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Regulating effect of baicalin on IKK/IKB/NF-kB signaling pathway and apoptosis-related proteins in rats with ulcerative colitis

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ABSTRACT

Objective: This study was aimed to explore effect of baicalin on IKK/IKB/NF-kB signaling pathway and apoptosis-related proteins in rats with ulcerative colitis (UC).
Methods: Histopathological observation and scores of colon tissue were performed in the UC rat model. IKK/IKB/NF-kB signaling pathway and apoptosis-related proteins were measured by Western blotting.
Results: Baicalin significantly increased the activity of SOD, CAT and GSH-Px in colon tissue of rats with UC, but significantly decreased the content of MDA, IL-1β, MPO, PEG2 and TNF-α in colon tissue of rats with UC. In the molecular mechanism, baicalin significantly decreased the expression of cleaved-caspase3, cleaved-caspase9, Bcl-2/Bax, cyt-c, NF-kB p-65, p-IKKβ/IKKβ and p-IKBα/IKBα. Baicalin could significantly inhibit p-IKBα/IKBα content change, but had no significant effect on p-IKKβ/IKKβ.
Conclusion: Baicalin may have a regulating effect on IKK/IKB/NF-kB signaling pathway and apoptosis-related proteins in UC rats.

1. Introduction

Ulcerative colitis (UC), also known as chronic non-specific UC, is an idiotypic inflammatory bowel disease and ulcerative lesion of the large intestine mucosa [1,2]. It is characterized by diarrhea, mucus pus and bloody stools and abdominal pain [3]. The pathogenesis of UC is complicated and related to the combination of various factors. As the research progresses further, it is considered that collective immune system disorders (such as cytokines, leptin and adiponectin) play an important role in UC [4–6]. There are genetic factors, environmental factors and microbial infections and other factors [7].

There is no effective cure for UC, but most patients can effectively control the condition through drug treatment. At present, aminosalicylic acid, glucocorticoids and immunosuppressive agents are commonly used in clinical practice, and incremental drug therapy strategies are often used [8–11]. The aminosalicylic acid is still the mainstay of UC treatment. Glucocorticoid has strong anti-inflammatory effect and rapid induction, but considering its adverse reactions and hormone dependence or resistance, clinical application should strictly control its indications and methods of use [12,13].

Baicalin, a flavonoid compound purified from the dry roots of *Scutellaria baicalensis Georgi*, has generally been used for the treatment of various allergic diseases [14]. Baicalin has been used for cancer therapy due to its multiple effects as an anti-cancer drug [15]. Baicalin is a promising antimycobacterial and anti-inflammatory agent which can be a novel candidate for the development of new adjunct drugs targeting host-directed therapy (HDT) for possible improved treatment [16]. Baicalin effectively suppresses the metastasis of breast cancer by reversing EMT, which may be mediated by down-regulation of 0205-catentin expression [17]. Baicalin induces apoptosis in human osteosarcoma cell through ROS-mediated mitochondrial pathway [18]. The mechanism for baicalin on abrogating experimental colitis was targeted inhibition of the TLR4/NF-κB pathway activation [19]. Baicalin could in vitro inhibit p-STAT4/STAT4 ratios, adjust p-STAT6/STAT6 ratios and related cytokines, thereby balancing the immunity and relieving inflammatory reactions of UC [20]. However, the mechanisms underlying the protective effects of baicalin on UC are still under investigation.

A rat model of UC was established in this study, and different doses of baicalin were administered, in order to explore effect of baicalin on IKK/IKB/NF-kB signaling pathway and apoptosis-related proteins in rats with UC.

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2. Materials and methods

2.1. Ethics statement

The study was approved and supervised by the Ethics Committee of Shuguang Hospital Affiliated to Shanghai Traditional Chinese Medical University. All protocols were conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. The animal experiments involved in this study were in compliance with the Laboratory Animal Management of National Animal Science and Technology Commission's Regulations.

2.2. Reagents and instruments

Baicalin was purchased from Sigma (572667, Sigma-Aldrich, St. Louis); RAW264.7 cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences; Bay 11-7082, EF-24, and LPS were purchased from sigma Co., Ltd. (USA); Primary antibodies of Caspase- 3. Caspase-9, Bcl-2, Bax, Cyt-c, NF-κB p-65, p-IKKβ, IKKβ, p-IKBα and IKBα, and fluorescent secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The BCA protein concentration assay kit was purchased from Sangon Biotech Bioengineering Co., Ltd. (Shanghai, China). The HE staining kit and the Masson staining kit were purchased from Beyotime Biotechnology Co., Ltd.

