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Gallic acid improved inflammation *via* NF-κB pathway in TNBS-induced ulcerative colitis

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ABSTRACT

Gallic acid (GA), as an active component, has been found in many fruits and plants, and it exhibits potential protective effects, such as anti-inflammatory, antioxidant, antiviral and anticancer. However, the effects of GA on ulcerative colitis (UC) remain unknown. The purpose of this study was to investigate the effects of GA on IL-1β-induced HIEC-6 cells and TNBS-induced UC in mice. Various biochemical analyses including proliferation and apoptosis were assessed in HIEC-6 cells. In addition, body weight of mice, the level of cytokines and histological changes were utilized to analyze the GA protecting mice with UC.

Our results showed that administration of GA significantly increased the expressions of IL-4, and IL-10, while down-regulated IL-1, IL-6, IL-12, IL-17, IL-23, TGF-β and TNF-α expressions compared with a model control group *in vitro* and *in vivo*. Moreover, flow cytometry and TUNEL analysis revealed that administration of GA significantly inhibited the apoptosis of HIEC-6 cells and mics in UC. Furthermore, pretreatment with GA obviously reversed the decrease in body weight, increase in colon weight, and attenuated the histological changes derived from UC. In addition, western blot analysis demonstrated that GA efficiently suppressed NF-κB signaling pathway in TNBS-induced UC. In conclusion, the findings of this study demonstrated that GA plays an anti-inflammatory role in UC *via* inhibiting NF-κB pathway.

1. Introduction

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD) and is presented as a chronic and relapsing inflammatory disease that affects the mucosal layer of the colon and rectum. UC is a common IBD, which is characterized by abdominal pain, bloody purulent stool, mucosal ulceration and recurrent diarrhea [1,2]. An increasing incidence has been reported that UC affects millions of people worldwide especially in China [3,4], which is associated with diet and lifestyle.

The pathogenesis of UC is incompletely understood. It is currently proposed that the pathogenesis of UC involves complex interactions of multiple factors, including inappropriate immune response to microbes in the gut and genetic, undetermined environmental and psychological factors [5,6]. Furthermore, it is remarkable that increased secretion of proinflammatory cytokines and intestinal immune system dysregulation may be the principal pathogenesis of UC.

The NF-κB pathway plays key roles in many physiological and pathological processes [7,8]. The NF-κB can be activated by various stimulus mediators, including chemokines, cytokines, bacterial products and adhesion molecules [9,10]. Following cell stimulation, IκBs are rapidly phosphorylated which mediated by IκB kinase 2 (IKK2) [11,12]. Then NF-κB heterodimers are released from the NF-κB/IκB complex and translocated to the nucleus which leads to a wide range of gene expression. Thus, targeting NF-κB pathway holds promise novel antiinflammatory therapies for UC.

Gallic acid (GA), as an active component, has been found in many fruits and plants such as strawberries, bananas, sumac, gallnuts and black tea [13–16]. There are several reports of GA exhibiting potential protective effects, such as anti-inflammatory, antioxidant, antiviral and anticancer [17–25]. To date, there are very few reports about the effects of GA on UC. Therefore, the aim of this study was to evaluate the effects of GA on human intestinal epithelial cell HIEC-6 *in vitro* and mice *in vivo*.

2. Material and methods

2.1. Induction of UC using TNBS and drug administration

UC was induced by 2, 4, 6-trinitrobenzene sulfonic acid (TNBS).

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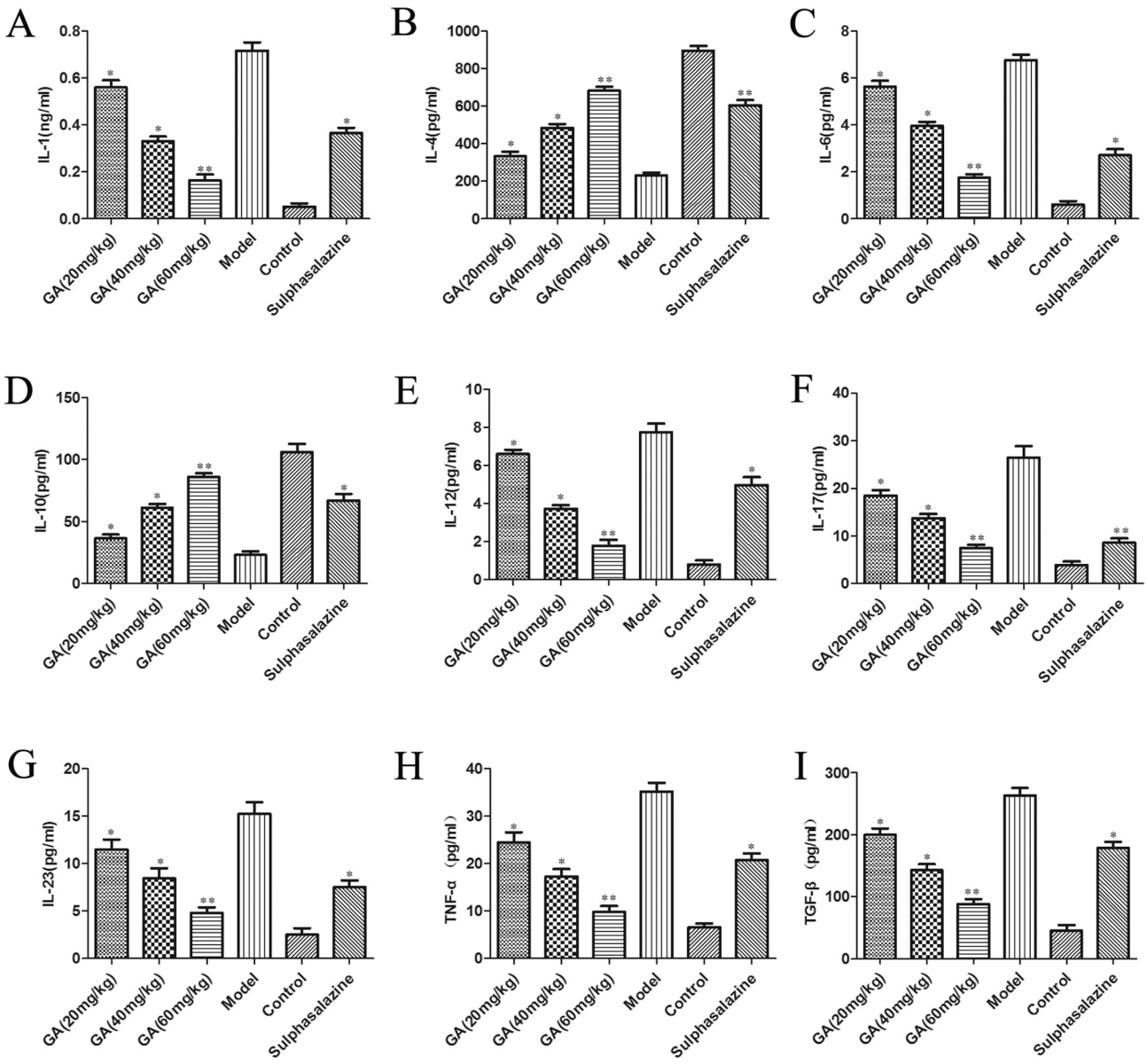

