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Pectic polysaccharides extracted from *Rauvolfia verticillata* (Lour.) Baill. var. hainanensis Tsiang increase IκB-α expression and ameliorate ulcerative colitis

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The therapeutic potential of pectic polysaccharides extracted from *Rauvolfia verticillata* (Lour.) Baill. var. *hainanensis Tsiang* in ulcerative colitis were investigated. This study showed that pectic polysaccharides extracted from *Rauvolfia verticillata* (Lour.) Baill.var.*hainanensis Tsiang* ameliorated ulcerative colitis and were proposed to exhibit anti-inflammatory effects *via* increased expression of IκB-α proteins and suppressing NF-αB translocation.

1. Introduction

Ulcerative colitis (UC) is a chronic, idiopathic, inflammatory bowel disease of unknown etiology. However, genetically susceptible individuals seem to have a dysregulated mucosal immune response to commensal gut flora, which results in bowel inflammation (Khor et al. 2011; Ordas et al. 2012). In addition to genetic factors, environmental factors and psychological state are considered to be causes of inflammatory bowel disease (Bernstein et al. 2006; Abraham et al. 2009; Lopez-Serrano et al. 2010).

UC is characterized by a contiguous mucosal inflammation starting in the rectum and proximally progressing in continuity in the colon for a different distance. Immune mechanisms play an important role in UC. Pectic polysaccharides have earlier been shown to possess an anti-inflammatory effect (Popov et al. 2005, 2007a; Galati et al. 2008). Popov et al. (2007b) found that pectin obtained from *Rauvolfia serpentina* L. could inhibit colitis in mice. Rauwolfia is commonly used in traditional Chinese medicine for lowering blood pressure, treating arrhythmia and tumors. However, rauwolfia is rarely used to cure UC. In this study, we extracted pectic polysaccharides from *Rauvolfia verticillata*(Lour.)Baill.var.*hainanensis Tsiang* and elucidated the effects of anti-inflammatory both *in vivo* and *in vitro* to make a preliminary study of the mechanism of anti-inflammatory in ulcerative colitis.

2. Investigations, results and discussion

The therapeutic potential of pectic polysaccharides extracted from *Rauvolfia verticillata* (Lour.) Baill. var. *hainanensis Tsiang* in ulcerative colitis were investigate. Pectic polysaccharides ameliorate dextran sulphate sodium (DSS)-induced colitis in mice.

Disease activity index (DAI) scores were monitored each day. Mice given 4% DSS in their drinking water for 7 d developed symptoms of colitis. Compared with control mice, the DAI value was significantly greater from day 2 to 14 in mice treated with DSS ($P < 0.05$, Fig. 1). Compared to the UC group, the colitis symptoms were relieved from day 12 to 14 in mice of the UC plus pectic polysaccharides group and UC plus salicylazosulfapyridine (SASP) group ($P < 0.05$, Fig. 1). There were no statistically significant differences between the two drug treatment groups. The histology of the murine intestinal mucosa in different experimental groups was examined (Fig. 2). The control group given normal saline showed no histological alterations. Similarly, mice given SASP exhibited virtually the same normal histology with no inflammatory cell infiltration, oedema or crypt abscesses. Severe submucosal oedema, erosion, ulceration, inflammatory cell infiltration and extensive destruction of the mucosal layer were observed in the mucosa of UC group animals. The UC plus

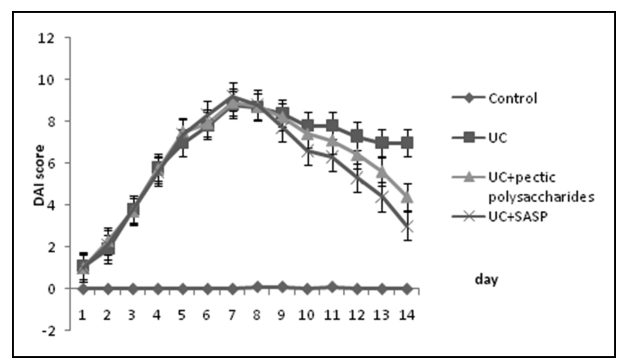

Fig. 1: Disease activity index (DAI) of colitis in mice from four groups. Female BALB/c mouse were given 4% DSS in their drinking water during the first 7 days, excepted control mouse. Subsequently, DSS was removed, and the animals received 200 μL of normal saline (control group and DSS group) or 100 mg/kg per 200 μL pectic polysaccharides or SASP (UC plus pectic polysaccharides group and UC plus SASP group, respectively) orally each day. The DAI value was calculated as described under Experimental (n = 10/group). $P < 0.05$, groups included UC mouse vs. the control group from day 2 to day 14; $P < 0.05$, UC plus pectic polysaccharides group or UC plus SASP group vs. the UC group from day 12 to day 14; $P > 0.05$, UC plus pectic polysaccharides group vs. UC plus SASP group.

