved in inhibiting dextran sulfate sodium (DSS)-induced ulcerative colitis in mice. Male C57BL/6 mice were randomly divided to three groups: control group, DSS group, and DSS plus ascorbic acid treated group. Several clinical and inflammatory parameters as well as oxidative stress were evaluated. The results demonstrated that ascorbic acid significantly reduced clinical signs, inflammatory cytokines, myeloperoxidase (MPO) and malonaldehyde (MDA) activities, whereas the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were increased in DSS-induced mice. In addition, ascorbic acid was capable of inhibiting NF- κ B, COX-2 and iNOS expression in the colonic. Taken together, these findings suggest that ascorbic acid contributes to the reduction of oxidative stress and inflammatory response in DSS-induced colitis and exerts the potential to prevent and clinical treatment of inflammatory bowel disease.

Keywords: Ascorbic acid, experimental colitis, oxidative stress, NF- κ B

Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), which is the chronic, relapsing and remitting inflammatory disease of the gastrointestinal tract [1]. IBD is characterized by acute pain, vomiting, weight loss, diarrhea and bloody stool symptoms [2]. Although great progress has been made in understanding the pathogenesis of UC, the exact mechanism remains unknown. Recent research has emphasized the development and course of UC are affected by several factors, including genetic susceptibility, environmental factors and immune system [3]. The treatment of IBD includes lifestyle alternations, pharmacological and surgical treatments [4]. Recently, immune-suppressants, such as anti-TNF- α agents; common agents, includes 5-ASA, steroid hormone are being used for treatment of IBD. Due to these therapies have significant and potentially serious side effects or high

cost, the discovery of efficacy and cost effective agents is emergent.

Oxidative stress is a key part of the chain of events in inflammatory bowel disease [5]. Scavenging the reactive oxygen species (ROS) moderates the damage to tissue and affects the signal transduction pathways. Ascorbic acid (Vitamin C) is one of the main antioxidant factors in biological systems [6]. Some studies have suggested that ascorbic acid, a vital cofactor that regulates enzymatic reactions, plays as a potent antioxidant against inflammation and oxidative stress through activating intracellular molecular pathways [7-9]. By trapping radicals, ascorbic acid prevents cell membrane oxidation and injury caused by ROS [10]. In addition, health benefits of ascorbic acid supplementation have provided positive advantages, such as prevention of metabolic disease or cancer [11, 12]. Furthermore, the intervention in the signal cascade of nuclear factor NF- κ B changes

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the expression of a series of genes connected with the inflammatory response and apoptosis [13-15]. However, the effect of ascorbic acid on inflammatory bowel disease and the special mechanism remains unknown.

In the present study, we used current biomedical approaches to investigate the therapeutic efficacy of ascorbic acid and possible protective mechanism involved in inhibiting DSS-induced chronic mouse model of ulcerative colitis.

Materials and methods

Reagents

DSS (MW 36,000-50,000) was obtained from MP Biochemicals (Aurora, USA). Ascorbic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies (p-65, p-p65, cox-2, iNOS) were purchased from Cell Signaling Technology (Danvers, MA, USA). Goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Abcam (Cambridge, UK). Monoclonal mouse anti- β -Actin was purchased from Sigma (St. Louis, MO, USA).

Animals and treatments

C57BL/6J male mice weighing 22-25 g were obtained from the Laboratory Animal Center of the Southern Medical University (Guangzhou, China) and maintained in a standard 12-h light/12-h dark cycle at a room temperature of $25 \pm 2^\circ\text{C}$ and a relative humidity between 50 and 60%. Mice ($n = 18$) were divided into three groups: control group, DSS group, and DSS plus ascorbic acid treated group. The DSS-induced ulcerative colitis mice were fed 4% dextran sodium sulfate dissolved in sterile, distilled water ad libitum for 7 days (days 1-7). The DSS+ Ascorbic acid treated group mice were intraperitoneally treated with Ascorbic acid (100 mg/kg) daily. The negative control group received a sham treatment. After 7 days, the DSS drinking water was change to normal water and the DSS+ Ascorbic acid treated group mice continue treating with ascorbic acid for 3 days (days 8-10). Body weight and food intake were measured daily. The daily weight changes were calculated by percent of the initial weight. The disease activity index (DAI) was calculated by grading on a scale of 0 to 4 using the following parameters: loss of body weight (0: normal; 1:

0-5%; 2: 5-10%; 3: 10-15%; 4: > 15%), stool consistency (0: normal; 2: loose stools; 4: watery diarrhea) and fecal occult blood (0: negative; 2: positive; 4: gross bleeding). At 11 days, mice were killed under anesthesia, the spleen weight and the colon length were measured.

