



Demethyleneberberine alleviates inflammatory bowel disease in mice through regulating NF- κ B signaling and T-helper cell homeostasis

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Abstract

Objective The activation of NF- κ B signaling and unbalance of T-helper (Th) cells have been reported to play a key role in the pathogenesis of colitis. Cortex Phellodendri Chinensis (CPC) is commonly used to treat inflammation and diarrhea. Demethyleneberberine (DMB), a component of CPC, was reported to treat alcoholic liver disease as a novel natural mitochondria-targeted antioxidant in our previous study. In this study, we investigated whether DMB could protect against dextran sulfate sodium (DSS)-induced inflammatory colitis in mice by regulation of NF- κ B pathway and Th cells homeostasis.

Methods Inflammatory colitis mice were induced by 3% DSS, and DMB were orally administered on the doses of 150 and 300 mg/kg. In vitro, DMB (10, 20, 40 μ M) and N-acetyl cysteine (NAC, 5 mM) were co-cultured with RAW264.7 for 2 h prior to lipopolysaccharide (LPS) stimulation, and splenocytes from the mice were cultured ex vivo for 48 h for immune response test.

Results In vivo, DMB significantly alleviated the weight loss and diminished myeloperoxidase (MPO) activity, while significantly reduced the production of pro-inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor- α (TNF- α), and inhibited the activation of NF- κ B signaling pathway. Furthermore, DMB decreased interferon (IFN)- γ , increased IL-4 concentration in the mice splenocytes and the ratio of IgG1/IgG2a in the serum. In vitro, ROS production and pro-inflammation cytokines were markedly inhibited by DMB in RAW264.7 cell.

Conclusions Our findings revealed that DMB alleviated mice colitis and inhibited the inflammatory responses by inhibiting NF- κ B pathway and regulating the balance of Th cells.

Keywords DMB · Colitis · NF- κ B · Th cell

Abbreviations

CMC	Carboxymethylcellulose sodium
CPC	Cortex Phellodendri Chinensis
DAI	Disease activity index
DMB	Demethyleneberberine
GSH	Glutathione
H&E	Hematoxylin and eosin
IBDs	Inflammatory bowel diseases
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MPO	Myeloperoxidase
NAC	N-acetyl cysteine
ROS	Reactive oxygen species
Th	T helper
Th1	Th cell type 1

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Th2	Th cell type 2
TNF- α	Tumor necrosis factor- α
UC	Ulcerative colitis

Introduction

Inflammatory bowel disease (IBDs), which include ulcerative colitis (UC) and Crohn's disease, are a group of chronic, multifactorial inflammatory disorders characterized by cytokine-driven and T lymphocyte-dependent inflammation of the intestinal mucosa [1, 2]. They have a high incidence of 24.5/100,000 in western countries [3], not only impacting quality of life, but also increasing the risk of colon cancer. A lot of evidence has shown that the risk of colorectal cancer development in patients with IBD is much higher than in the general population [4]. Immunosuppressants, such as prednisone, azathioprine and methotrexate, are used to treat IBD. Because of the potential side effects of immunosuppressants, including steroid dependence [5] and serious infections [6], there is an urgent need to develop new therapeutic options and approaches for IBD.

Reactive oxygen species (ROS) plays a pivotal role in the pathogenesis of colitis and innate immune response activation [7]. Once the innate immune response is initiated, inflammatory factors derived from intestinal epithelial cells and innate immune cells such as macrophages, including increased TNF- α , IL-6 and so on, lead to exaggerated adaptive immune responses [8]. Among these signals, NF- κ B is a key transcription factor responsible for inflammation and immunity of mammals and human beings [9]. Recently, it has been reported that dampening NF- κ B signaling pathway attenuated immune responses and ameliorated colitis [10]. Moreover, an imbalance of Th cell type 1 (Th1) versus type 2 (Th2) appears to be a key pathogenic mechanism in colitis [11, 12]. Th1 cells produce IFN- γ and positively contribute to the humoral (antibody) response to a limited extent by supporting the production of the IgG2a antibody subclass. On the contrary, the cytokines, which include IL-4, IL-5 and IL-10, are produced in Th2 cells and involved in the stimulating IgG1 production by B cells. Furthermore, IL-4 was found to be reduced in IBD, which may cause deflection of immunosuppressive and anti-inflammatory mechanism [13].

Traditional Chinese medicine Cortex Phellodendri Chinensis (CPC) has been reported with several pharmacological activities, including anti-microbial, anti-inflammatory and anti-diarrheal [14–17]. Demethyleneberberine (DMB), a component in the CPC, has been showed an obvious therapy effect to alcoholic liver disease in our previous study [18]. DMB (9, 10-dimethoxy-5, 6-dihydroisoquinolino [2, 1-b] isoquinolin-7-ium-2, 3-diol)

6-dihydroisoquinolino [2, 1-b] isoquinolin-7-ium-2, 3-diol, structure was shown in Fig. 1) is an isoquinoline alkaloid, which is a natural mitochondria-targeted antioxidant with both cationic and catechol groups [18]. Research has showed that antioxidant agents ameliorate experimental colitis [19]. However, the anti-colitic effect and mechanism of action of DMB are unknown. Thus, the aim of this study was to determine the anti-oxidant and anti-inflammatory effect of DMB on the experimental colitis induced by DSS and Raw264.7 cell stimulated by lipopolysaccharide (LPS).