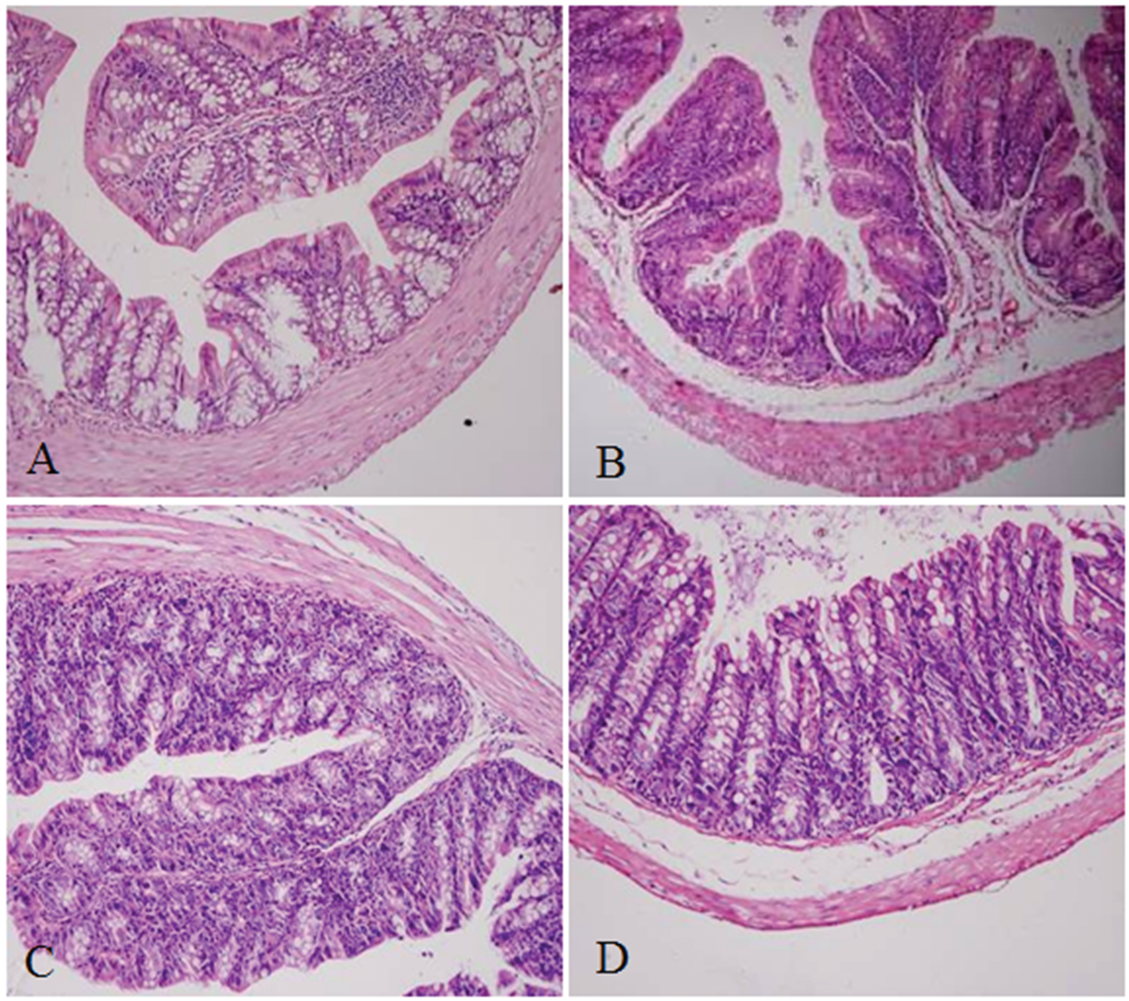

Fig. 2: Histological analysis of mice. Representative images show the histological structures of the colon tissues in four groups (HE, Original magnification: × 200). A: Control group (NS treated) animals showing normal colon tissue architecture. B: UC group animals showing severe submucosal oedema, erosion, ulceration, inflammatory cell infiltration and extensive destruction of mucosal layer. C: UC plus pectic polysaccharides group showing attenuation in inflammation, characterized by suppression of erosion, ulceration, reduction in inflammatory cellular infiltrate, and protection against epithelium damage in the mucosa, although oedema was still exsisted. D: UC plus SASP group animals showing a same normal histology as control group.

pectic polysaccharides group revealed attenuation in inflammation, characterized by suppression of erosion, ulceration, reduction in inflammatory cellular infiltrate, and protection against epithelium damage, although oedema still existed.