Assessment of MDA, SOD, CAT and GPx

The colon tissues were homogenized in lysis buffer (Beyotime, China) supplemented with phenylmethanesulfonyl fluoride (PMSF). The homogenate was centrifuged at 15000 g for 10 min and the supernatant were collected for detecting the MDA content, SOD, CAT and GPx activities by using commercial kits (Beyotime, China). The protein concentration was measured by the bicinchoninic acid (BCA) protein assay (Beyotime, China) according to the manufacturer's manuals. All results were normalized to the protein content and expressed as nmol/mg protein or U/mg protein.

Histopathological analysis

The colon was isolated and 1 cm of the distal colon was fixed in 4% paraformaldehyde. Paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E). The stained samples were visualized using an optical Leica DM6000 B microscope and analyzed using the Leica V3.8 (Leica Microsystems, Wetzlar, Germany). A histological score reflecting infiltration of inflammatory cells and epithelial structure was given on a scale as described previously [16]. These criteria were scored as follows: inflammation severity (0: none; 1: slight; 2: moderate; 3: severe), inflammation extent (0: none; 1: mucosal; 2: submucosal; 3: transmural), and crypt damage (0: none; 1: damage to the basal third of the crypt; 2: damage to the basal two-thirds of the crypt; 3: only surface epithelium intact; 4: loss of entire crypt and epithelium).

Myeloperoxidase activity assay

The colon tissue was homogenized on ice in 5 volumes of PBS and centrifuged at 15000 g at 4°C for 10 min. The myeloperoxidase (MPO) activity in colon tissue was measured using a MPO assay kit (Nanjing Jiancheng, China) following the manufacturer's instructions. Results of MPO activity are expressed as fold to control group.

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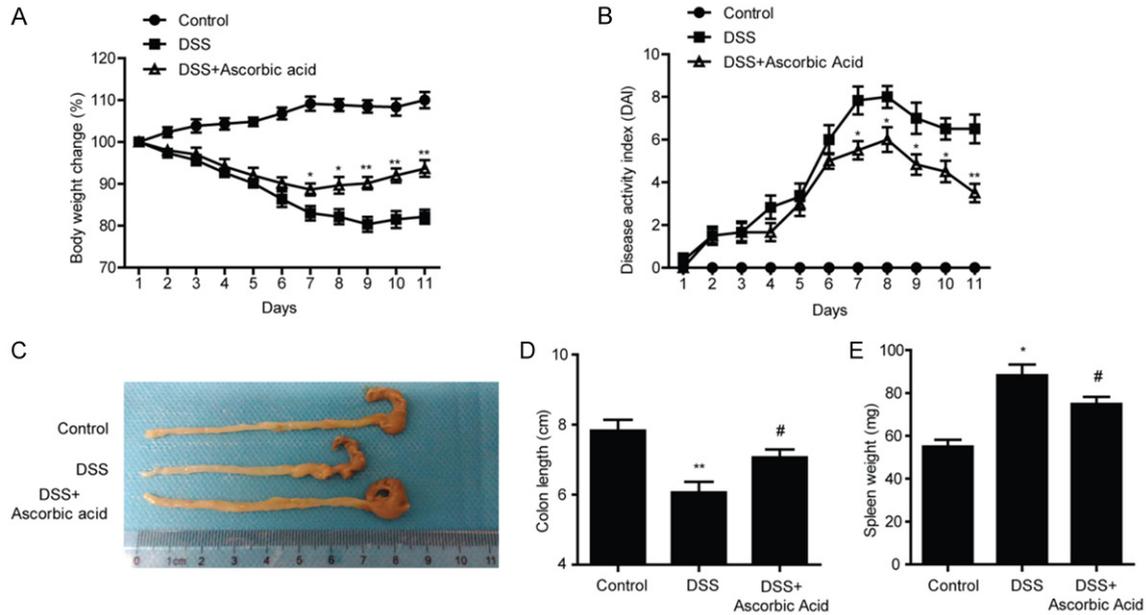