2.3. Preparation, grouping and administration of animal model

50 SPF male SD rats weighing 180–220 g were purchased from Nanjing Junke Biological Engineering Co., Ltd. After 7 days of adaptive feeding in a room temperature (25 °C) environment, constant humidity (50% ± 5%), rats were randomly divided into 5 groups (10 in every group): control group, model group, low-dose group (BA-L, 30 mg/kg of baicalin), medium-dose group (BA-M, 60 mg/kg of baicalin) and high-dose group (BA-H, 90 mg/kg of baicalin). The UC rat model was established as follow. Rats were fasted for 24 h before modeling, but were free to drink water. After mild anesthesia with ether, 20 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS) was dissolved in 0.8 ml of 50% ethanol, the mixed solution was slowly injected into 7–8 cm of the proximal end of the descending colon, and the rats were kept in the vertical position for 60 s. The rats in the blank group were clysis with the same amount of physiological saline in the same manner. On the second day of modeling, the groups were intragastrically administered; the baicalin groups were given the corresponding dose of baicalin solution for 4 weeks; the blank group and the model group were given the corresponding dose of tap water.

2.4. Determination of relevant biochemical indicators

After the rats were anesthetized with ether, the rats were sacrificed by decapitation, and the colon tissue was quickly taken. The tissue was homogenized by adding 10% of the homogenate of tissue homogenate to the homogenate medium at 4 °C sterile physiological saline, and thoroughly ground under ice bath. Centrifugation was performed at 4 °C, 3500 rpm/min for 10 min, the supernatant was taken, and then the SOD, GSH-Px, CAT activity and MDA levels in the colon tissue homogenate were measured, and all operations were performed according to the kit instructions.

2.5. Determination of IKK/IKB/NF-kB signaling pathway and apoptosis-related proteins

The appropriate amount of colon tissue was weighed, and ground with liquid nitrogen. The tissue protein lysate in a ratio of 10:1 was added, and then a broad spectrum of phosphatase inhibitors (1:100) and PMSF (1:100) was added. It was placed on an ice bath for 2 h, and was continuously shaken. The supernatant was centrifuged (4 °C, 3500 rpm, 10 min), and the protein concentration was measured by BCA method. The corresponding sample volume was calculated and the corresponding buffer salt was added. It was heated denature for 10 min, SDS-PAGE electrophoresis was performed, the protein was transferred to PVDF membrane after electrophoresis, and blocked with 5% fetal bovine serum (BSA) for 2 h. The primary antibodies were added, and incubated overnight at 4 °C. The membrane was washed with TBST for 5 min × 3 times. Then, a fluorescent secondary antibody was added for exposure, and the relative gray value of the target protein was analyzed using Image Studio software.

2.6. RAW264.7 cell culture

RAW264.7 cells were cultured in a cell culture incubator at 37 °C, 5% CO_2 and saturated humidity using a cell culture medium. The culture medium was replaced once every 2–3 days. After the cells grew to 80–90% of the bottom area of the bottle, they were digested with 0.25% trypsin and subcultured, and the cells with good growth in the log phase were taken for subsequent experiments. The cells were seeded at different concentrations in a 96-well plate. After the cells were attached to the wall, the culture solution was discarded. Total of 200 μl culture solution with different concentrations of LPS (50, 100, 200, 300, 400, 500, 700, 1000 ng/ml) were added to each well, and incubated for 24 h, 48 h, and 72 h, respectively. The control group was added with 200 μl of the culture medium without LPS. After the completion of the culture, the culture solution was discarded, 100 μl of fresh culture solution was added, and 10 μl of CCK solution was added to each well. The plate was incubated in an incubator for 2 h, placed in a microplate reader, and the absorbance was measured at 450 nm. Cell viability = absorbance value of the administration group / absorbance value of the control group × 100%.

2.7. Statistical analysis

Data was presented in (mean ± standard deviation) and analyzed by SPSS 20.0. Student's *t*-test was used to compare differences between two groups. One-way ANOVA with Duncan's post-hoc test was used for comparing multiple groups. $P < 0.05$ indicated that difference was statistically significant.

3. Results

3.1. Effect of baicalin on oxidative stress related indexes in rats with UC

The results showed that the activities of SOD, CAT and GSH-Px in the model group were significantly lower than that in the control group, and the content of MDA was significantly increased, indicating that the rats in the model group were in the state of peroxidative stress. When the baicalin was administered, the SOD, CAT, GSH-Px activities were all increased and MDA content was decreased in the medium-dose group and the high-dose group, indicating that baicalin had a protective effect on oxidative stress injury in rats of UC model (Fig. 1).

3.2. Effect of baicalin on the contents of PEG2, MPO, IL-1β and TNF-α in colon tissue of rats with UC

As shown in Fig. 2, compared with the control group, the contents of PEG2, MPO, IL-1β, and TNF-α in the colon tissue of the model group were significantly increased ($P < 0.05$). After administration of baicalin, the contents of PEG2, MPO, IL-1β and TNF-α in colon tissue the administration group were significantly decreased ($P < 0.05$), indicating that baicalin had anti-inflammatory effects.