Fig. 1. Effects of GA on cytokine production in cells. (A–J) The levels of pro-inflammatory cytokines and anti-inflammatory cytokines were assessed by ELISA. *P < 0.05, **P < 0.01 *vs.* the model group.

Briefly, TNBS was dissolved in 50% ethanol and instilled into the lumen of the colon using medical-grade rubber in fusion tube which was positioned 8 cm into the anus. The mice were kept vertically for 5 min after administration of TNBS in order to ensure that TNBS entered entire colon. A total number of 60 male 6–7-weeks Balb/c mice were randomly allocated into six groups, A: model group (TNBS induced colitis without treatment), B: control group, C: GA (20 mg/kg) treated group (TNBS + GA group), D: GA (40 mg/kg) treated group (TNBS + GA group), E: GA (60 mg/kg) treated group (TNBS + GA group) and F: sulphasalazine treated group (TNBS + sulphasalazine group). As control, the mice were treated with physiological saline with an equal volume instead of the TNBS solution. For drug administration, 1% (w/v) GA at a dose of 20 mg/kg, 40 mg/kg or 60 mg/kg was administrated to mice in group C, D, E twice daily for another 7 days by intragastric injection.

2.2. Cell culture and ulcerative colitis induced by IL-1β

The HIEC-6 cells were procured from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere with 5% CO_2 at 37 °C.

The cells were plated to six well plates and cultured for 24 h. Then, these cells were stimulated with IL-1β (10 ng/mL, Peprotech, US) to produce a UC model. After stimulation for 1 h, the cells were treated with IL-1β, GA (20 mg/mL), GA (40 mg/mL), GA (60 mg/mL) and sulphasalazine and incubated at 37 °C for 48 h. The cells without any treatment were used as control. After that, cells were washed and collected for further proliferation, cell cycle and apoptosis analyses.

2.3. Cell viability assay

The proliferation of HEC-6 cells was measured using MTT and colony formation. 1×10^4 cells were seeded into 96-well plates for 24 h and subsequently treated with different concentrations of GA for 24, 48, 72 or 96 h. MTT was added to each well and the cells were incubated for 4 h. The absorbance value of each well was performed at the wavelength of 490 nm. For colony formation assay, cells were seeded in a

10-cm dish and maintained in complete culture medium. After 7 days, cells were fixed with methanol and subsequently stained with 0.1% crystal violet. The colonies were counted manually.

2.4. Western blot

Proteins were extracted in RIPA lysis buffer. Equal amounts of proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and blocked in 5% milk with PBS at room temperature for 2 h. The membranes were incubated with the primary antibodies at 4 °C overnight, followed by washing three times with 1 × TBST. Then the membranes were incubated with secondary antibodies at room temperature for 1 h. The signals were visualized by ECL system (Amersham Pharmacia). The densities of the bands were analyzed using ImageJ software. Each experiment was repeated at least three times.

2.5. ELISA for cytokines

Colonic tissue specimens of mice were homogenized in PBS containing protease inhibitors. After centrifugation, the culture supernatants were collected. The levels of cytokines including IL-1, IL-4, IL-6, IL-10, IL-12, IL-17, IL-23, TNF-α and TGF-β were determined with ELISA kit (R&D Systems, USA). The absorbance was measured at 450 nm.

2.6. Histological studies

The colon tissues were fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin (H&E) and observed under light microscopy at 200 × magnification. The criteria for injury score was determined by damage scores which were as follows: 0, no damage; 1, indicating mild (≤25%); 2, moderate (26%–50%); 3, severe (51%–75%) and 4, extremely severe (≥75%). Five fields were randomly selected of each sample.