Figure 1. Ascorbic acid alleviated DSS-induced experimental colitis. (A) Body weight changes after DSS induction of colitis. (B) Disease activity index (DAI). * $P < 0.05$; ** $P < 0.01$, versus DSS-treated group. (C) Macroscopic appearances and (D) the length of colons. E. Spleen weight. Data are presented as mean \pm SEM of 6 mice in each group. * $P < 0.05$; ** $P < 0.01$, versus control group and # $P < 0.05$ versus DSS-treated group.

Enzyme-linked immunosorbent assay

Levels of inflammation in DSS-induced ulcerative colitis mice with or without ascorbic acid treatment were examined by detecting TNF- α , IL-1 β , IL-6 and IL-17 in distal colon tissue for using enzyme-linked immunosorbent assay (ELISA) kits (Boster, Wuhan, China). Distal colon tissue from each group was homogenized with lysis buffer to extract total protein. The homogenate was centrifuged at 15000 g at 4°C for 10 min. The concentration of total protein was determined by BCA protein assay kit (Beyotime, China). The amount of TNF- α , IL-1 β , IL-6 and IL-17 in distal colon tissue was quantified according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA from the colon was extracted using Trizol reagent (TaKaRa, Japan) following the manufacturer's instructions. The cDNA was synthesized using a reverse transcription kit (TaKaRa) according to the manufacturer's protocol. Real-time quantitative PCR was carried out using CFX96 real-time system (Bio-Rad, USA) with a cDNA sample, and amplification was carried out in a 20 ml reaction volume containing 1 \times SYBR Green PCR Master Mix

(TaKaRa). The primers used were as follows: TNF- α (forward: TGAAGTTCGGGGTATCGGTC; reverse: AGCCTTGTCCCTGAAGAGGAAC), IL-1 β (forward: TGGTGTGACGTTCCCATTA; reverse: CAGCACGAGGCTTTTTGTTG), IL-6 (forward: TCCAGTTGCCTTCTTGGGAC; reverse: AGTCTCCTCTCCGACTTGT), IL-17 (forward: CGTGGCCTCGATTGTCGCC; reverse: GGTTTCTTAGGGGTCAGCCGCG) and GAPDH (forward: TGGCCTCCAGGAGTAAGAAAC; reverse: AGTTGGGATAGGGCCTCTCTTG). The relative expression of the target genes was analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to the endogenous GAPDH.

Protein extraction and western blot analysis

Total proteins extracted from colon tissues were lysed and homogenized in lysis buffer (Beyotime, China) supplemented with protease inhibitor cocktail. The homogenates were centrifuged at 15,000 g for 15 min at 4°C, the protein concentration in the supernatants was measured using the BCA kit. The extracts were heated in 97°C for 5 min and 30 μ g of protein was separated by 10% SDS-PAGE, then transferred onto a nitrocellulose membrane. After blocking with 5% non-fat milk, the membranes were incubated with antibodies against p-65, p-p65, COX-2 and iNOS overnight at 4°C.