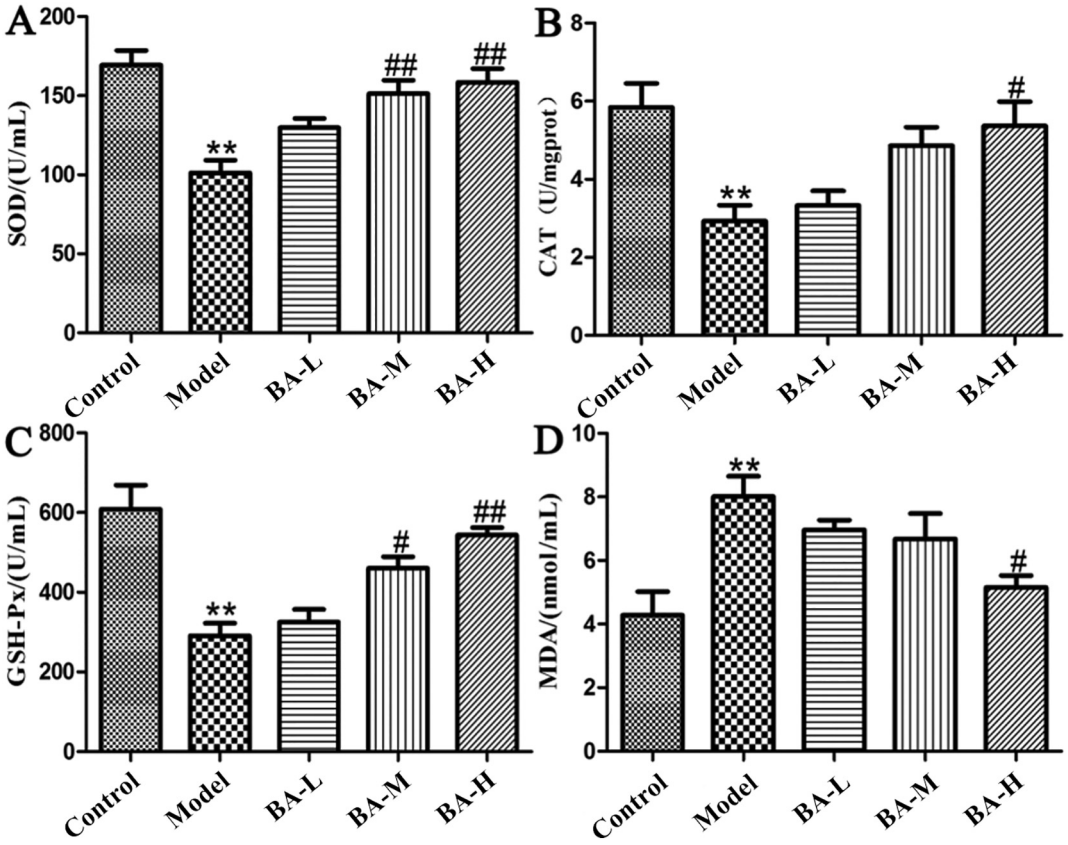

Fig. 1. Effect of baicalin on SOD (A), CAT(B), GSH-Px(C) activities and MDA(D) content in colon tissue of rats with UC. ** was $P < 0.01$ versus (vs) control group, # was $P < 0.05$ and ## was $P < 0.001$ vs model group.