2.7. Immunohistochemistry

Immunohistochemical studies were used to detect the expressions of p-IkBα, IkBα, p-NF-κB (p-p65) and NF-κB (p65). The tissues were fixed in 4% paraformaldehyd and rehydrated in gradient concentration of ethanol (100–50%). Endogenous peroxidases were removed by incubation with 0.3% H_2O_2 for 10 min at room temperature. Afterwards, the tissues were blocked with bovine serum albumin (BSA). The tissues were incubated with primary antibodies for 30 min at 37 °C in a wet chamber, washed three times with TBS and then incubated with the secondary antibody for 2 h at room temperature. The tissues were stained with DAB and counterstained with hematoxylin.

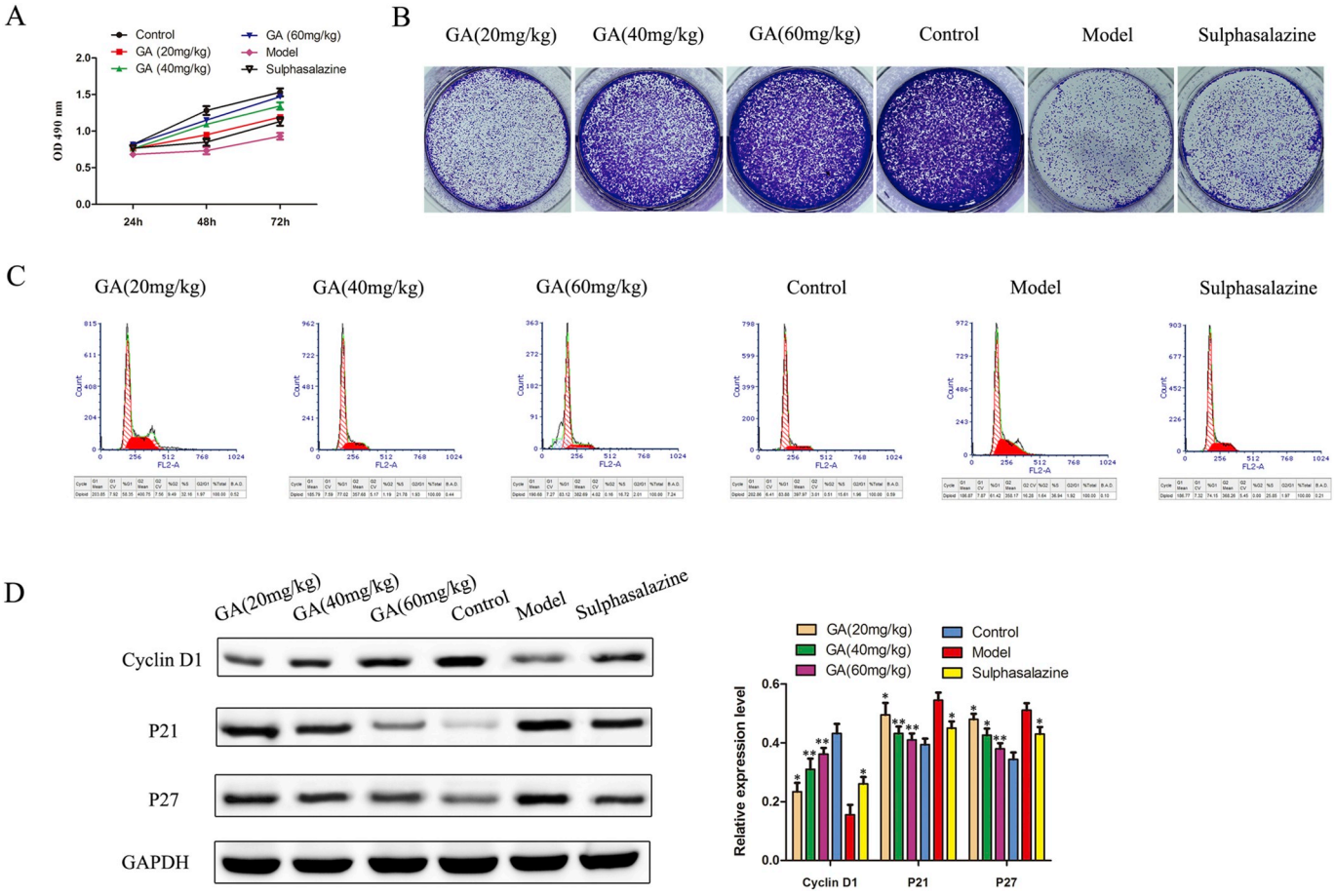

Fig. 2. GA promoted the proliferation of HIEC-6 cells. (A, B) The growth of cells was suppressed by GA. (C) Cell-cycle analysis showed that cells treat with GA had decreased numbers of cells in S phase. (D) Western blot analysis for the protein levels of Cyclin D1, p21 and p27 in all groups. GAPDH was used as an internal control. * $P < 0.05$, ** $P < 0.01$ compared with the model group.