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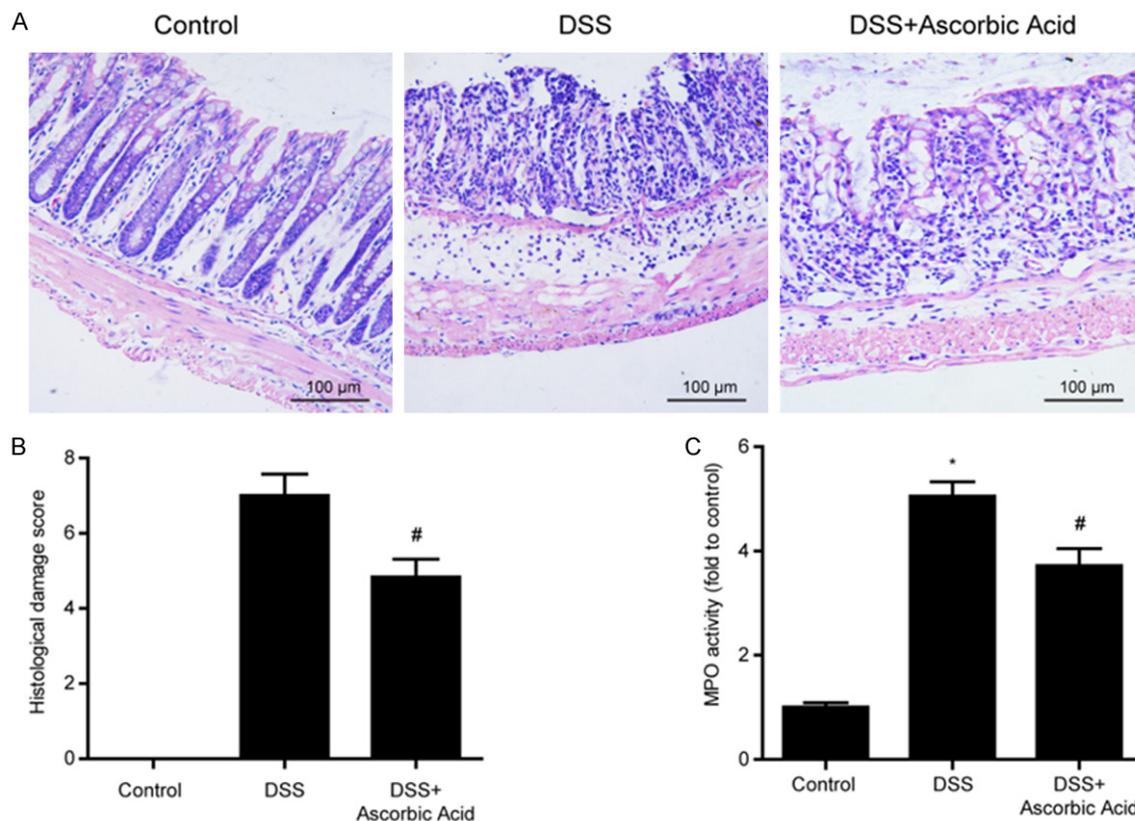


Figure 2. Ascorbic acid treatment protected against DSS-induced colon damage in mice. A. Colon tissues were stained with hematoxylin and eosin (H&E). B. Histopathological scores were analyzed from slides. C. MPO activity. Data are presented as means \pm SEM. * $P < 0.05$ compared with control group; # $P < 0.05$ compared with DSS-treated group.

Protein bands were visualized and analyzed using western blotting detection system according to the manufacturer's instructions (Bio-Rad, USA).

Statistical analysis

Data were expressed as the mean \pm SEM of at least 3 independent experiments and analyzed with PRISM 5.0 software (Graphpad Software, San Diego, CA). Unpaired student's t-test was used to compare the means of two groups and $P < 0.05$ was considered to be statistically significant.

Results

Ascorbic acid alleviated DSS-induced experimental colitis.

DSS administration for seven days induced acute colitis characterized by decreased body weight starting on day 3 compared with the

control mice (Figure 1A). Ascorbic acid (100 mg/kg) suppressed DSS-induced body weight loss were obtained starting on day 7 and lasted up to day 11. Colitis mice also exhibited features of loose feces and hematochezia, thus disease activity index (DAI) was evaluated. The score of DAI significantly increased after DSS intake, however, ascorbic acid treatment markedly attenuated the increased score (Figure 1B). The shortening of the colon and the swelling of the spleen have been identified as index to reflect the severity of colorectal inflammation. After DSS treatment, significant shortening of the colon length was observed in the DSS-induced mice, and this reduction was abrogated by treatment with ascorbic acid (Figure 1C and 1D). The spleen weights of the mice administered DSS were significantly higher compared with those of the control group, and ascorbic acid treatment significantly decreased the spleen weights (Figure 1E). The severity of colonic inflammation and ulceration was evalu-