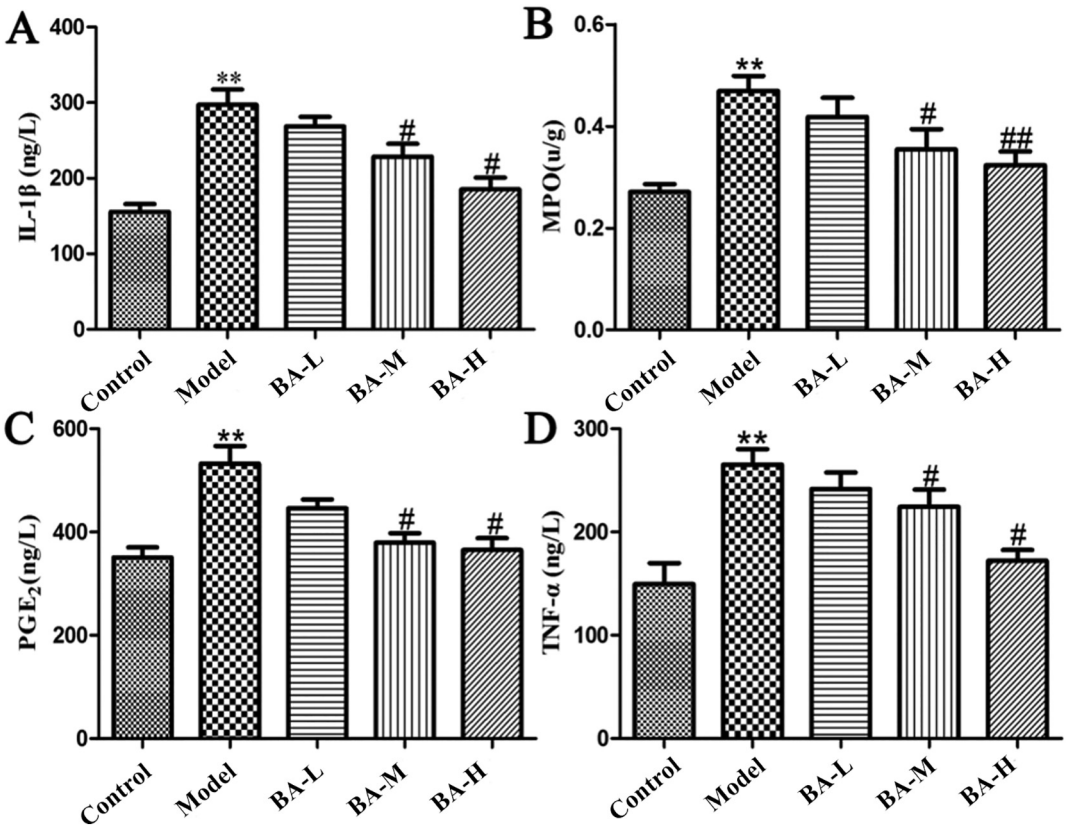

Fig. 2. Effects of baicalin on the contents of IL-1β (A), MPO(B), PEG_2(C) and TNF-α(D)in colon tissue of rats with UC. ** was $P < 0.01$ vs control group, # was $P < 0.05$ and ## was $P < 0.001$ vs model group.