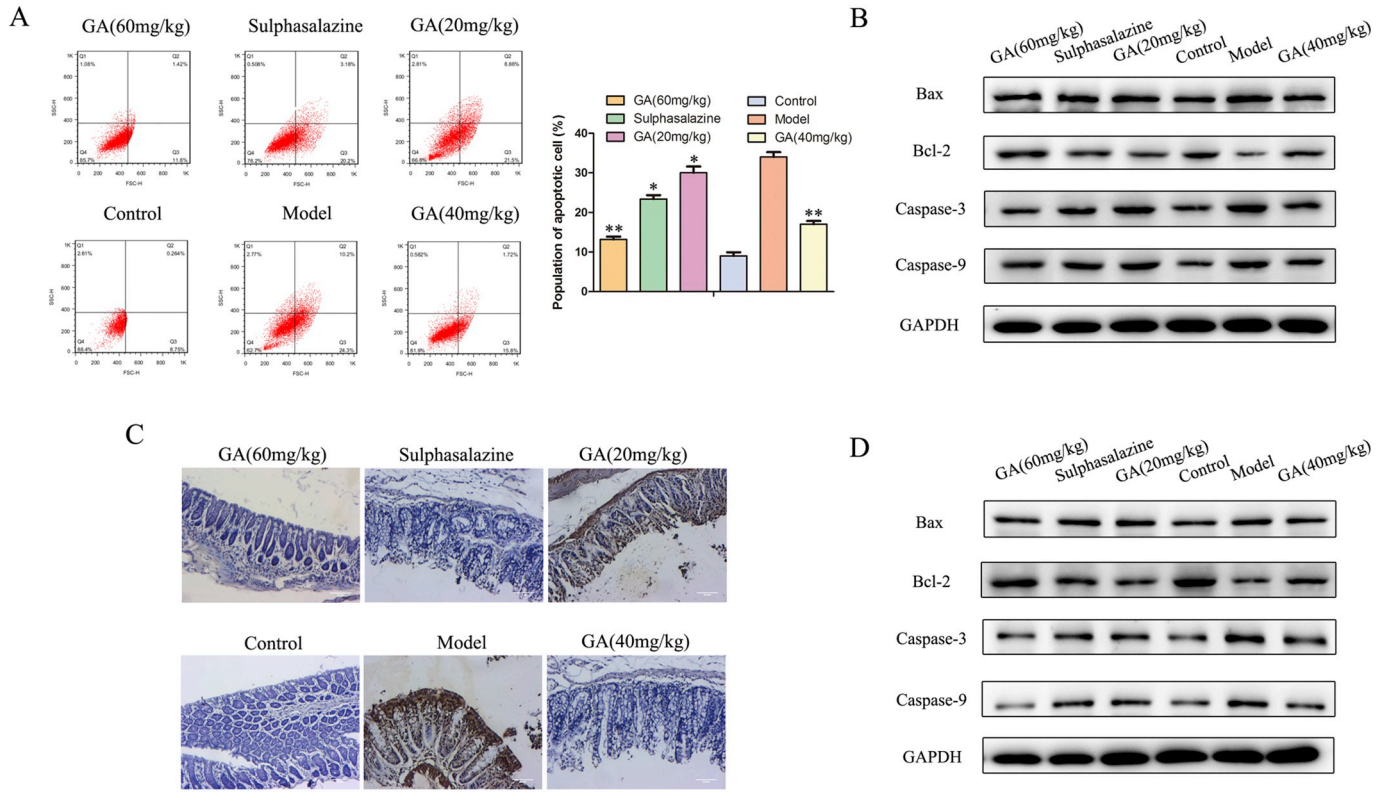

Fig. 3. GA reduced apoptosis *in vitro* and *in vivo*. (A, C) Apoptosis was analyzed by flow cytometric analysis and TUNEL assay. (B, D) Western blot analysis was performed to analyze the protein expression. The data are presented as the means ± SD, and P-values were calculated using Student's *t*-test. * $P < 0.05$, ** $P < 0.01$ compared with the model group. The representative pictures were taken at 200× magnification of microscopic field from each group. The scale bar represents 50 μm.

2.8. Statistical analysis

All the data are presented as the mean ± standard. Statistical analysis was performed with one-way ANOVA using SPSS statistical software 19.0 (Chicago, IL, USA). All the experiments were performed in triplicate. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of GA on inflammation in vitro and in vivo

To investigate the potential anti-inflammatory effects of GA on UC *in vitro* and *in vivo*, we employed the IL-1β and 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis model and treated with GA. We assessed the levels of pro-inflammatory and anti-inflammatory factors, including IL-1, IL-4, IL-6, IL-10, IL-12, IL-17, IL-23, TNF-α and TGF-β in cells and colon tissues. As shown in Figs. 1 and 6, the levels of pro-inflammatory factors IL-1, IL-6, IL-12, IL-17, IL-23, TGF-β and TNF-α were significantly increased, while the anti-inflammatory factors IL-4 and IL-10 were decreased in the model group relative to those in the control group. However, there was a significant reduction of pro-inflammatory in the GA treatment group and the sulphasalazine group. Besides, the anti-inflammatory cytokines were markedly induced when treated with GA. All these experiments show that GA reduces release of proinflammatory cytokines while increases release of anti-inflammatory cytokines. Therefore, these results confirmed that GA have anti-inflammatory effects on TNBS-induced UC.

3.2. GA induced the viability of intestinal epithelial cells

To determine anti-proliferative effects of GA on intestinal epithelial cells, MTT and clone formation assays were performed. We observed that GA significantly increased cell viability as compared with model group at 24, 48, 72 and 96 h (Fig. 2A). The function of GA for proliferation in HIEC-6 cells was further confirmed by colony formation assay (Fig. 2B). In addition, flow cytometry was used to detect cell cycle distribution in HIEC-6 cells and we observed that the GA altered the cell cycle by decreasing S-phase significantly (Fig. 2C). Moreover, we found that p21 and p27 protein expressions were decreased in the GA group than the model group (Fig. 2D). From these results, it could be concluded that GA has concentration-dependent proliferative function in UC.