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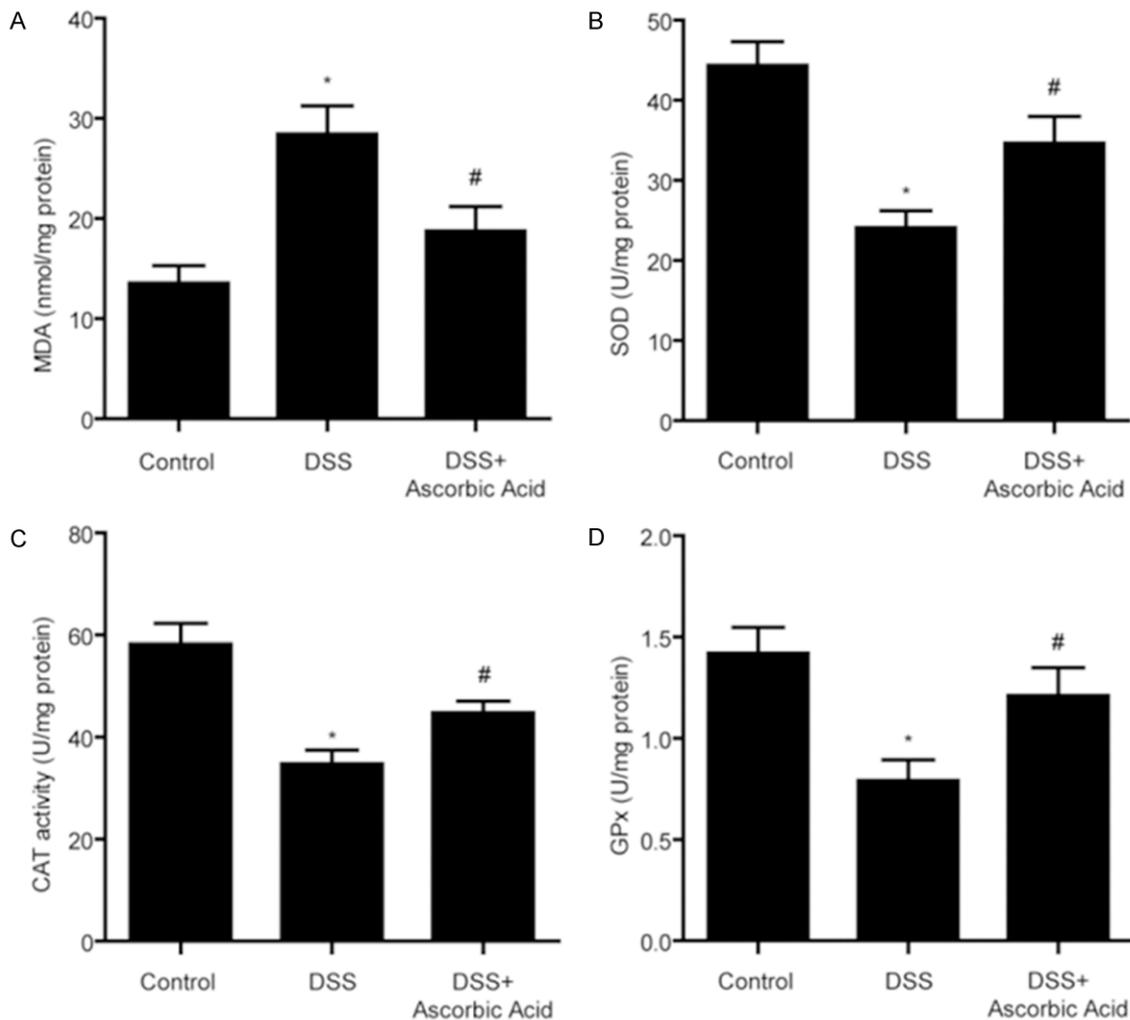


Figure 3. Ascorbic acid decreased the levels of oxidative stress in DSS-induced colitis mice. A. MDA content. B. SOD activity. C. CAT activity. D. GPx activity in the colonic tissue. Data represent the means \pm SEM. * $P < 0.05$ compared with control group; # $P < 0.05$ compared with DSS-treated group.

ated by histopathological analysis using H&E staining (**Figure 2A**). DSS-exposed mice exhibited distortion of crypts, loss of goblet cells, severe epithelial injury and massive inflammatory cells infiltration in mucosa and submucosa. Interestingly, ascorbic acid-treated mice exhibited less inflammatory cell infiltration and only mild evidence of crypt distortion. Ascorbic acid treatment resulted in a significant reduction of the histological damage scores induced by DSS (**Figure 2B**). Furthermore, ascorbic acid significantly attenuated DSS-induced MPO activity (**Figure 2C**). Taken together, our results suggested that ascorbic acid could alleviate DSS-induced colitis.