Methods

Materials

Synthesized DMB from berberine (BBR) with slight modification from Zuo's method in our laboratory fulfilled the requirement for cell and animal test [18, 20]. DSS: molecular weight of 36–50 kDa (MP Biomedicals, USA). Antibodies: NF- κ B kit, including antibodies against p65, p-p65, IKK β , I κ B, p-I κ B, was purchased from Cell Signaling Technology, Inc (China) (Shanghai, China) and GAPDH was purchased from Santa Cruz Biotechnology, Inc. (Texas, USA). ELISA kits for murine IL-6, TNF- α , IL-4, IFN- γ , IgG1 and IgG2a were purchased from Beijing 4A Biotech (Beijing, China). MPO kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). LPS was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd (Shanghai, China). DMEM and FBS were purchased from Gibco. All other chemicals were analytical grade.

Animals

Female C57BL/6 mice (6–8 weeks, 18–22 g) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China) and group-housed under controlled temperature (22 ± 2 °C) and photoperiods (12:12-h light–dark cycle). Mice were acclimated to these conditions

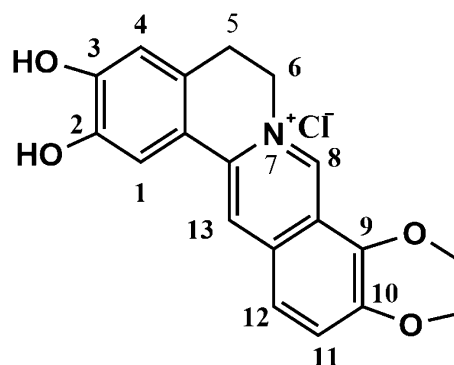


Fig. 1 The chemical structure of DMB (9, 10-dimethoxy-5, 6-dihydroisoquinolino [2, 1-b] isoquinolin-7-ium-2, 3-diol)

for 7 days and then matched by age and body weight for each group. All procedures were approved by the Institutional Animal Care and Use Committee at China Pharmaceutical University and adhered to the Jiangsu Provincial Guidelines for the use of experimental animals.

Induction of colitis

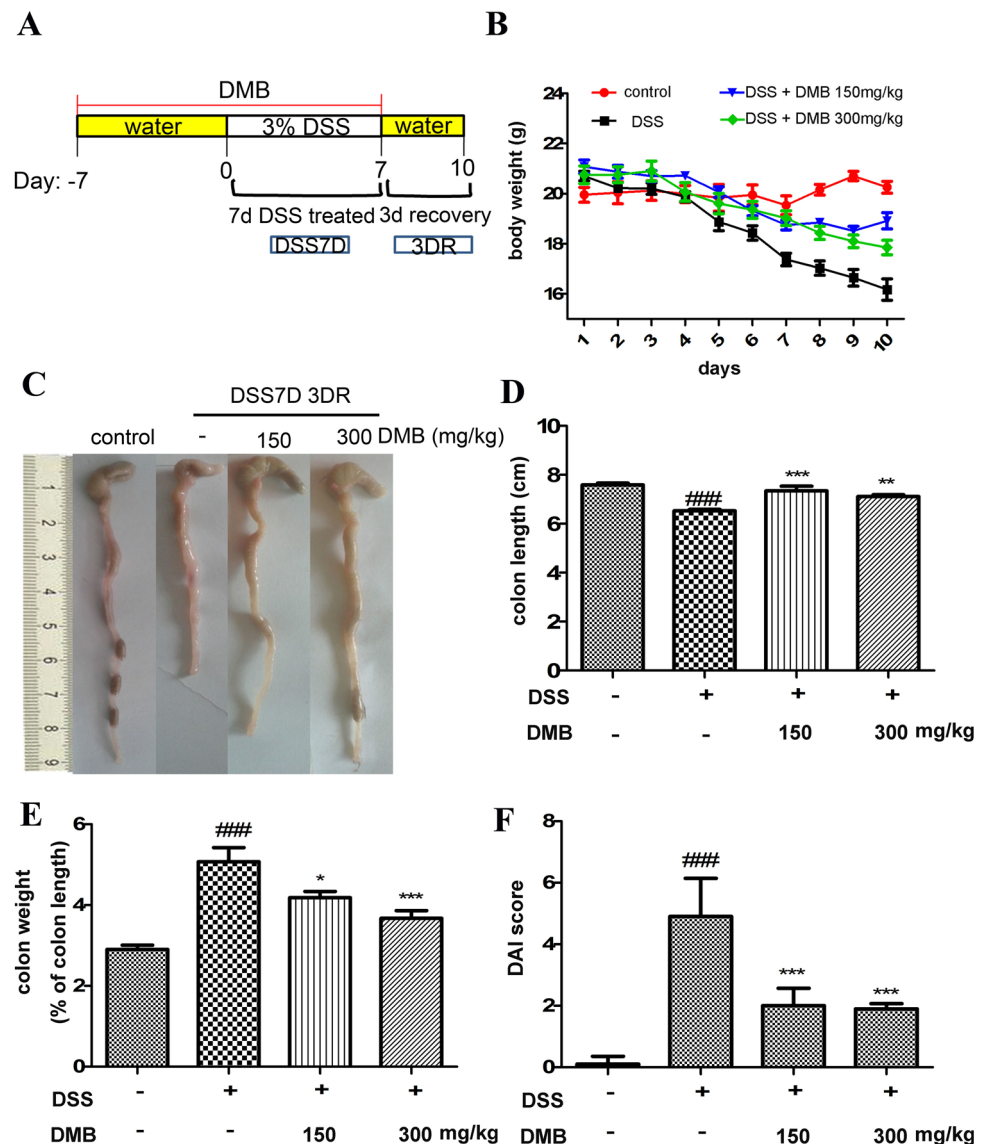
Acute colitis mice were induced by 3% (wt/vol) of DSS dissolved in drinking water continuously from day 0 to day 7, while mice in control group received the same drinking water without DSS ($n = 7$ mice in each group). DMB (150, 300 mg/kg) were orally administered once a day from day -7 to day 7 (Fig. 2a). To prepare the suspension of DMB, DMB powder was weighed, then suspended in carboxymethylcellulose sodium (CMC) to the concentration of 15 mg/ml and 30 mg/ml. Mice were observed daily for

