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Original Research Article

Rhizoma atractylodis suppresses TNBs-induced colitis through NF-κB signaling pathway

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Abstract

Purpose: *Colitis is a kind of chronic non-specific inflammatory disease with increasing morbidity worldwide. Rhizoma atractylodis is the dry rhizome of Atractylodes lancea, which is often used to treat inflammations, but its connection with colitis is yet to be understood. Thus, the study was to confirm the functions of Rhizoma atractylodis on colitis cell progression.*
Methods: *Cells of human colonic epithelial cell line, NCM460 were induced by trinitrobenzene sulfonic acid (TNBs) to create inflammatory TNBs-NCM460 model groups, while untreated NCM460 cells were taken as normal control group. Varying concentrations (0, 20, 50 and 100 µmol/L) of Rhizoma atractylodis were added to pretreat the NCM460 cells before exposure to TNBs. RT-qPCR and Elisa methods detected the mRNA expression and protein concentrations of IL-6 and TNF-α and Western blot analyses were utilized to measure protein levels of NF-κB.*
Results: *Rhizoma atractylodis suppressed the Il-6 and TNF-α, which were induced by TNBs. Furthermore, the NF-κB pathway was inactivated in the cells with the pretreatment of hizoma atractylodis.*
Conclusion: *Rhizoma atractylodis inhibited inflammatory cytokines IL-6 and TNF-α through NF-κB inactivation. Therefore, rhizoma atractylodism might be a complementary medication in colitis.*

Keywords: *Cell progression, Colitis, NF-κB, Rhizoma atractylodis*

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INTRODUCTION

Inflammatory bowel disease (IBD) contains Crohn's disease (CD) and ulcerative colitis (UC) [1]. UC is a kind of chronic non-specific disease caused by inflammation, which is limited to the rectum and colon mucosa [2]. The major clinical feature of UC is mucosa ulcer, which causes pain in abdomen, diarrhea and mucopurulent bloody faeces[3, 4]. Studies proved that morbidity of UC in China has increased gradually, which severely impacted patients in their qualities of life[5]. The pathogenesis of UC is now believed to be on the basis of genetic and environmental factors[6]. Due to stimulations of microbial antigens, immune system of bodies is activated, which causes damage to cytokine network balance and activates inflammatory

cells[7]. Adhesion molecules are induced to express abnormal and cluster to inflammatory sites and release a variety of inflammatory factors[8]. This progression can lead to chronic inflammation of colon tissues[9]. In recent year, incidence rate of colitis has increased annually[10]. Therefore, it is essential to find new medications to treat this disease.

Rhizoma atractylodis is the dry rhizome of *Atractylodes lancea* (Thunb.) DC. or *A. chinensis* (DC.) Koidz, which can cause dry dampness, strengthen the spleen, relieve wind and disperse cold and improve eyesight[11, 12]. Traditional Chinese medicines (TCM) have documented its use in preventing, treating and curing symptoms and diseases. TCM was the main treatment in Chinese communities before western medicines were introduced into China [13]. Chemical constituents of *Rhizoma atractylodis* are Sesquiterpenes, eneyne, triterpene, steroids and Aromatic glucosides[14]. These compositions were proven to have abilities of hepatoprotection, antibiosis, antivirus and anti-cancer, and so on [15, 16]. Trinitrobenzene sulfonic acid, a skin contactant could couple with proteins with high molecular weight to cause immune response and development of Th1 inflammation[17]. *Rhizoma atractylodis* is a common medicine in inflammation, but its connection with colitis is rare. Thus, this study was to evaluate the role of Rhizoma atractylodis on colitis and also gaining an insight on the underlying mechanism.

METHODS

Cell culture

A human colon epithelial cell line, NCM460 was obtained from ATCC, USA. The cell line was incubated in DMEM with 10 % FBS (Thermo Fisher, USA) at 37 ℃, 5 % CO_2. After the confluence of the cells reached 80 %, they were collected, washed using PBS and digested with 0.25 % trypsin (Beyotime, Shanghai, China). Thereafter, DMEM was used to re-suspend cells before sub-culturing. Cells at log phage were collected for further study.

TNBs treatment

Trinitrobenzene sulfonic acid (TNBs) was bought from MP Biomedicals, USA. The cells were seeded onto a 12-well plate (2×10^5 cells each well). After incubation for 24 hr, 0.5, 1.0 and 1.5 mg/L TNBs were respectively added onto the plate, while culture medium was added to normal cells (control). Then, culturing of cells continued for 24 hr at 37 ℃, 5 % CO_2. Normal cells and inflammatory cells were then collected and placed into 6-well plates at 5×10^5 cells each well.