Ascorbic acid decreased the levels of oxidative stress in DSS-induced colitis mice

Oxidative stress and its associated inflammatory activation play an important role in inflammatory bowel disease. To evaluate the antioxidant capacity of ascorbic acid, we measured several oxidative stress parameters in the colon. The MDA levels in colonic tissues were found to be significantly increased in DSS-induced mice compared to untreated control group. Treatment with ascorbic acid significantly decreased MDA levels compared to those in DSS-treated mice (**Figure 3A**). In addition, our results show that the activities of SOD, CAT and GPx were significantly decreased in the colon

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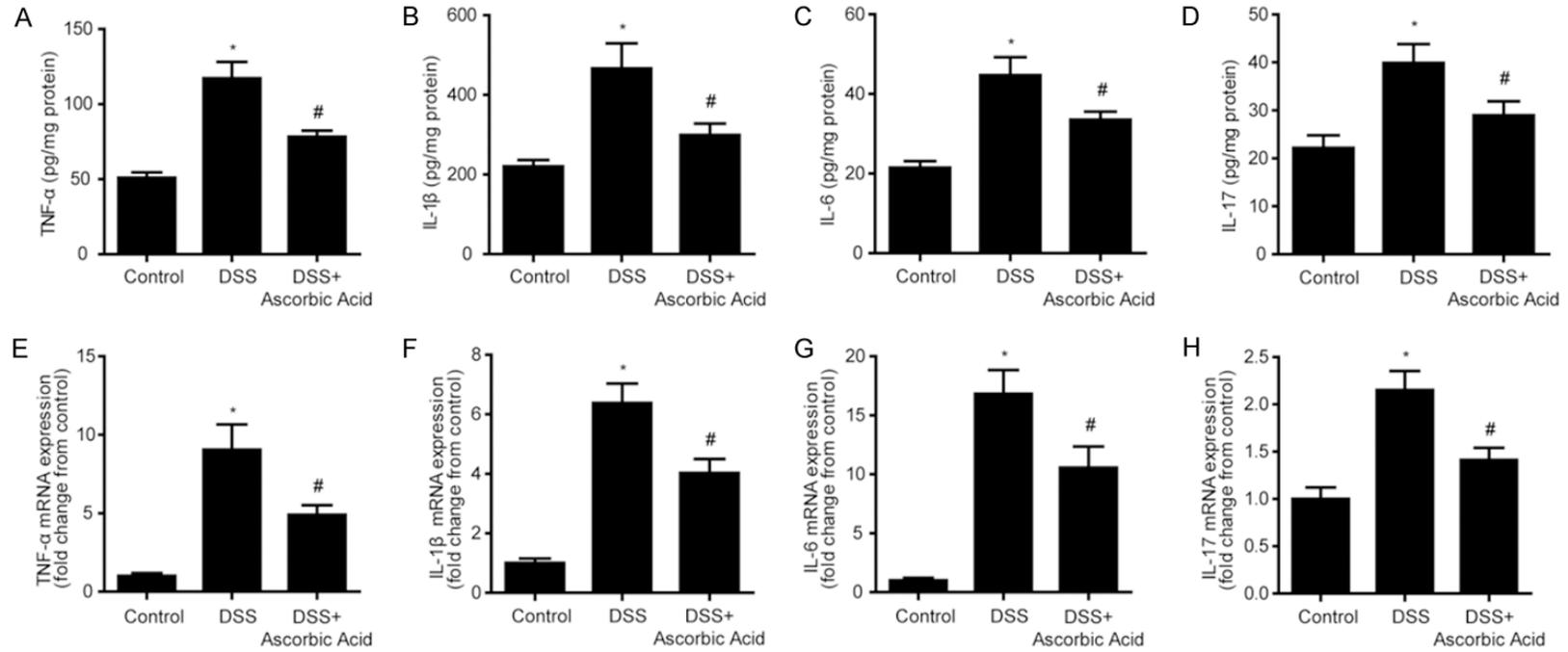


Figure 4. Ascorbic acid decreased pro-inflammatory mediators production and mRNA levels in DSS-induced colitis mice. TNF- α (A), IL-1 β (B), IL-6 (C) and IL-17 (D) in colon were determined by ELISA, respectively. The mRNA levels of TNF- α (E), IL-1 β (F), IL-6 (G) and IL-17 (H) in colonic tissue were determined by RT-PCR. Data represent the means \pm SEM. *P < 0.05 compared with control group; #P < 0.05 compared with DSS-treated group.