IκB-α protein was decreased in the UC group when compared with control group, increased in the UC plus pectic polysaccharides group and the UC plus SASP group, when compared with UC group either in murine colon tissues or cultured colon cells of UC patients by Western blot ($P < 0.05$, Fig. 3A and3B).

Pectic polysaccharides reduced the production of pro-inflammatory cytokines that were increased in the DSS-induced colitis in mouse, including TNF-α, IL-lβ, IL-6 and IL-4 (Fig. 4A). Likewise, TNF-α and IL-6 were down-regulated in colon culture supernatants of UC patients by cultured with pectic polysaccharides (Fig. 4B).

Following normal stimulation, colon cells release of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-4 and IL-6 is protective in fighting pathogens like bacteria. However, under pathological conditions, including oxidative stress, toxicity, colon cells can be overstimulated and produce excess pro-inflammatory cytokines which result in inflammatory bowel disease like UC (Goyal et al. 2014). This study investigates whether pectic polysaccharides inhibit production of pro-inflammatory cytokines in UC. Our data showed that pectic polysaccharides reduced protein expression of TNF-α, IL-1β,

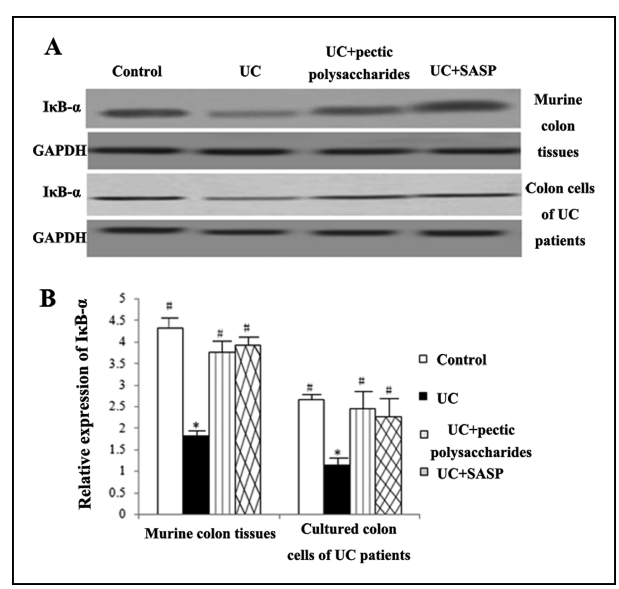

Fig. 3: Pectic polysaccharides increased IκB-α protein expression in colitis. IκB-α protein were analyzed by Western blotting. Detection of GAPDH was estimated as protein-loading control for each lane. $^{\#}P < 0.05$ compared with UC group, $^{*}P < 0.05$ compared with control group.

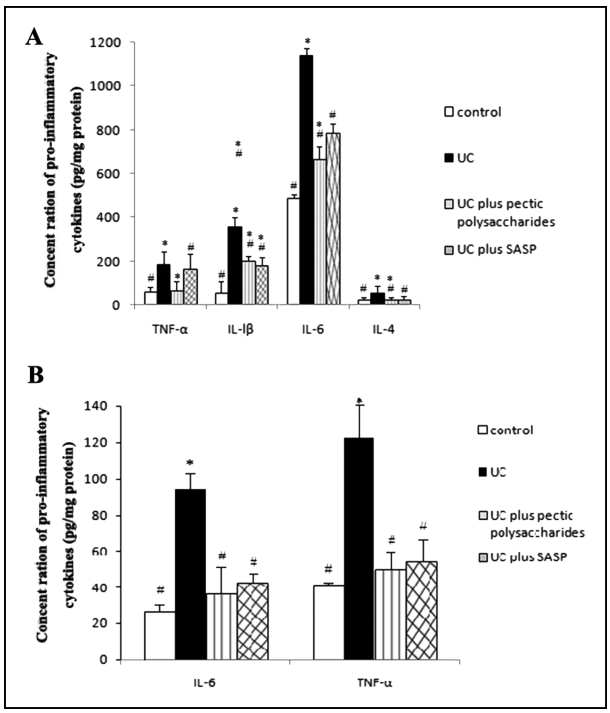

Fig. 4: Pectic polysaccharides reduced proinflammatory cytokines production in colitis. A: After 14 d, mice were sacrificed and colon tissues were used for evaluating the levels of proinflammatory cytokines by ELISA analysis. B: Cultured colon tissues of UC patients were incubated with 10 μmol/l pectic polysaccharides or 20 μmol/l SASP, while control group and UC group were incubated with NS. After 24 h, supernatants were collected and proinflammatory cytokines levels were estimated by ELISA. $^{\#}P < 0.05$ compared with UC group, $^{*}P < 0.05$ compared with control group.