weight, morbidity, stool consistency, and gross blood in feces and at the anus. Then they were killed and rapidly dissected under anesthesia by ether inhalation. Blood sampling for serum IgG1 and IgG2a test was drawn from the posterior vena cava. Then blood samples were centrifuged $5000\times g$ for 10 min at 4°C , and serums were stored at -70°C until analysis. The entire colon was quickly removed, washed with ice-cold 0.9% physiological saline then saved in the liquid nitrogen container.

Calculation of DAI

Disease activity index(DAI) is a useful composite score for following mice with colitis, which was calculated as described previously [21]. Briefly, the following parameters were used for calculation: (a) diarrhea (0 points = control, 2 points = loose stools, 4

Fig. 2 Effect of DMB on the recovery of DSS-induced colitis in mice. **a** The model of acute colitis in mice induced by DSS. **b** Body weight was measured daily throughout the experiment from day 1 to day 10. **c** Representative photograph showing the shortening of the colon in mice treated with DSS. **d** Lengths of the freshly removed colons were measured from rectum to ileocecal junction. **e** Colon weight was weighted and calculated by per cm of colon length. **f** The severity of colitis was macroscopically assessed every day. Values were shown as the mean \pm SEM of seven mice. *, # <0.05, **, ## <0.01 and ***, ### <0.001. * p compared with DSS group and # p compared with the control group



points = watery diarrhea); (b) hematochezia (0 points = no bleeding, 2, slight bleeding, 4 points, gross bleeding).

Histopathological assay

Segments of colons were fixed in 10% formaldehyde solution, embedded in paraffin, sectioned to 4 μm thickness, and then stained with H&E for light microscopic examination. Histological scores were calculated to assess colitis by a modified combined scoring system, which includes degree of inflammation (scale of 0–2), depth of inflammation (0–3), crypt damage (0–4) and percentage of area involved by inflammation (0–4) [11]. The total score is 0 (normal) to 13 (severe colitis).

Assay for MPO and antioxidant activity

Colon tissues were homogenized in 10 mM PBS (pH 7.4) and then centrifuged at $10,000\times g$ at 4 °C for 15 min. The supernatants were used to determine MPO level according to manufacturer's instructions. The MDA concentration was estimated as described by Ohkawa et al. [22], while the amount of GSH was measured according to the method of Paglia and Valentine [23].

Culture and treatment of RAW 264.7 cells and splenocytes

RAW 264.7 macrophage cells were obtained from the Institute of Chinese Academy of Sciences in Shanghai, and cultured in DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37 °C with 5% CO₂. DMB (10, 20, or 40 μM) were dissolved in DMSO (0.4%) and added 2 h prior to 100 ng/ml LPS for 24 or 6 h. After incubation for 24 h, the culture mediums were collected for ELISA.

Splenocytes were obtained from spleens and then dispersed by tapping gently with a rubber rod on a sterile stainless sieve. The red blood cells in the suspended cells were lysed with lysis buffer (Sigma, USA) in an ice bath for 10 min. Washed twice with PBS, and then cells were cultured for 4 h in DMEM containing 10% FBS, 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Splenocytes were plated into 24-well plates at a density of 1×10^6 cells/ml, and treated with DMB (10, 20, or 40 μM) for 48 h.

Measurement of intracellular ROS

Intracellular oxidative stress was detected using DCFH-DA as described by Lemmens KJA et al. [24]. Briefly, RAW264.7 cells (2×10^5 cells/well) were inoculated in a 24-well plate

and incubated to reach 80% confluence. The cells were pre-cultured for 2 h with DMB (10, 20, or 40 μM) and N-acetyl cysteine (NAC) (5 mM), and then stimulated with 100 ng/ml LPS for 24 h or 2 mM H₂O₂ for 2 h. Then washed twice with PBS, the cells were incubated with 10 μM DCFH-DA and 10 $\mu\text{g}/\text{ml}$ Hoechst in the dark for 30 min. The harvested cells were immediately analyzed by the flow cytometry or fluorescent microscope after washed twice.

NO assay

NO productions in supernatants of RAW264.7 cell and colon tissues were determined with a colorimetric assay based on the Griess reaction [25]. The supernatants (100 μl) were reacted with 100 μl Griess reagent (6 mg/ml) at room temperature for 10 min, and then NO₂⁻ concentration was determined by measuring absorbance at 540 nm. A standard curve was constructed using known concentrations of sodium nitrite.

ELISA of colon tissues, serum and cells

The colon homogenate and culture mediums of splenocytes and RAW264.7 cells were collected and centrifuged ($12,000\times g$, 4 °C) for 15 min. The production of IL-1 β , IL-6, TNF- α and IL-10 in colon tissues and RAW264.7 cell, IFN- γ and IL-4 in the splenocytes and IgG1 and IgG2a in the serum were determined by commercial ELISA kits.