3.3. GA reduced apoptosis in cells and colon of UC

We used flow cytometry analysis to further characterize the positive or negative effects of GA on cell apoptosis. As shown in Fig. 3A, when HIEC-6 cells were treated with GA, the number of apoptotic cells was visibly decreased compared with the model group. Next, we examined the consequence of GA on colon apoptosis in TNBS-induced UC using TUNEL assay. We found that percentage of TUNEL positive cells significantly decreased in the GA group compared with the model group (Fig. 3C). The apoptosis-related protein expressions were detected by western blot. As shown in Fig. 3B and Fig. 3D, the expressions of Bax, Caspase-3 and Caspase-9 in HIEC-6 cells treated with GA were

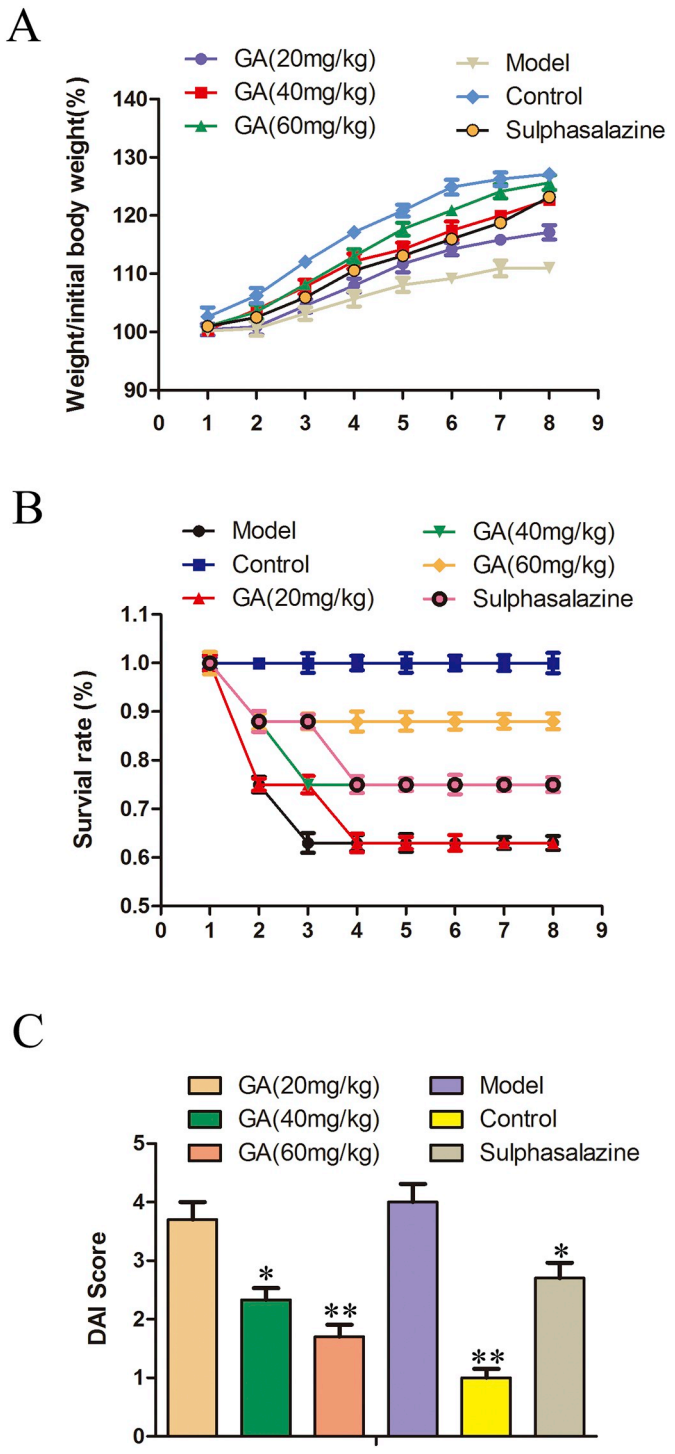

Fig. 4. Effect of GA on clinical signs in TNBS-induced colitis. (A, B) Effect of GA on survival rate and body weight in mice. (C) Disease activity index score in the six groups. * $P < 0.05$, ** $P < 0.01$ compared with the model group.

obviously decreased compared with the model group. On the contrary, Bcl-2 expression was obviously increased. Similar results were obtained in colon tissues. These results indicated that GA plays important roles in regulating apoptosis in UC.

3.4. GA improved clinical symptoms in TNBS-induced UC of mice

In order to investigate the effects of GA on TBNS-induced UC, survival was assessed. As shown in Fig. 4B, the survival rate in the model group was lower than that in the GA group. The mice in the control group had a normal diet and their body weight increased during experiments. TNBS caused loss of body weight of mice. However, GA had a protective effect on UC induced weight loss (Fig. 4A). As shown in Fig. 4C, the administration of GA to mice resulted in an increased disease activity index (DAI) compared with the model group.

3.5. Effect of GA on histological alteration

All colons were collected and we further measured the length and weight of colon tissues. Compared with the control group, the length of colons in the model group was shortened. The colons in the GA group were less shortened in length and less reduced in weight compared with the model group (Fig. 5A, B). The mouse treated with 60 mg/kg GA seems to be smaller than other mice in Fig. 5A owing to the fact that the mice were positioned sideways during taking this photo. In fact, the body weight of mice treated with 60 mg/kg GA was increased. Furthermore, we confirmed the anti-inflammatory effects of GA at the histological level by H&E staining (Fig. 5C). GA treatment showed that colonic inflammation was dramatically reduced compared with TNBS-induced colonic histological damage. Thus, these data indicated that GA plays a critical role in improving the damage in TNBS-induced colitis.