IL-4, and IL-6 in mice with DSS-induced colitis, meanwhile the expression of TNF-α, and IL-6 decreased in cultured colon supernatant of UC patients. These results suggested that pectics exerted anti-inflammatory effects in UC. The results are consistent with previous studies (Popov et al. 2005, 2007a, 2007b; Galati et al. 2008).

To further characterize the nature of the inhibitory effect of pectic polysaccharides on pro-inflammatory proteins production, the NF-κ B signal transduction pathway was examined. NF-κB is the major transcription factor which mediates inflammatory signaling (Imanifooladi et al. 2010; O'connor et al. 2010; Pasparakis 2012). A large number of studies showed that NF-κB played a broad regulatory role in ulcerative colitis, promoted expression of various pro-inflammatory cytokines including TNF-α, IL-1β, IL-4 and IL-6 (Kundu et al. 2004; Karin et al. 2005). NF-κB exists mainly as a heterodimer composed of subunits of the Rel family, p50 and p65 (Verma et al. 1995). In resting stage, NF-κB is normally localized to the cytoplasm, where it is bound by IκB-α proteins. During inflammatory stimuli, IκB-α is phosphorylated by IκBkinase, subsequently degraded by proteasome, then NF-κB gets released and translocates into the nucleus, where it triggers the transcription of multiple genes involved in the inflammatory cascade (Brown et al. 1995; Whiteside et al. 1995). The results of this study suggested that pectic polysaccharides increased the production of IκB-α protein *in vivo* and *in vitro*. These indicated that pectic polysaccharides exhibit anti-inflammatory effects on ulcerative colitis which might be due to the inhibition of NF-κB signal transduction pathways.

Furthermore, the results presented herein clearly indicated that pectic polysaccharides efficiently relieved the symptoms of DSS-induced colitis in mice. In this study, we showed that pectic polysaccharides decreased DAI scores in mice with DSS-induced colitis, mitigated colitis-induced histological damage by suppression of erosion, ulceration, reduction in inflammatory cellular infiltrate.

In summary, the results of this study provided evidence that pectic polysaccharides extracted from *Rauvolfia verticillata* (Lour.) Baill. var. *hainanensis* Tsiang ameliorated ulcerative colitis and might exhibit its anti-inflammatory effects *via* increased expression of IκB-α proteins and suppressing NF-αB translocation.

3. Experimental

R. verticillata (Lour) Baill. var. *hainanensis Tsiang* was obtained from the Jianfengling National Forest Park of Ledong li autonomous county, Hainan province, China. Pectic polysaccharides were extracted according to Popov et al. (2007b). Briefly, the rootstock (5 g) dried at 40 °C was treated with diluted HCl (up to pH 4.4) at 50 °C for 3 h and the material obtained was extracted with 0.7% aqueous ammonium oxalate. A polysaccharide fraction was precipitated with four volumes of 96% ethanol. After centrifugation at 8000 g for 10 min, the precipitate was collected and dried at 40 °C. Subsequently, the precipitate was dissolved in distilled water and stored at -20 °C.

A total of 40 female BALB/c mice (6–8 weeks old, 17-22 g) were purchased from the Huaxi Medical Animal Center of Sichuang University (Sichuang, China). The mice were bred under standard conditions and maintained in a 12-h light/12-h dark cycle at 23 ± 1 °C and had access to food and water *ad libitum*. The mice were randomly divided into four groups: control, UC, UC plus pectic polysaccharides and UC plus salicylazosulfa pyridine (SASP, used as a positive control drug) (n = 10/group). Colitis was induced in BALB/c mice by adding dextran sulphate sodium (DSS, molecular weight: 36-50 kDa; MP Biomedicals) to drinking water at a level of 4% for 7 days. Control animals received water only. After 7 d, DSS was removed from the drinking water and the animals received 200 μL of normal saline (control group and UC group) or 100 mg/kg per 200 μL pectic polysaccharides or salicylazosulfapyridine (SASP, UC plus pectic polysaccharides group and UC plus SASP group, respectively) orally each day. All of the mice were sacrificed by cervical dislocation after 14 days. On the day of sacrifice, the colons were removed and rinsed clean. One sample from each colon was fixed in formalin, and another fresh colon sample was snap frozen and used for western blotting and ELISA analysis. The experiments were approved by the Institutional Animal Care Committee, and were conducted following the guidelines of the animal care policy.