Immunofluorescence histochemistry assay

Cryosections (4 μm) of colons were fixed in acetone, stained with anti-p65 (1:400) at room temperature for 2 h and then stained with DAPI and imaged with a confocal laser scanning microscope (Olympus, Japan).

RNA isolation and RT-PCR assay

RAW264.7 cells were seeded at $1 \times 10^6/\text{ml}$ in a 6-well plate, and then treated with DMB (10, 20, or 40 μM) for 2 h prior to LPS (100 ng/ml) stimulation for an additional 6 h when reached 80% confluence. RNA isolation and RT-PCR assay were conducted according to Wen et al. [26]. The primer sequences used in RT-PCR were listed in Table 1. Gene expression levels were calculated by the $\Delta\Delta\text{Ct}$ method and the values were normalized to endogenous reference cyclophilin.

Western blotting analysis

Colons were homogenized in 1 ml of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail (Roche, Switzerland) and centrifuged ($12,000\times g$, 4 °C) for 15 min.

Table 1 Primers used for RT-PCR

Target gene	Primer sequence(5'-3')
IL-6	F: ACGATGATGCACTTGC R: CAATCCAGAAACCGT
IL-10	F: ATTCCCTGGGTGAGAAGCT R: AGCTCTGTCTAGGTCCTGG
TNF- α	F: GTGTGGATCCCAAGCAATAC R: GTTGTTTCCAGGAAGACAG
IL-1 β	F: ACAAGGAGAACCAAGCAACGC R: GCACTACAGGCTCCGAGATGAA
iNOS	F: CCTTCCGAAGTTTCTGGCAGCAGC R: GGCTGTCAGAGCCTCGTGGCTTTGG
Cyclophilin	F: CCATCGTGTCATCAAGGACTTCAT R: CTTGCCATCCAGCCAGGAGGTCTT

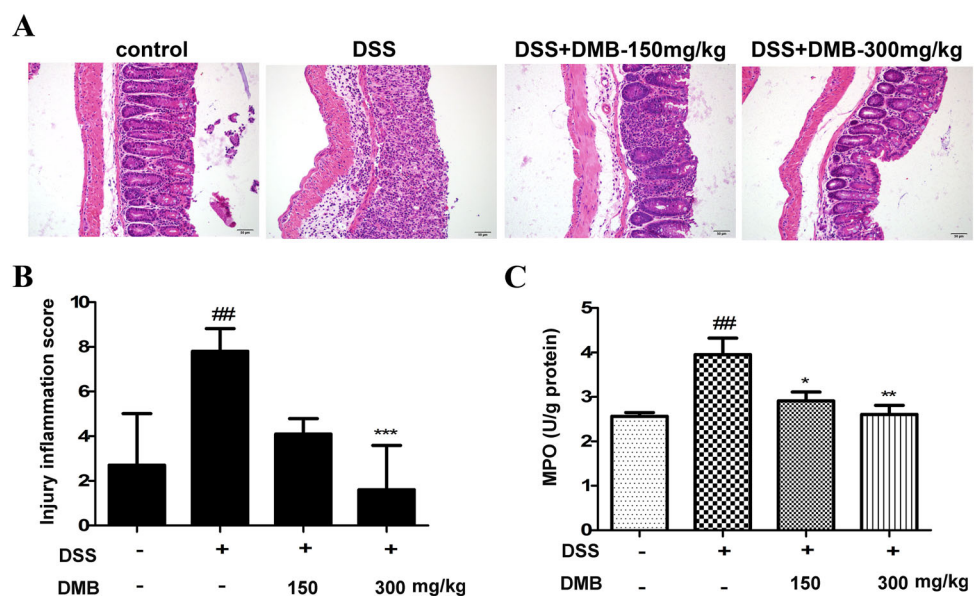
F forward, R reverse

The protein concentration was determined with bicinchoninic acid (BCA) method. Equal amounts of total protein (100 μ g) were subjected to electrophoresis on 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, USA). Levels of I κ B, p-I κ B, IKK β , p-p65, p65 and GAPDH were assayed as previously described [26]. ImageJ 64 software was used to analyze the relative density of each band.

Statistical analysis

Statistical significance for multiple comparisons in each study was determined by one-way ANOVA followed by Newman-Keuls analysis using Prism 5.0 (GraphPad Software). *P* value <0.05 was defined as statistically

Fig. 3 Effect of DMB on the colon damage in mice induced by DSS. Sections of the colon were collected and stained with H&E. Representative histological photo (a) and histological score (b) for mice in each group were presented. Protein from colon tissue was extracted and MPO activity was determined (c). Values were shown as the mean \pm SEM of seven mice. *, # <0.05, **, ## <0.01 and ***, ### <0.001. **p* compared with DSS group and #*p* compared with the control group



significant. All data presented are representative of at least five repeat experiments with means SE.

Results

Effect of DMB on the recovery of DSS-induced colitis in mice

DSS-induced mice lost weight significantly starting from the fourth day. However, the oral administration of DMB significantly alleviated weight loss and the body weight began to recover from day 7 to day 10 in the DMB group (Fig. 2b). Contrast to the obvious increase in the colon wall thickness and DAI scores in the DSS group, a remarkable attenuation of these responses was found in the colons of mice treated with either 150 or 300 mg/kg of DMB (Fig. 2f). Moreover, DMB exerted preventive effects on the colon, including the colon weight and colon length (Fig. 2c-e).