3.6. GA ameliorated UC by NF-κB signaling pathway

In order to investigate the possible mechanisms that GA attenuated TNBS-induced UC, we detected the p-IκBα, IκBα, p-NF-κB (p-p65) and NF-κB (p65) protein levels in colon using western blot and immunohistochemical assays. We have validated that GA treatment can inhibit the expressions of p-IκBα and p-NF-κB (p-p65) when compared with the model group (Fig. 7). Moreover, IκBα protein level had less extend of degradation, but NF-κB (p65) level seemed comparable. Taken together, GA ameliorates UC by regulating NF-κB signaling pathway.

4. Discussion

UC is an inflammatory bowel disease that represents clinicopathologic features of inflammation of colonic mucosa. Some evidences suggested that the imbalance of mucosal immune system may cause generation of inflammatory mediators, leading to lesions of the colonic mucosa, chronic inflammation, ulceration and edema [26,27].

The methods for attenuation of UC have many types, such as drugs, small interfering RNA, endocrine hormones and erythropoietin. However, these clinical applications were prevented by various drawbacks. It is necessary to find a safe, convenient and effective method to treat UC. Thus, the efficacy of GA against UC was explored in our study. To our knowledge, this study is the first to provide *in vitro* and *in vivo* evidence of the potential therapeutic value of GA in UC.

In the present study, we evaluated the effects of GA in HIEC-6 cells and a TNBS-induced mouse model of UC. We detected the serum levels of inflammatory cytokines using ELISA. The results showed that GA treatment could increase the levels of IL-4 and IL-10 and decrease the expressions of IL-1, IL-6, IL-12, IL-17, IL-23, TGF-β and TNF-α in HIEC-6 cells and in mice, in which UC was induced by IL-1β and TNBS. Apoptosis is a programmed cell death occurring in various

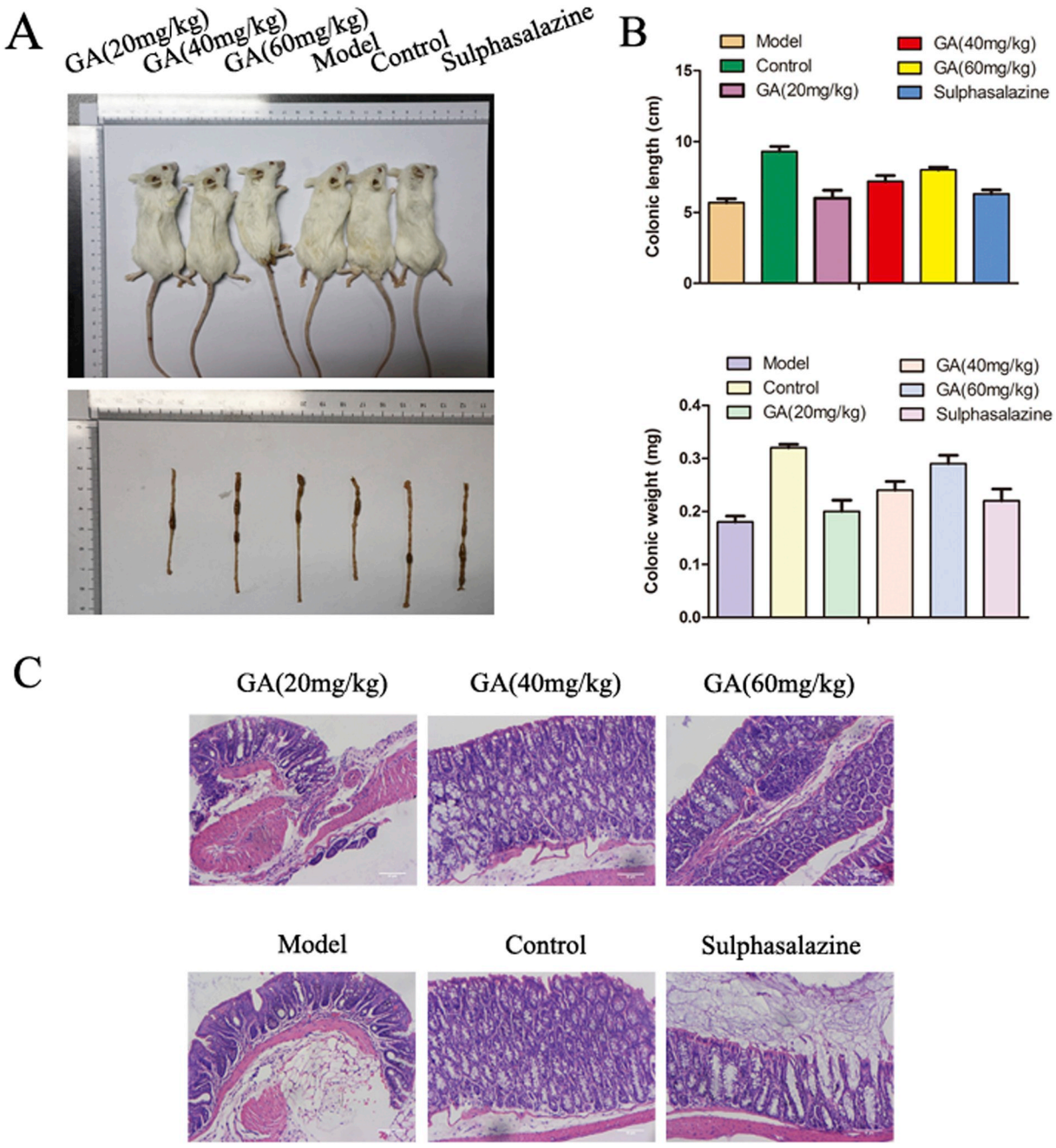

Fig. 5. Effect of GA on histological alteration. (A, B) Effect of GA on colon length and weight. (C) The histological level by H&E staining. The scale bar represents 50 μm. * $P < 0.05$, ** $P < 0.01$ compared with the model group.