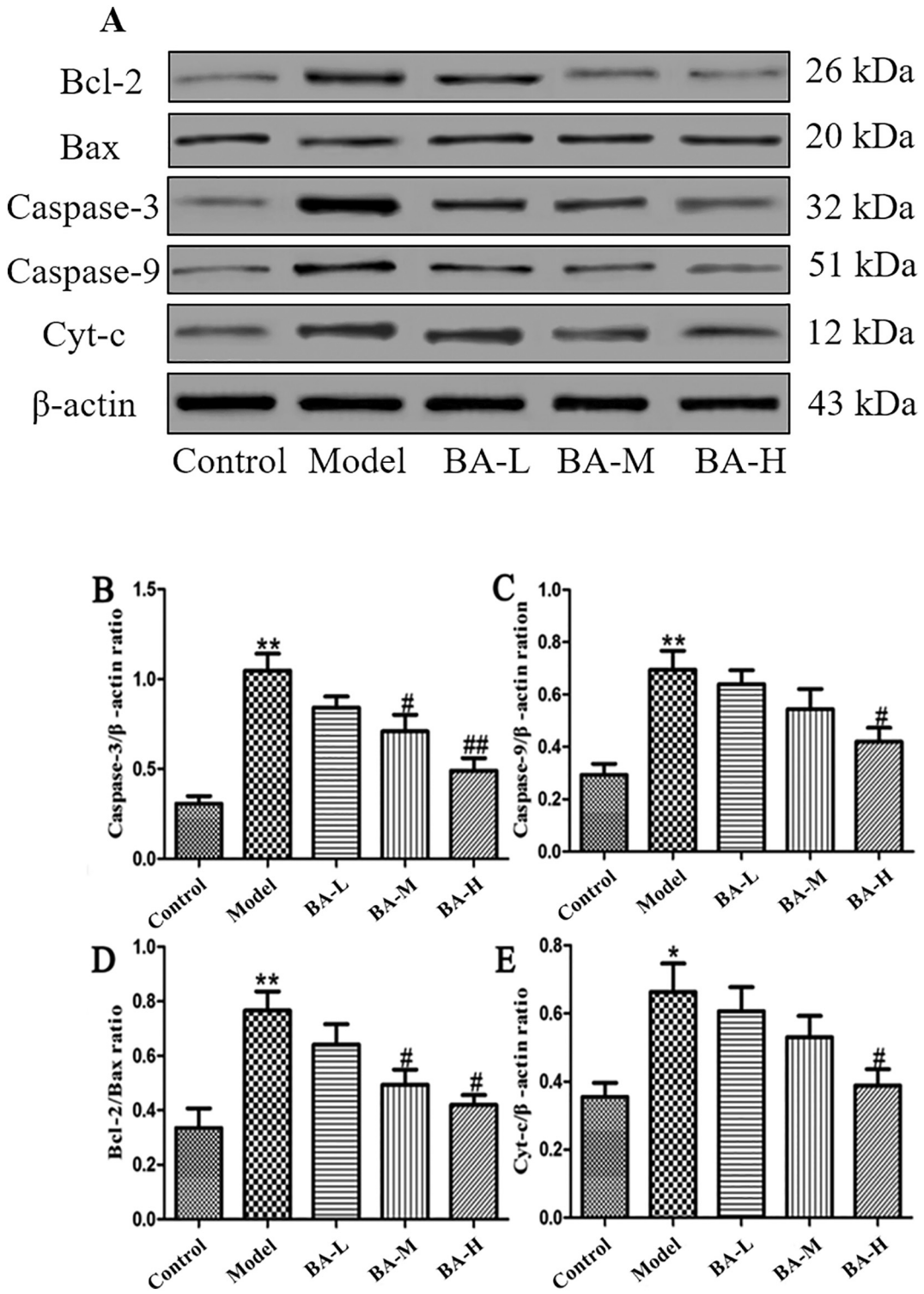

Fig. 3. Effect of baicalin on Caspase-3 (panel A/B), Caspase-9 (panel A/C), Bcl-2/Bax (panel A/D) and Cyt-c (panel A/E) in colon tissue of rats with UC. * was $P < 0.05$ and ** was $P < 0.01$ vs control group; # was $P < 0.05$ and ## was $P < 0.001$ vs model group.

3.3. Determination of apoptosis-related proteins in colon tissue of rats with UC

The contents of Caspase-3, Caspase-9, Bcl-2/Bax ratio and Cyt-c in the colon tissue of the model group were higher than the control group, indicating a significant increase in colonic apoptosis-associated protein expression in colonic model rats ($P < 0.05$). When baicalin was administered, the Bcl-2/Bax ratio and the changes of Cyt-c, Caspase-3 and Caspase-9 levels in the medium-dose group and high-dose group were significantly decreased, indicating that baicalin had significant effect on colonic cell apoptosis induced by UC (Fig. 3).