Effect of DMB on the DSS-induced colon injury in mice

The results of H&E staining indicated that DSS treatment accelerated initial damage to the mucosa, characterized by the loss of goblet cells and the occurrence of a more diffuse crypt architecture compared to the colon of control group (Fig. 3a). Additionally, the severity of inflammation seemed to be significantly lower after treated with DMB (Fig. 3b). Furthermore, increasing colonic MPO activity with the neutrophil infiltration in the colon is treated as an inflammatory marker for colitis. The MPO activity was reduced significantly in a dose-dependent manner in the mice with the treatment with DMB (Fig. 3c).

Effect of DMB on the oxidative damage in colon and ROS in the Raw264.7 cell

MDA is an end product of lipid peroxidation induced by ROS, while GSH is the most important intracellular defense against oxidative stress and is essential for both the functional and structural integrity of the gut [27]. Our current data showed that DMB treatment reduced the concentration of MDA, but increased the GSH content (Fig. 4a, b). To further investigate the antioxidant activity of DMB, we evaluated the generation of ROS in the RAW264.7 cell. When stimulated with LPS or H₂O₂, the intracellular ROS level in RAW264.7 cell increased with the time, but pretreatment

with DMB notably prevented the LPS-induced ROS generation (Fig. 4c–e). Moreover, DMB play an important antioxidant effect in T-AOC test and LPS-induced RAW264.7 cell at the concentration of 5 mM (Fig. S1a–c). As a well-established ROS scavenger, NAC treatment also showed a significant inhibitory LPS or H₂O₂-induced ROS overproduction.

Effect of DMB on the NF-κB signaling of the colon

The transcription factor NF-κB is a central mediator in inflammation and several other cellular responses. Western blotting analysis was used to further confirm our results.

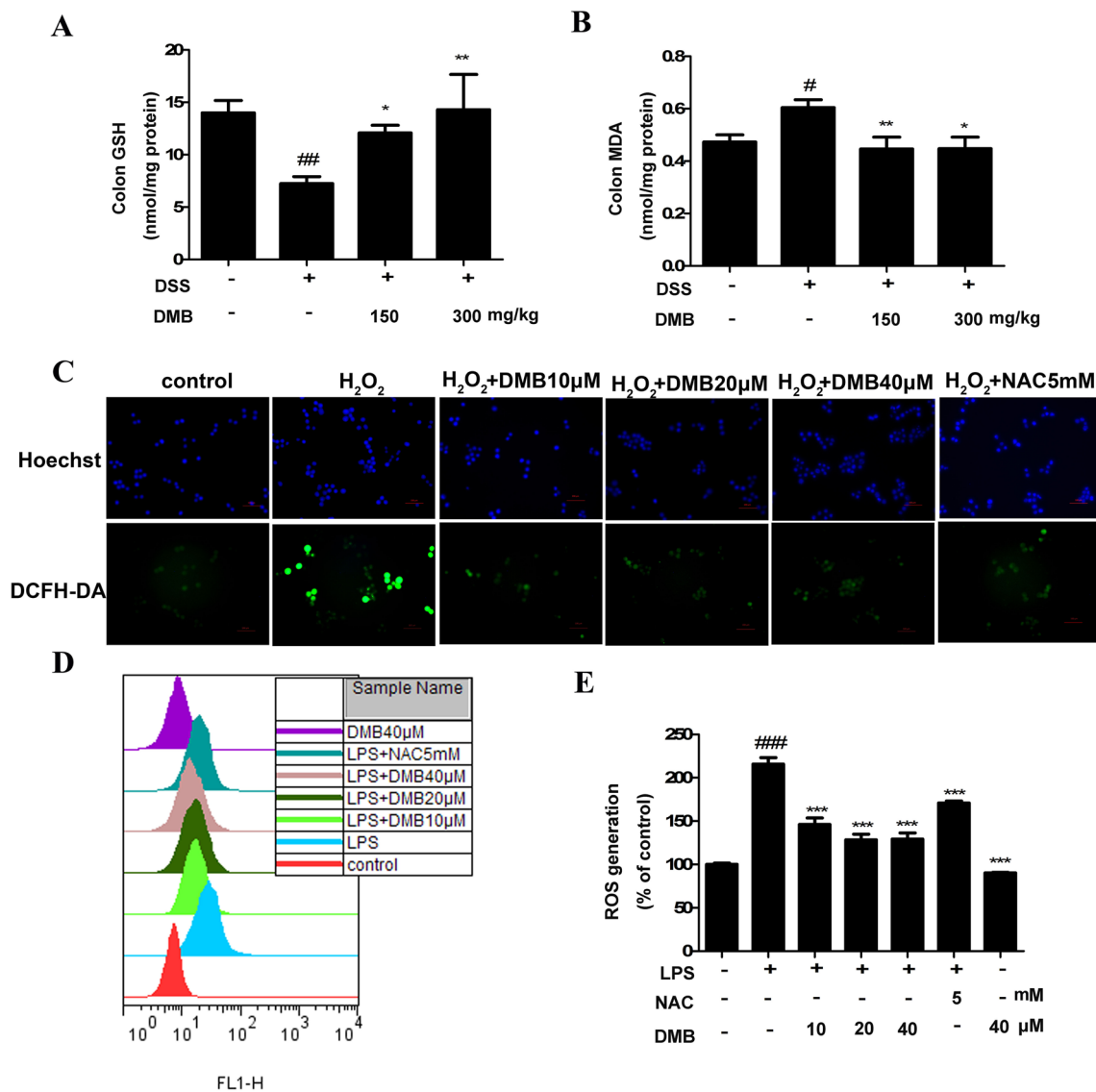


Fig. 4 Effect of DMB on the oxidative damage in colon and ROS in the vitro experiment. GSH and MDA were measured in colon homogenates. The results were shown in (a) and (b) respectively. The ROS productions in RAW264.7 cell were measured by fluorescent microscope with Hoechst and DCFH-DA (c). The ROS generations