inflammatory diseases including UC [28,29]. Moreover, in our study, we observed significantly reduced apoptosis in HIEC-6 cells and colons in GA pretreatment group compared with the model group using flow cytometry and TUNEL evaluation. These results indicated that the protective effects of GA against TNBS-induced colitis might be, at least in part, mediated by its anti-apoptotic effects. Furthermore, we have investigated the ameliorative effect of GA in the mouse model of TNBS-induced colitis by assessing histological change. The results presented that GA administration improves clinical symptoms and significantly attenuates the colonic inflammation.

NF-κB signaling is an important pathway that subsequently controls the expressions of proinflammatory cytokines and chemokines [30]. NF-κB signaling components are regulated by a number of molecules and play essential roles in cellular biological processes. IκBα is an important inhibitor for NF-κB. The degradation of IκBα is in response to certain pro-inflammatory cytokines and IκBa phosphoration

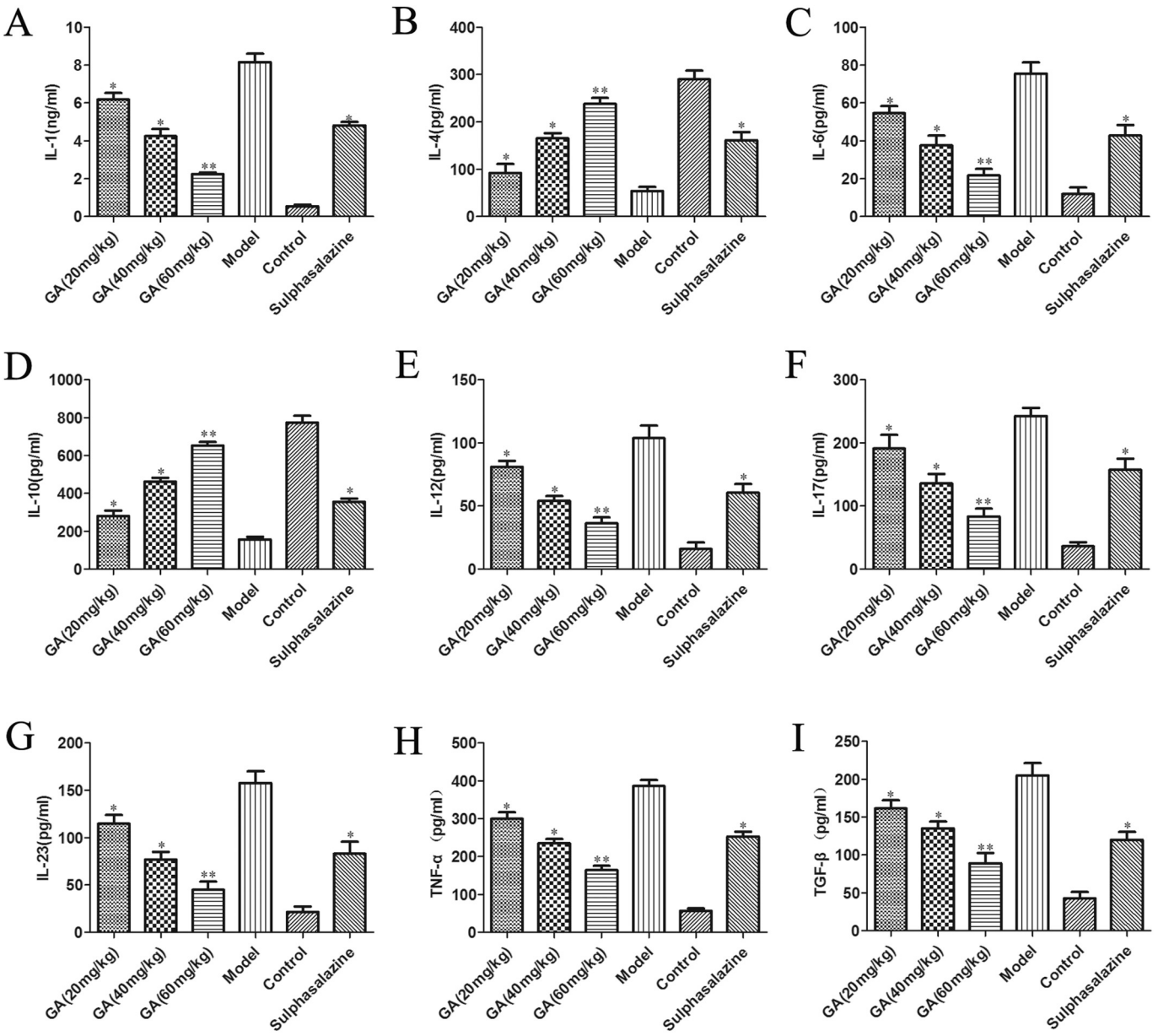

Fig. 6. Effects of GA on cytokine production in colon tissues. (A–J) The levels of pro-inflammatory cytokines and anti-inflammatory cytokines were assessed by ELISA. * $P < 0.05$, ** $P < 0.01$ compared with the model group.

consequentially results in activation of NF-κB signaling. Subsequently, phosphorylated NF-κB is translocated into the nucleus, following regulates the productions of inflammatory cytokines and chemokines [31].