3.4. Effect of baicalin on IKK/IKB/NF-κB signaling pathway in colon tissue of rats with UC

The contents of NF-κB p-65, p-IKKβ/IKKβ and p-IKBα/IKBα in the

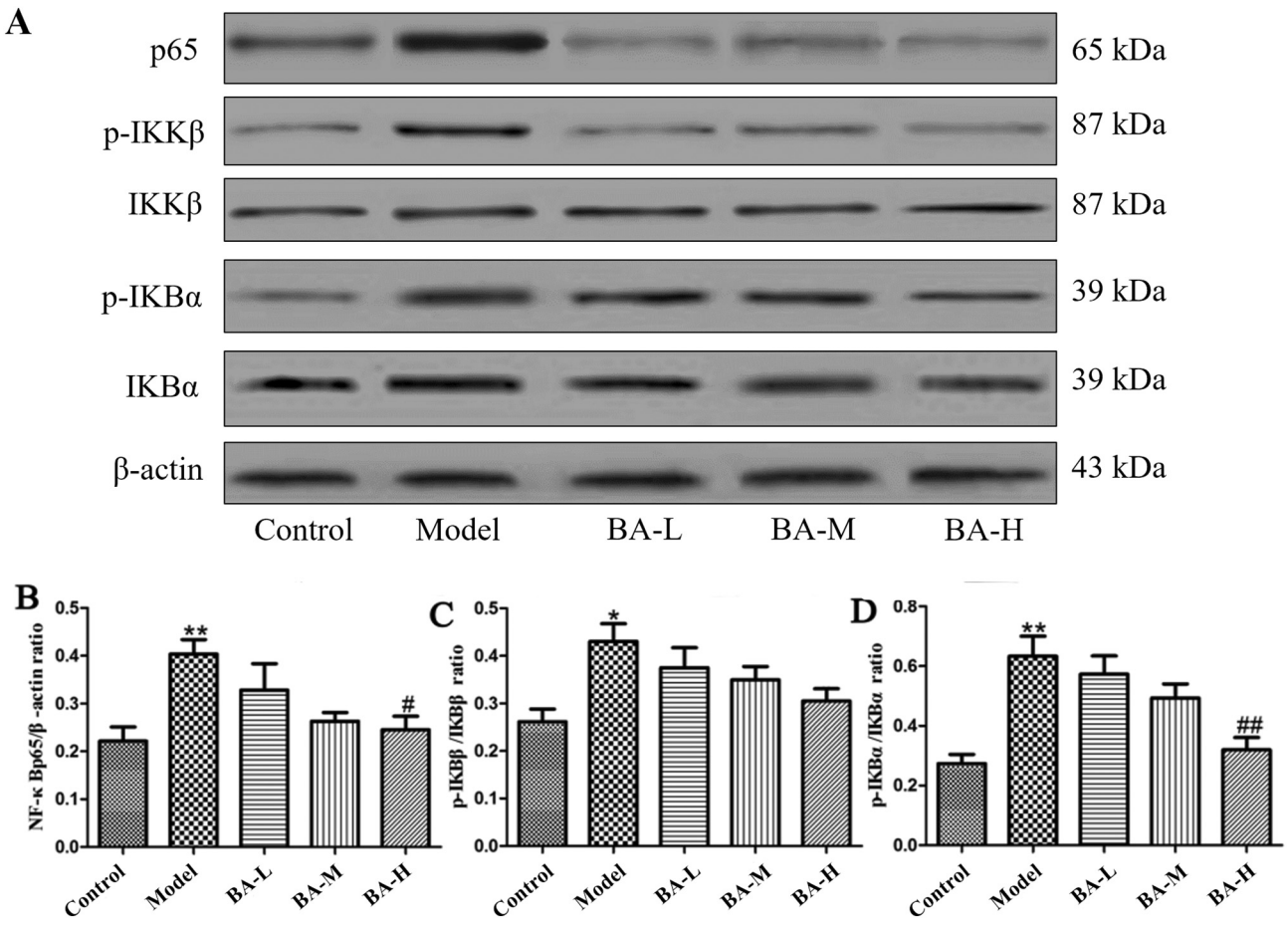

Fig. 4. Effect of baicalin on p-65 (panel A/B), p-IKKβ/IKKβ (panel A/C) and p-IKBα/IKBα (panel A/D) in colon tissue of rats with UC. * was $P < 0.05$ and ** was $P < 0.01$ vs control group; # was $P < 0.05$ and ## was $P < 0.001$ vs model group.

colon tissue of the model group were higher than the control group ($P < 0.05$), indicating that the colon tissue of the UC model rats was in an inflammatory state, and the body inflammation-related signaling pathway was activated. When baicalin was administered, the contents of NF-κB p-65, p-IKKβ/IKKβand p-IKBα/IKBαin the medium-dose group and high-dose group were significantly decreased, indicating baicalin had a significant effect on colonic tissue inflammation in rats of UC model (Fig. 4).

3.5. Effect of baicalin on IKK/IKB/NF-kB signaling pathway in RAW264.7 cells

Cell results showed that 500 ng/ml LPS could significantly reduce cell viability of RAW264.7 cells within 48 h. Therefore, 500 ng/ml LPS were used to establish an inflammatory cell model. In order to investigate the mechanism of action of baicalin, this study separately administered LPS-induced RAW264.7 cells with Bay 11-7082 (IκBα inhibitor), EF-24 (IKK inhibitor) and baicalin. The results showed that baicalin could significantly inhibit p-IKBα/IKBα content changes, but have no significant effect on p-IKKβ/IKKβ, suggesting that the anti-inflammatory effect of baicalin may be through inhibition of changes in p-IKBα/IKBα content (Fig. 5).

4. Discussion

UC is an idiopathic, chronic inflammatory disorder of the colonic mucosa, which starts in the rectum and generally extends proximally in a continuous manner through part of, or the entire, colon [21]. Although etiology and pathogenesis of UC are not completely clear, it has been found to be related to heredity, infection, intestinal mucosal barrier function, environment, and immune factors [22,23]. There are many methods for the treatment of UC. At the same time, with the deep exploration of the etiology and pathogenesis of UC, new treatment methods have emerged, and the situation of UC treatment is improving over time. The aim of this study was to explore effect of baicalin on UC rats.

Oxidative stress is a risk factor for the development of chronic inflammatory diseases, such as UC, and is the main driving inflammation towards DNA damage, which in turn, leads to carcinogenesis [24]. Oxidative stress is involved in both pathogenesis and exacerbation of UC [25]. Changes in SOD, CAT, GSH-Px activities and MDA content in the body are important indexes of oxidative stress in the body. Previous study found that epicatechin significantly improved antioxidant activity by increasing the levels of GSH-Px, CAT and SOD in the colon tissue and by reducing the production of MDA [26], which was similar with our results. Another study found that Data of MTT reduction assay and flow cytometry revealed that viability loss and apoptotic rate were reduced by pre-treatment of PC12 cells with baicalin for 24 h, and baicalin was also found to increase SOD, GSH-Px activities and to decrease MDA level [27]. Analyses of SOD, GSH-Px, MQAE and GABAA suggested baicalin played an antioxidant role in chick embryos possibly through suppression of outwardly rectifying Cl(−) in the high-glucose microenvironment [28]. In this study, oxidative stress related indexes such as SOD, CAT, GSH-Px activities and MDA content were studied. The activities of SOD, CAT and GSH-Px in the model group were significantly lower than and the content of MDA was significantly higher than the control group, indicating that the rats in the model group were in the state of peroxidative stress. In the medium-dose group and the high-