Rhizoma atractylodis treatment

The *Rhizoma atractylodis* was bought from a local traditional drugstore. DMSO was used to dilute and form *Rhizoma atractylodis* solution with different concentrations (0, 20, 50 and 100 μmol/L). Then *Rhizoma atractylodis* solution was added to pretreat the normal NCM460 cell line for 24h and thereafter treated with TNBs as indicated above.

MTT assay

Cell viabilities were determined using the MTT assay. Normal and inflammatory cells were gathered and inoculated in a 96-well plate (5×10^3 cells per well). Cells were separated into negative control (NCM460) and model groups (TNBs-NCM460 (0.5 mg/L); TNBs-NCM460 (1.0 mg/L) and TNBs-NCM460 (1.5 mg/L) with or without the pretreatment of *Rhizoma atractylodis*. Optical density (OD) was detected using microplate reader (MK3, Thermo Fisher, CA, USA) at 490 nm.

RT-qPCR

mRNA expressions of IL-6 and TNF-α were assessed by RT-qPCR method. TRizol (Beyotime, Shanghai, China) was used to isolate total cellular RNAs from each group, followed by purification and removal of genomic DNA. PrimeScript RT Master Mix Kit (Takara Bio Goteborg Sweden) was used for reverse transcription into cDNA. The RT-qPCR was conducted. The temperatures for the reverse transcription reaction were: 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. The PCR conditions were: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 sec, 55 °C for 40 sec and 72 °C for 30 sec. GAPDH was used as internal control. Primer sequences used were: IL-6, forward 5'- AGACAGCCACTCACCTCTTC-3' and reverse; 5'- AGTGCCTCTTTGCTGCTTTC-3' and TNF-α, forward 5'- AGGACCAGCTAAGA GGGAGA -3'and reverse 5'- TTCAGTGCTCAT GGTGTCCT-3'. GAPDH served as the internal control. The $2^{-\triangle\triangle Ct}$ method was used for expression analysis.

Elisa (Enzyme Linked Immunosorbent Assay)

The supernatant in each group was collected for Elisa detection. The Elisa kits, TNF alpha ELISA Kit (#88-7346-88, Invitrogen, Shanghai, China), IL-6 Human ELISA Kit (EH2IL6, Invitrogen,

Shanghai, China). Reagents were prepared and experimental procedure was conducted by following the instructions. Standard curve was formed according to OD values read at 450 nm. IL-6 and TNF-alpha concentration was calculated.

Western blot method

Protein extraction was obtained from the cells using RIPA buffer. The protein concentrations were analyzed using a BCA protein kit assay (Beyotime, Shanghai , China). An amount of 30 µg protein was electrophoresed in SDS-PAGE (Bioss, Beijing, China). The proteins were immobilized onto PVDF membranes, which were further sealed with 10 % skimmed milk and antibodies were diluted as instructed by manufacturers. Then diluted antibodies were added on PVDF membranes and the membranes were incubated at 4 ℃ for a night. Primary antibodies used included: anti-NF-κB (1:1000; ab16502) and GAPDH (1:2000; ab181602) as the internal reference (Abcam, Shanghai, China). By the following day, the membranes were then incubated with goat anti-Rabbit IgG (HRP) (1:800; ab150077) at room temperature for an hour. Finally, ECL kit (Bioss, Beijing, China) was used and images of the bands were captured after exposure in dark room. Relative values of the blotting were analyzed by Image Pro Software (Media cybernetics, USA).

Statistical analysis

Experiments were completed in triplicate. Experimental results were analyzed and statistical data were presented in figures as mean ± SD and analyzed by GraphPad 8.0 version (CA, USA). Differences between results in groups were evaluated by methods of one-way analysis of variance (ANOVA). $P<0.05$ was considered significant.

RESULTS

TNBs induced pro-inflammatory cytokines in NCM460 cells

The mRNA expression of IL-6 and TNF-α were detected in normal NCM460 control and TNBs-treated NCM460 cells using RT-qPCR assay. Expression of both IL-6 and TNF-α (Fig. 1A&B) were elevated in the cells treated by TNBs with higher concentrations 1 and 1.5ug/ml. Further, the culture supernatant was collected and underwent Elisa detection for IL-6 and TNF-α concentrations. It was found that IL-6 concentration also increased in groups treated with 1 and 1.5 ug/ml TNBs (Fig. 1C). Similarly, TNF-α concentration was elevated in cells after treatment with 1 and 1.5 ug/ml TNBs (Figure 1D).