induced by LPS in the RAW264.7 cell was calculated with the flow cytometry (d, e). Values were shown as the mean ± SEM of five independent tests. *, # < 0.05, **, ## < 0.01 and ***, ### < 0.001. **p* versus control group and #*p* versus LPS/DSS group

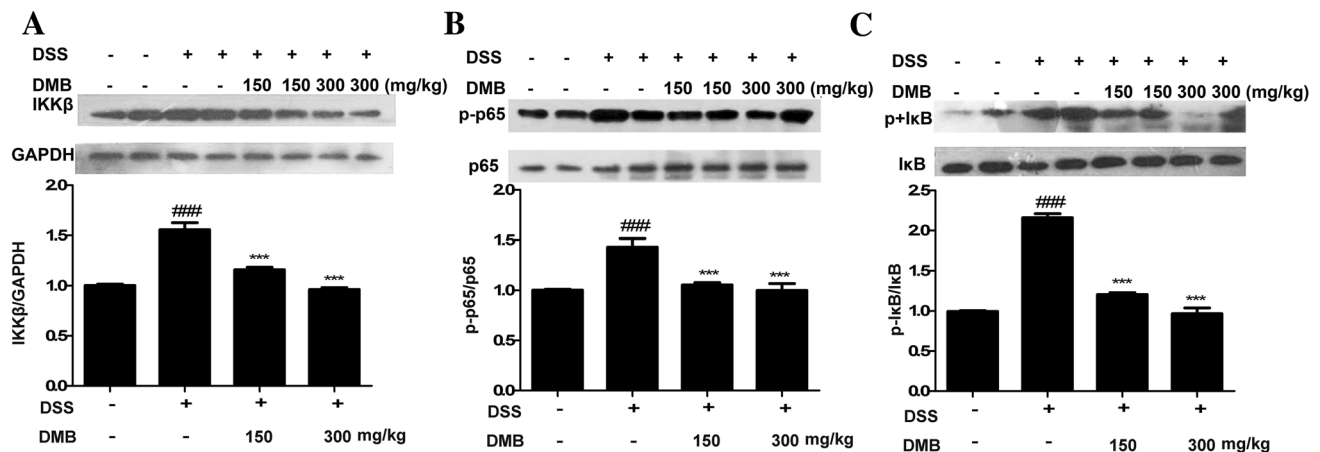


Fig. 5 Effect of DMB on the activation of NF- κ B signaling in mice colon. **a–c** The key protein expressions of NF- κ B signaling were examined by Western blotting, including IKK β , p65 and p-p65, I κ B and p-I κ B. The relative densities of western blotting band were

compared with the GAPDH band in each group. *, # <0.05, **, ## <0.01 and ***, ### <0.001. **p* versus Control group and #*p* versus DSS group

DMB treatment caused a dose-dependent decrease in the key proteins expression of NF- κ B signaling pathway in colon tissues, including IKK β , p-p65 and p-I κ B (Fig. 5a–c). In addition, the expression of I κ B protein increased after DMB treatment (Fig. 5c).

Effect of DMB on the production of inflammatory cytokines in mice and RAW264.7 cell

The DSS-induced acute colitis is well-characterized by increased epithelial injury and production of inflammatory cytokines [9]. Intestinal bacterial endotoxin LPS penetrate the epithelial barrier and directly stimulate the mucosal immune system [28]. The results demonstrated elevated levels of pro-inflammatory cytokines production, including TNF- α , IL-6 and NO in the colon and TNF- α , IL-6, IL-1 β and NO in the RAW264.7 cell, were reduced significantly (Fig. 6a–b). Consistent with the increased content of cytokines in the cell supernatant, stimulation with 100 ng/ml LPS for 6 h caused a significant increase in the mRNA expression of pro-inflammatory cytokines (Fig. 6c). However, Pretreatment with DMB diminished the mRNA expression of pro-inflammatory cytokines, while it remarkably increased IL-10 expression in RAW264.7 cells in dose dependent (Fig. 6c).

Effect of DMB on the homeostasis of Th cells in splenocytes

To investigate the function of DMB on Th cells, we analyzed the effects of DMB on the production of cytokines such as IFN- γ and IL-4 in splenocytes. Mice in DMB group showed lower production of IFN- γ and higher production of IL-4 compared with mice in control group. Furthermore,

DMB regulated the production of IFN- γ , IL-4 and the ratio of IgG1/IgG2a (Fig. 7b–d). To the immune tissues, DMB treatment decreased the spleen weight, while increased the thymus weight (Fig. 7a).

Discussion

IBD is a chronic inflammatory disorder in the gastrointestinal tract. In our research, DMB showed a significant antioxidant effect in LPS/H₂O₂-stimulated RAW264.7 cell and DSS-induced mice. Furthermore, DMB inhibited NF- κ B signaling and helped homeostatic maintenance of Th cells in colitis mice. These findings indicate that DMB alleviates colitis through antioxidant effect and ultimately diminishing the inflammatory activity.