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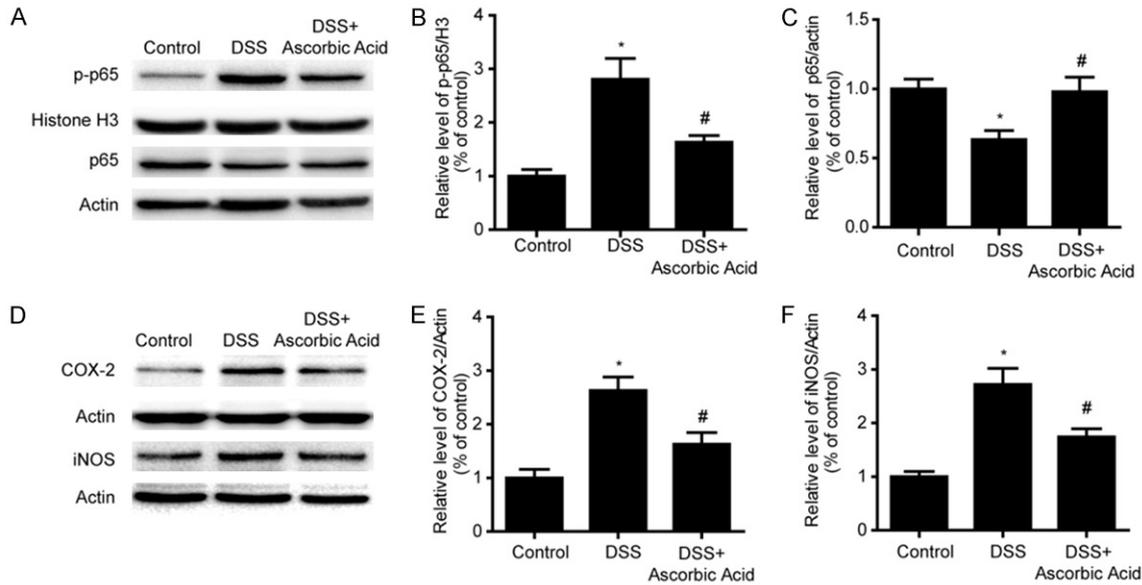


Figure 5. Ascorbic acid inhibited the activation of NF- κ B and decreased inflammatory proteins expression. A. One representative image of p-p65 and p65 expression is shown. B, C. Quantification of the protein expression was performed by densitometric analysis of the blots. D. A representative immunoblot of COX-2 and iNOS. E, F. Quantification of the protein expression. The results are expressed as a percentage of the control, which is set at 100%. Data were expressed as means \pm SEM of three independent experiments. * $P < 0.05$ compared with control group; # $P < 0.05$ compared with DSS-treated group.

tissues of DSS-induced mice as compared to the control mice. However, ascorbic acid treatment significantly elevated the SOD, CAT and GPx activities in DSS-treated mice (**Figure 3B-D**).

Ascorbic acid decreased pro-inflammatory cytokines production and mRNA levels in DSS-induced colitis mice

It has been demonstrated that the pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-17, play central roles in the development of inflammatory diseases [17, 18]. The anti-inflammatory effects of ascorbic acid were further confirmed by measuring protein and mRNA expression of the inflammatory cytokines in the colon. The DSS group significantly increased the levels of TNF- α , IL-1 β , IL-6 and IL-17 in the colon compared with the untreated control group. Ascorbic acid treatment remarkably decreased the production of TNF- α , IL-1 β , IL-6 and IL-17 in the colon induced by DSS (**Figure 4A-D**). Consistent with these findings, ascorbic acid treatment could also decrease mRNA expression of pro-inflammatory cytokines in the colon induced by DSS (**Figure 4E-H**). These results suggested that ascorbic acid may play a

role in reducing pro-inflammatory cytokine levels in DSS-induced colitis.