For DSS colitis mice, body weight, stool consistency, and blood in the stool were monitored daily to assess the severity of colitis. The disease activity index (DAI) was assessed in accordance with the method described by Murano et al. (2000). The DAI was assessed by an investigator who was blind to the experimental groups.

Ten patients with UC participated in this study, 6 male, 4 female, aged from 29 to 54 years (median age: 36.5 years), 8 to 45 months duration of UC (mean duration: 18 months), hospitalized at the Hainan Provincial People's Hospital, were recruited from August to December, 2012. The diagnosis of UC was endoscopically and histologically confirmed, and activity of UC was evaluated using the Mayo Disease Activity Index (0: normal mucosa, 1: erythema, 2: erosion, 3: ulcer) (Dieleman et al. 1998; Mirbagheri et al. 2008). Subjects with Mayo Disease Activity Index levels ≥ 2 were considered to have active UC. All of the ten patients had active UC according to the classification.

From each of four patients (randomly selected), one colonic biopsy specimen was taken from the non-inflamed region as a sample of control group. In six UC patients, biopsy specimens from the same patient including two inflamed areas of the colonic mucosa were obtained as UC tissue samples. The control group included four non-inflamed biopsy specimens, the inflamed colonic biopsy specimens randomly divided into three groups: UC, UC plus pectic polysaccharides, and UC plus SASP (n = 4/group).

Written informed consent was obtained from all the patients before enrollment in the study. This study was performed and approved by the institutional Ethics Committees of Hainan Provincial People's Hospital.

Biopsy specimens placed in 0.09% normal saline supplemented with 500U/ml penicillin and 500U/ml streptomycin at 4 °C for tissue culture. After collection, the biopsy specimens were transferred to the laboratory. The tissue was gently washed ten times in normal saline with penicillin and streptomycin, and placed in tissue culture flasks in RPMI 1640 medium (Gibco BRL, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (Gibco BRL), 250U/ml penicillin, 250U/ml streptomycin and 50 μg/ml gentamycin. After 1 h of culture at 37 °C in a humidified 95% O_2, 5% CO_2 atmosphere, pectic polysaccharides and SASP were added in the culture medium of UC plus pectic polysaccharides group and UC plus SASP group, respectively, while equal volumes of normal saline were added

in control group and UC group. The final pectic polysaccharides concentration was 10 μmol/l, the SASP concentration was 20 μmol/l. After 24 h the supernatants and cells were harvested separately, and stored at -80 °C until analysis.

For microscopic histological evaluation, formalin-fixed murine colon tissues were embedded in paraffin and 4 μm sections were stained with hematoxylin and eosin and evaluated using light microscopy.

Frozen murine colon tissue and cultured colon cells of UC patients were used for IκB-α protein expression Western blotting analysis. Total protein extract was prepared using a Mammalian Cell Extraction Kit (Biovision, USA) according to the manufacturer's instructions. Concentration of the protein was measured by the bicinchoninic acid method. A total of 100 μg of protein was resolved onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto immunoblot polyvinylidene difluoride membranes (Chemicon International, Millipore, Billerica, MA, USA). The blots were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween (TBS-T) for 1 h, washed three times with TBS-T, and incubated overnight at 4 °C with primary antibodies IκBα (1:1000, Cayman Chemical Inc., USA). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used to verify equal loading of proteins(1:2000). Blots were then washed four times for 15 min each in TBS-T and incubated with horseradish peroxidase-labeled secondary goat anti-mouse (1:2000; Santa Cruz Biotechnology) for 2 h at room temperature. Blots were again washed four times for 15 min each in TBS-T. Membranes were visualized with enhanced chemiluminescence (Pplygen Co., China). Finally, the densitometry values of each protein analyte normalized to GADPH were compared.

The murine colon tissues and supernatants of cultured colon tissue of UC patients were used for cytokines ELISA analysis. In addition, the murine colon homogenates were obtained by handwork, and centrifuged at 3000 rpm, 10 min. The supernatants were reserved for analysis. The amounts of cytokines in the supernatants for TNF-α, IL-1β, IL-4 and IL-6 were determined by ELISA (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. Three replicates were carried out for each of the different treatments.

All data were expressed as means ± SD. Multiple group analysis was performed using a oneway analysis of variance with a post-hoc Tukey's test. Comparison of 2 groups in all other data was analysed by Student's t-test. Differences were considered statistically significant if the P value was less than 0.05. Data were analyzed using SPSS 19.0 (Chicago, IL, USA).

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