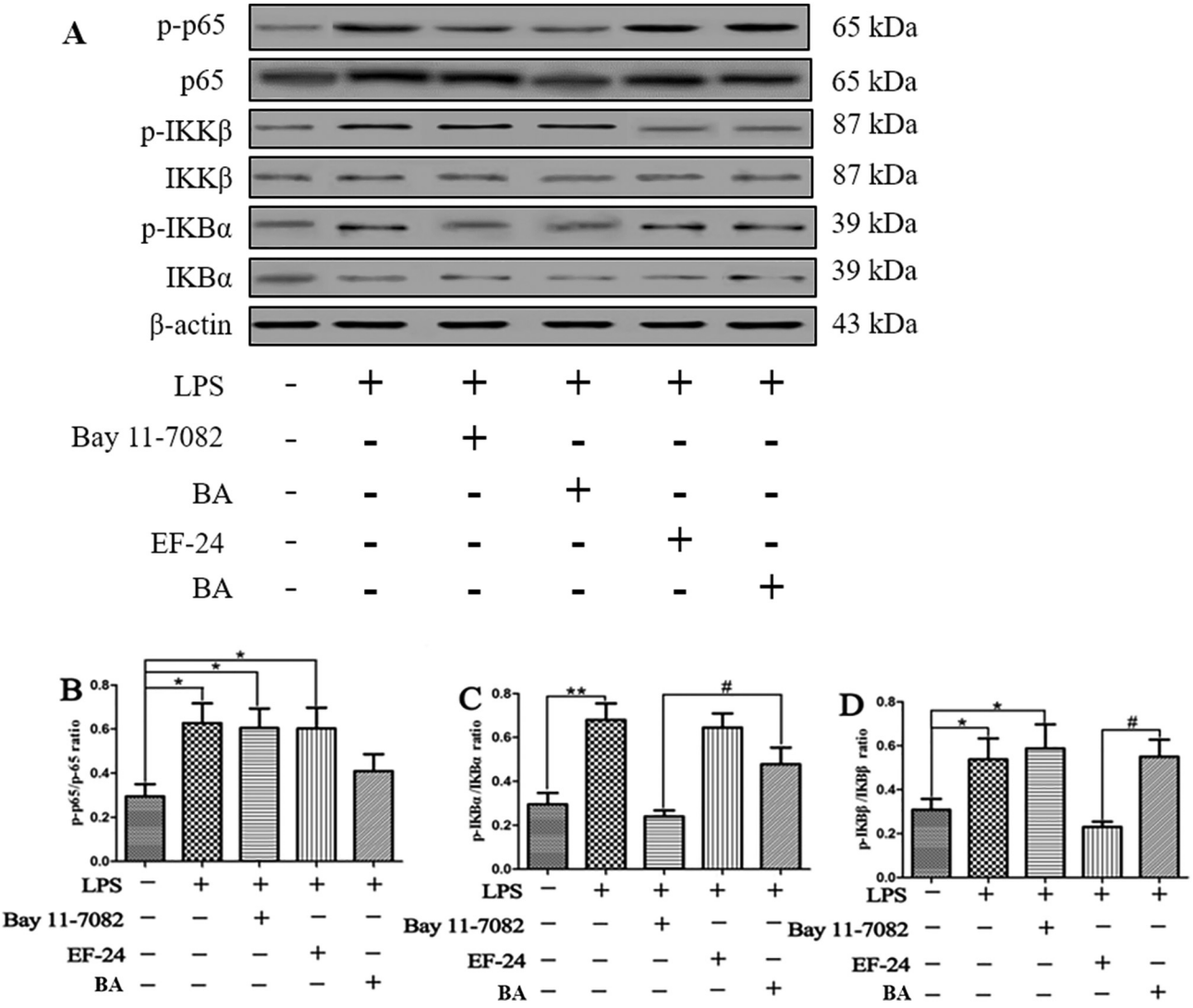

Fig. 5. Effect of baicalin on p-65 (panel A/B), p-IKBα/IKBα (panel A/C) and p-IKKβ/IKKβ (panel A/D) in RAW264.7 cells. * was $P < 0.05$ and ** was $P < 0.01$ vs control group; # was $P < 0.05$ vs model group.

dose group, the SOD, CAT, GSH-Px activities were all increased and MDA content was decreased, indicating that baicalin had a protective effect on oxidative stress injury in rats of UC model.