Most studies conducted in the last thirty years have focused on the role of adaptive immune responses in the pathogenesis of IBD. In particular, while CD has long been considered to be driven by a Th1 response, UC has been rather associated with a non-conventional Th2 response [29]. In mice, production of IgG1 versus IgG2a is widely interpreted as a reflection of differential Th2–Th1 reactivity [30]. To our surprise, we first found the imbalance of Th cells in the spleen of DSS-induced mice colitis. One of the characteristic macroscopic changes associated with DSS colitis is enlargement of the spleen. A study suggest a close correlation between spleen size and severity of DSS colitis [31]. In our results, oral administration of DMB obvious inhibited the spleen enlargement. In order to clarify whether treatment with DMB regulated the balance of Th cell, we analyzed cytokine production in the splenocyte. Contrary with previous study in the colon, our results showed that IFN- γ concentration was higher,

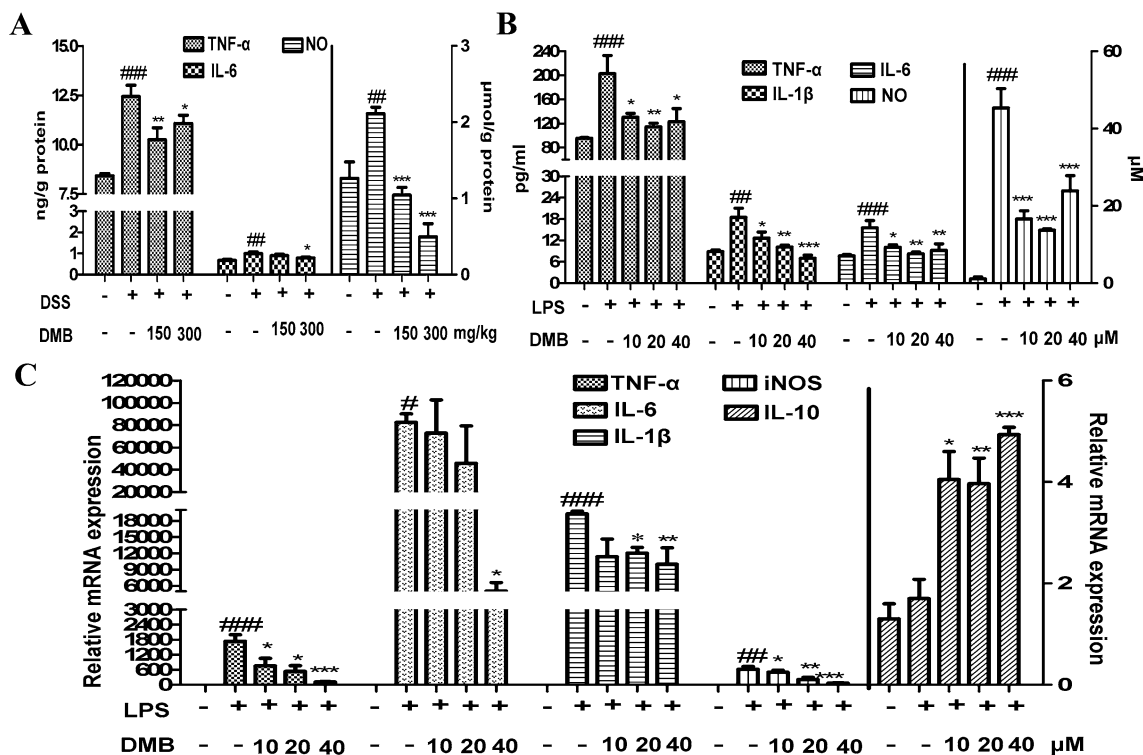


Fig. 6 Effect of DMB on the production of inflammatory cytokines in DSS-induced colitis mice and LPS-stimulated RAW264.7 cell. The pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β) in supernatants of colon tissues and RAW264.7 cells were collected and measured by ELISA, while NO expression measured by experiment kit (a, b). The

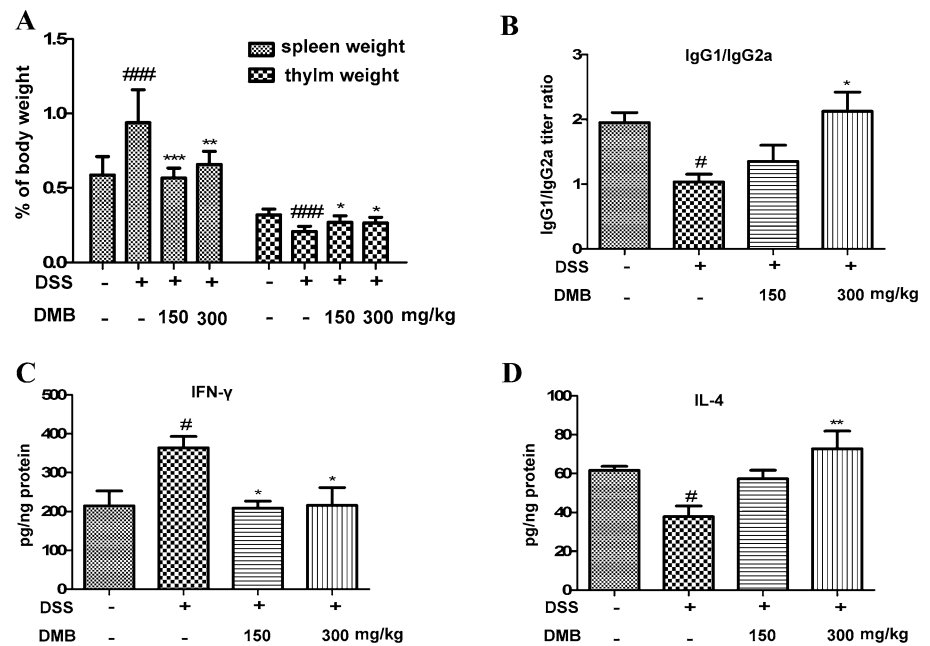
mRNA expression of pro-inflammatory cytokines, including TNF- α , IL-6, iNOS, COX-2 and IL-10, were measured by RT-PCR (c). Values were shown as the mean \pm SEM of five independent tests. *, # < 0.05, **, ## < 0.01 and ***, ### < 0.001. **p* versus Control group and #*p* versus LPS or DSS group