Cells were induced by TNBs (0.5,1,1.5ug/ml) for 24hrs, generating groups,0.5, 1 and 1.5. The untreated cells served as control (Ctrl). A&B. RT-qPCR measured mRNA expression of IL-6 and TNF-α in each group with GAPDH as an internal control. C&D. Elisa methods calculated the IL-6 and TNF-α concentrations in supernatant of each group. Experiments were performed in triplicate. {$P<0.05$).

Effects of *Rhizoma atractylodis* on IL-6 and TNF-α in TNBs-induced NCM460 cells

Cells pretreated using different concentrations of *Rhizoma atractylodis* (20, 50 and 100 µmol/L) were further exposed to 1.5ug/ml TNBs for 24 hrs. RT-qPCR test showed that IL-6 and TNF-α expression was inhibited in the pretreatment groups of rhizoma atractylodis (Figure 2A-B). Elisa methods further confirmed that IL-6 and TNF-α concentrations were decreased in the cells with rhizoma atractylodis pretreatment (Fig. 2C-D). However, no significant change was noted between the 100 and 50µmol/L rhizoma atractylodis groups (Figure 2).

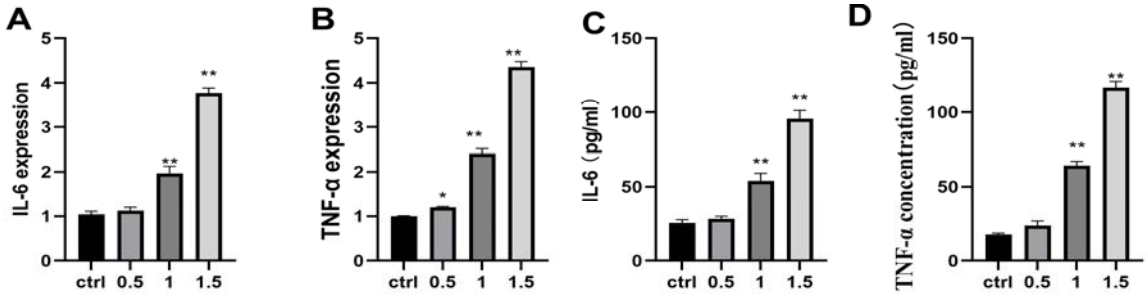

Figure 1: TNBs induced inflammatory factors in NCM460 cells

Rhizoma atractylodis down-regulated protein expression of NF-κB

Protein level of NF-κB was also measured using Western blotting. NF-κB protein expression in the TNBs-treated NCM460 cells was higher than the normal control group (Figure. 3A-B). In cells with the *Rhizoma atractylodis* pretreatment, NF-κB protein expression was suppressed (Figure 3A&B).

DISCUSSION

Colitis is a common normal chronic disease in gastroenterology. It is a multifactorial non-specific inflammation [18]. Pathogeneses of colitis are focused on colic mucosa and sub mucosa [19]. Patients with colitis can even have parenteral responses like bile duct diseases, ocular damage and even skin injuries [18]. Chinese medicine has been reported for their complementary therapeutic effects in different diseases [20]. Previous reports revealed that various Chinese medicines including wogonin and baicalin could target the inflammation-related signaling pathway NF-kB in colitis in animal models and in vitro[21]. However, in colitis models, the potential role of rhizoma atractylodis remains to be unveiled. The present study investigated the inhibitory effect of *Rhizoma atractylodis* in TNBs-provoked inflammatory features in NCM460 cells. There have been several reports that *Rhizoma atractylodis* has anti-diarrhea and anti-inflammatory properties and that the mechanism of *Rhizoma atractylodis* underlying anti-diarrhea activity is the induction of anti-inflammation [22].