Ascorbic acid inhibited the activation of NF- κ B and decreased inflammatory proteins expression

It has been confirmed that NF- κ B is the central transcription factor in the regulation of pro-inflammatory cytokines and chemokines [19, 20]. The activation of NF- κ B is a crucial step in the development of inflammatory bowel disease [14, 21, 22]. To investigate the effects of ascorbic acid on NF- κ B activation, we first assess the functional status of NF- κ B expression in colon tissues. The results showed that DSS-induced phosphorylation of NF- κ B p65 compare to the control group and were reversed by ascorbic acid (**Figure 5A-C**). As the important downstream target gene of NF- κ B, the expression of COX-2 and iNOS were measured in colon tissues. We found an increased expression of COX-2 and iNOS in DSS-induced mice and markedly blocked by ascorbic acid treatment. The quantification of the immunoblots is shown in **Figure 5D-F**. On the basis of these findings, we proposed that ascorbic acid inhibits cytokines and chemokines by inhibiting NF- κ B pathway activation.

Discussion

Conventional therapies for treating inflammatory bowel disease were pharmacological and surgical interventions and usually associated with complications or side effects, such as infection, perforation and refractory rectal bleeding [23, 24]. Therefore, the development of safer and efficacy agents for the management of IBD patients is emergent. Recent studies have shown that some plant compounds, including allicin [25], cardamonin [15], boehmeria nivea [16] and cranberry [17] exhibit antioxidant and anti-inflammatory properties to ameliorate the severity of inflammatory bowel disease. Here, we are the first time to show that ascorbic acid is a feasible treatment option for DSS-induced colitis in mice. We demonstrated that ascorbic acid markedly ameliorated DSS-induced body weight loss, colon shortening, spleen swelling and histological damage. In addition, ascorbic acid significantly decreased the activity of myeloperoxidase and the production of TNF- α , IL-1 β , IL-6 and IL-17 in the colon.

Oxidative stress is one of the most crucial factors in the development of inflammatory bowel disease. Oxidative stress can damage cellular macromolecules including DNA, lipids and proteins [26]. The oxidative stress could easily overwhelm the host defenses and cause tissue and organ damage. MDA, an end product of lipid peroxidation is harmful and contributes to the pathogenesis of IBD [27]. In present study, the level of MDA was markedly increased in DSS-induced mice. However, treatment with ascorbic acid significantly decreased the level of MDA in colon. It has been demonstrated that antioxidant enzymes such as SOD, CAT, and GPx play an important role in lipid peroxidation [28]. Consistent with previous studies [25, 29], our data showed that DSS exposure induce a depletion of enzymatic antioxidants. Notably, ascorbic acid treatment significantly elevated the SOD, CAT and GPx activities in DSS-induced mice. Taken together, ascorbic acid can strengthen the enzymatic defense system and alleviate the severity of DSS-induced colitis.

NF- κ B is a nuclear transcription factor that has the ability to induce the expression of inflammatory mediators and plays an important role in diverse immune responses [20, 22]. It is well known that NF- κ B can regulate inflammatory proteins expression, such as COX-2 and iNOS

[30]. NF- κ B activation requires I κ B phosphorylation. The phosphorylated NF- κ B then translocate to the nucleus and initiates the transcription of target genes [29]. In our study, we observed that DSS exposure induce phosphorylation of NF- κ B and inflammatory proteins expression. When treated with ascorbic acid, the p65-NF- κ B expression was markedly blocked. Moreover, the COX-2 and iNOS expression was reduced by ascorbic acid in DSS-induced mice. These results indicate that ascorbic acid inhibits cytokines and chemokines by inhibiting NF- κ B pathway activation.

To summarize, our study suggest that ascorbic acid contributes to the reduction of oxidative stress and inflammatory response in DSS-induced colitis. In addition, ascorbic acid can decrease the pro-inflammatory proteins iNOS and COX-2 expression through inhibiting NF- κ B pathway activation. Ascorbic acid exerts the potential to prevent and clinical treatment of inflammatory bowel disease.

Disclosure of conflict of interest

None.

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