whereas IL-4 concentration was lower in mice spleen with DSS-induced colitis than that in control mice. We demonstrated that increased IFN- γ and decreased IL-4 could be reversed by treatment with DMB. Furthermore, DMB evoked dramatically higher IgG1/IgG2a ratios relating to increased activity of the Th2-pathway. These dates may suggest that DMB suppressed the Th1 responses and enhanced Th2 responses and prevented the unbalance of Th1 and Th2 in the spleen of DSS-induced colitis.

With an approximately 17% sulfur, DSS is associated with a marked increase in the measurable ROS and the activation of an inflammatory cascade [32]. Researchers have showed agents capable of eliminating reactive oxygen species ameliorate experimental colitis [33]. To ensure the antioxidant capacity of DMB in vitro, RAW264.7 cells were induced by LPS or H₂O₂. Of note, we found that pre-incubated DMB reduced ROS production in dose-dependent in LPS/H₂O₂-stimulated RAW264.7 cells. We also observed the same anti-oxidant and anti-inflammatory effect of DMB on the LPS/H₂O₂-induced human colonic epithelial cell HT29 (The results were not shown). Furthermore DMB was more effective than NAC at the concentration of 40 μ M in the RAW264.7 cell induced by LPS. The part reasons maybe LPS triggers the production

of ROS and promotes acute inflammation while NAC is known to act by raising intracellular concentrations of cysteine and scavenging ROS [34, 35]. Based on the antioxidant effect of DMB on macrophage and anti-diarrhea of CPC, we propose a hypothesis that DMB may alleviate acute mice colitis induced by DSS by ameliorating oxidant stress. Consistent with our vitro results, the current data show DMB plays a significant in antioxidant effect in colitis mice through marked decreased MDA content and increased GSH activity. DMB not only scavenges the production of ROS, but plays a crucial role to the reduction of inflammatory cytokines in the Raw264.7 cell. To all known, production of cytokines activate transcription factors such as NF- κ B [36]. It was also reported that ROS mediated activation of NF- κ B in macrophage and colitis [37]. In addition, inhibited NF- κ B helps the prevention of colitis [38]. Our date showed DMB reduced colon inflammation, such as neutrophil infiltration and multiple erosions, and inhibited the activation of NF- κ B signaling in colon in response to DSS-induced mice colitis. As NF- κ B is the down signaling of TLR4 receptor [39, 40], DMB treatment down regulated the expression of TLR4 in mice colon (Fig. S6 a, b). Although we still did not know the time course of activation of NF- κ B in colitis mice, the

Fig. 7 Effect of DMB on the weight of thymus and spleen and disturbance in splenocytes. The test agents were orally administered once every day for 7 days prior to DSS treatment. The mice were anesthetized with ether and killed 3 days after DSS treatment. The spleen and thymus were isolated and weighed, meanwhile splenic lymphocytes were collected, and IFN- γ , IL-4, IgG2a and IgG1 levels were measured by ELISA. The results were shown in (a), (c) and (d) respectively. The ratio of IgG2a and IgG1 was calculated and shown in (b). *, # <0.05, **, ### <0.01 and ***, ### <0.001. **p* versus control group and #*p* versus LPS



time course of p-p65 expression and inflammatory cytokines expression in RAW264.7 cells were explored (Figs. S2, S3a, b). Consistent with the vivo results, DMB decreased the nuclear transcript of p65 (Fig. S4a, b) and the expression of inflammatory cytokines in Raw264.7 stimulated by LPS significantly, such as TNF- α , IL-6, IL-1 β , NO and so on. As DMB was absorbed in cells quickly with the HPLC analysis (Table S1, Fig. S5a–e), we also researched the toxicity in mice (Table S2) and tissue distribution of DMB in rat (Tables S3, S4, Fig. S7a–d). The data indicated that DMB distributed in the brain and lung as short as 5 min, and then mainly accumulated in the liver at the time of 30 min (Fig. S7a–d). In the toxicity experiment, part of mice appeared with convulsions, shortness of breath, dark limbs and tail skin immediately and then died within 5–10 min. However, no abnormal organs were found with the naked eye observation.

In summary, our data first indicated that DMB exerts therapeutic effect on DSS-induced mice colitis. The potential mechanisms may contribute to inhibiting oxidant stress and NF- κ B signaling pathway, as well as partly regulating the balance of Th1 versus Th2 in spleen. These findings provide new point on DMB and its possible use in intestinal inflammatory disorder.

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Compliance with ethical standards

Conflict of interest There are no competing interests to declare.

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