IL-6 is a pro-inflammatory cytokine, mainly produced by macrophages [23]. It is associated with adhesion molecules and could activate NF-kB pathway and IL-6 is also closely related to neutrophil granulocytes and epithelial cells in IBD patients [24]. TNF-α is mainly secreted by macrophages and T-cells, which could elevate inflammatory reactions and immunoregulations

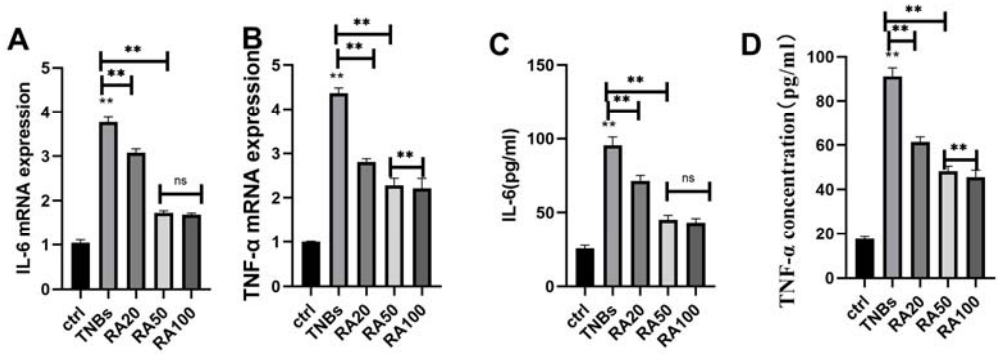

Figure 2: Effects of rhizoma atractylodis on IL-6 and TNF-α in TNBs-induced NCM460 cells.
Cells were pretreated with or without *rhizoma atractylodis* (RA: 20, 50 and 100 μmol/L) and then the cells were treated with 1.5ug/ml TNBs for 24hrs, forming groups, RA20, RA50 and RA100. The normal cells served as control(ctrl). A&B. RT-qPCR measured mRNA expression levels in each group with GAPDH as an internal control. C&D. Elisa methods were used to calculate the concentration of IL-6 and TNF-α in culture supernatant of each group. Experiments were performed in triplicate. (P<0.05).

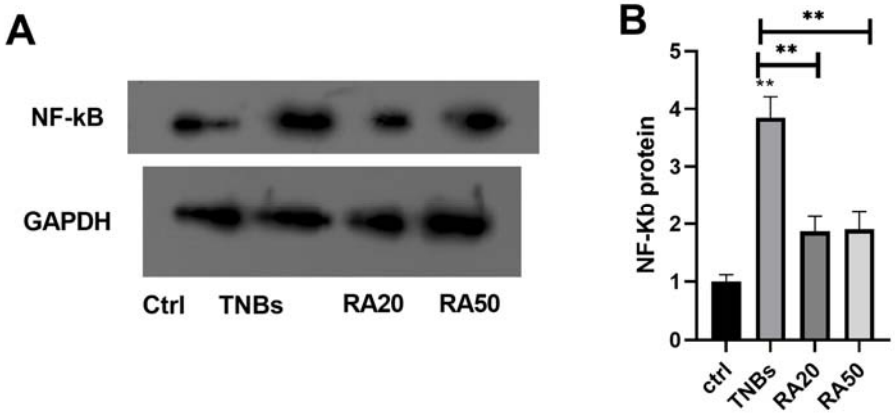

Figure 3. Effect of Rhizoma atractylodis on protein NF-κB in inflammatory cells of colitis. A. Western blot method was used to determine NF-κB protein level in cells. B. Relative protein expression was analyzed on Image Pro Software.

[25]. TNF-α plays an important role in intestinal mucosa injury by promoting platelet activating factors and producing leukotriene and oxygen free radicals to induce damages of thrombus[26]. TNF-α could aggravate injuries of intestinal mucosa through the interaction with inflammatory cells. In this study, protein levels of IL-6 and TNF-α were detected in both normal and TNBs-induced cells. Our results revealed that the mRNA expressions and protein concentrations of IL-6 and TNF-α were down-regulated in the cells pretreated with *Rhizoma atractylodis*. In colitis, IL-6/STAT3 was also discovered to regulate the epithelial homeostasis[27].

NF-κB could facilitate IL-8, TNF-α, IL-1β and improve the expressions to bring out occurrences and severity of colitis[28]. In this study, the pretreatment with *Rhizoma atractylodis* in cells could help to inactivate NF-kB pathway, which might be closely related with the downregulation of IL-6 and TNF-α.

CONCLUSION

Rhizoma atractylodis inhibited cell viability of inflammatory TNBs-NCM460 cells by suppressing pro-inflammatory factors IL-6 and TNF-α, thereby inhibiting the expression of NF-κB. Our findings suggest that *Rhizoma atractylodis* could be a potential useful medicine in treating colitis.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Tong Dong and Wen Jiang are co-first authors.

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