Constant activation of NF-κB activity has been a well-proven molecular event for UC [32–34]. To characterize the potential mechanism regulated by GA in UC, we measured the NF-κB activation by western blot and immunohistochemical assays for p-NF-κB and p-IκB and found that pre-treatment with GA reduced the degradation of IκBα and nuclear translocation of NF-κB (p65), while increasing the expression of IκB and NF-κB. Our results suggested that GA inhibits NF-κB activation in UC.

Taken together, our study demonstrated that GA exhibited the protective effects on TNBS-induced colitis by inhibiting inflammation and induced apoptosis *via* the NF-κB pathway. Therefore, we propose that GA may contribute to the pathogenesis of UC owing to anti-inflammatory effects.

Conflict of interest

The authors have no conflict of interest pertaining to this manuscript.

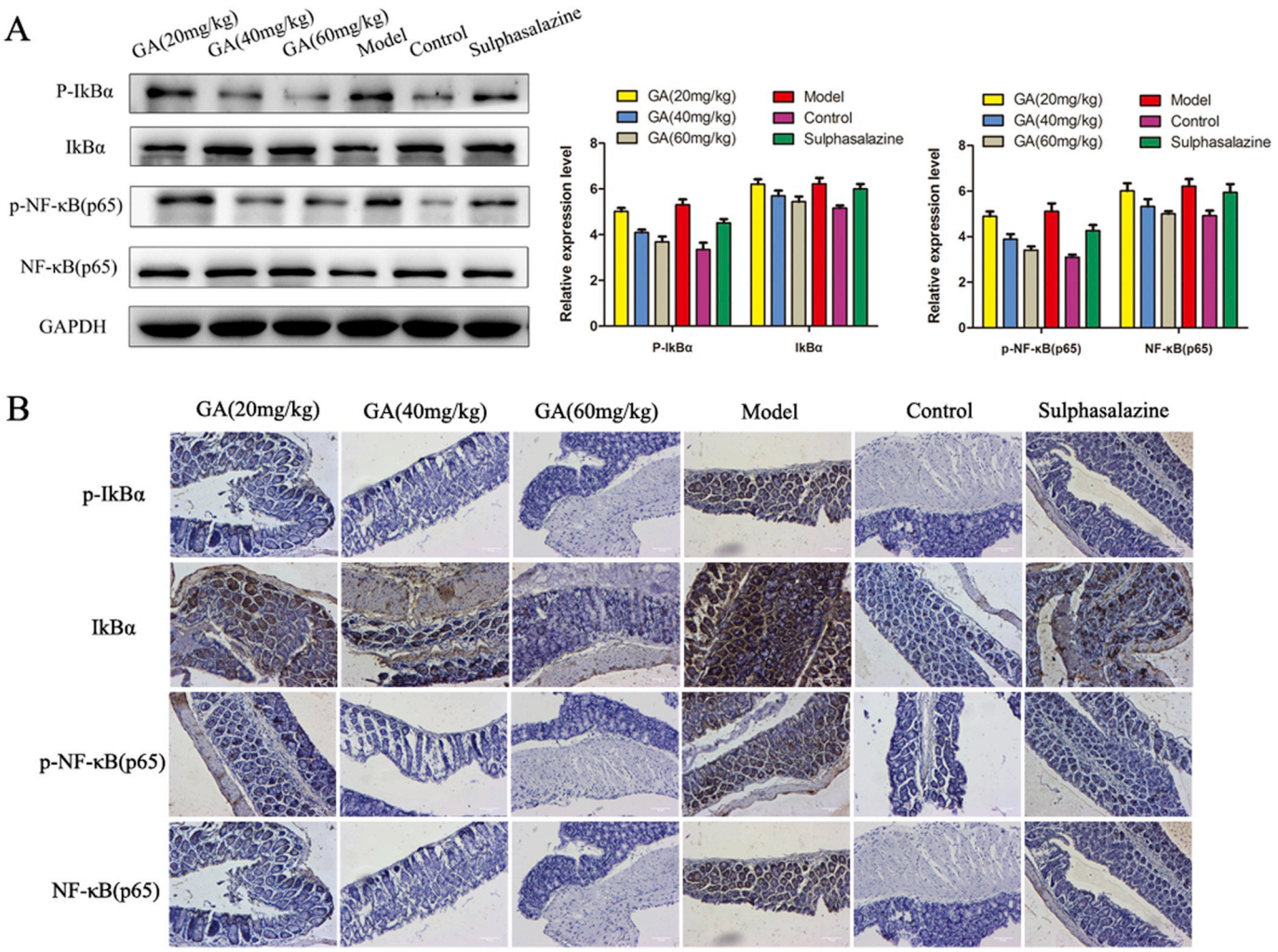

Fig. 7. GA ameliorated UC by NF-κB signaling pathway. (A, B) The protein expression of p-IκBα, IκBα, p-NF-κB(p65) and NF-κB(p65) using western blot and immunohistochemical analysis. The GAPDH was used the internal control. Data are presented as the mean of three independent experiments. The scale bar represents 50 μm. * $P < 0.05$, ** $P < 0.01$ compared with the model group.

Acknowledgements

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