The inflammatory response is an important indicator of the inflammatory state of the body. The determination of PEG_2, MPO, IL-1β and TNF-α plays important guiding role in evaluating the inflammatory state of the body [29]. Najafi et al. found that treatment with sodium valproate at the doses of 100 and 300 mg/kg decreased the MPO activity and colonic concentrations of IL-1β, IL-6 and TNF-α in UC rats [30]. The production of pro inflammatory cytokines TNF-α, IL-1β, and IL-6 was significantly reduced in UC rats after K68 was administered [31]. In our study, the contents of PEG2, MPO, IL-1β and TNF-α in the baicalin-administrated group were significantly decreased, indicating that baicalin had anti-inflammatory effects on UC rats.

The contents of Caspase-3, Caspase-9, Bcl-2/Bax ratio and Cyt-c as apoptosis-related proteins were measured in this study. When paeoniflorin was administered, the Bcl-2/Bax ratio, Cyt-c, Caspase-3 and Caspase-9 in the medium-dose group and high-dose group were significantly decreased, indicating that paeoniflorin had significant effect on colonic cell apoptosis induced by UC. Previous study found that paeoniflorin had the anti-inflammatory effect on UC via inhibiting MAPK/NF-kappa B pathway and apoptosis in mice [32]. In this study, paeoniflorin could decrease the contents of NF-κB p-65, p-IKKβ/IKKβ and p-IKBα/IKBα, indicating paeoniflorin had a significant effect on colonic tissue inflammation in rats of UC model.

RAW264.7 cells are a mouse monocyte-macrophage cell leukemia [33]. In this study, 500 ng/ml LPS was used to establish an inflammatory cell model, and 500 ng/ml LPS could significantly reduce cell viability of RAW264.7 cells within 480 h. LPS-induced RAW264.7 cells were separately administered with Bay 11-7082 (IκBα inhibitor), EF-24 (IKK inhibitor) and paeoniflorin. The results found that paeoniflorin could significantly inhibit p-IKBα/IKBα content changes, but have no significant effect on p-IKKβ/IKKβ, indicating that the anti-inflammatory effect of paeoniflorin may be through inhibition of changes in p-IKBα/IKBα content. This was the first time that found paeoniflorin exerted anti-inflammatory effect via IKK/IKB/NF-kB signaling pathway. However, the detail mechanisms are still needed further study.

In conclusion, by using TNBS induced-UC rat model and LPS-induced RAW264.7 cells, it was found that paeoniflorin may have a regulating effect on IKK/IKB/NF-kB signaling pathway and apoptosis-related proteins in UC rats (Fig. 6). But this study was only performed in animal level and cell level, we hope that a clinical study will be performed as soon as possible, and paeoniflorin can exert an anti-inflammatory effect in UC patients.

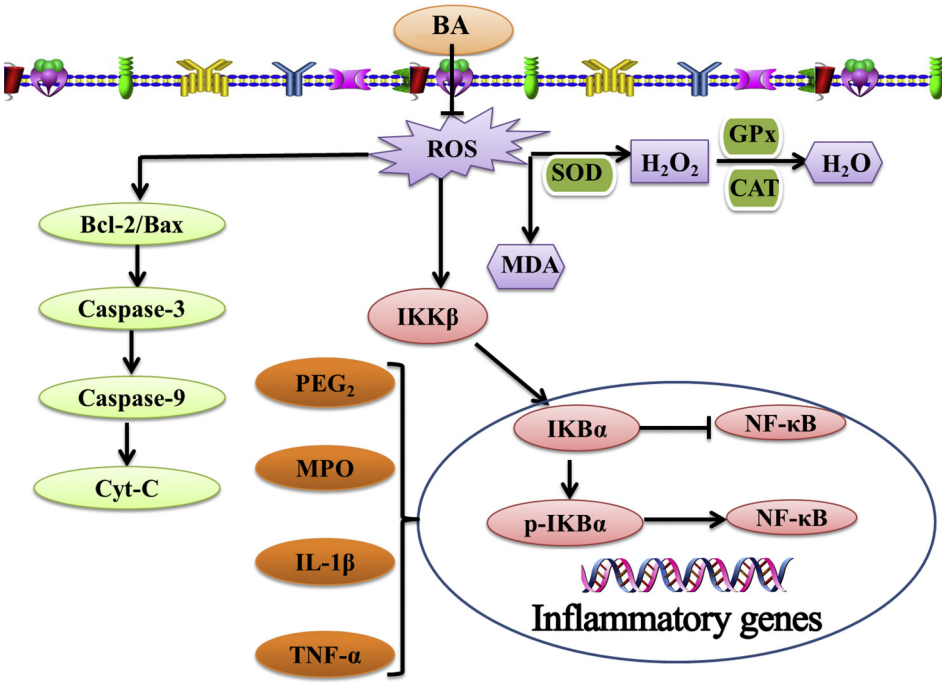

Fig. 6. Schematic diagram of research ideas.

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Disclosure of conflict of interest

None.

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