Original Article
Therapeutic efficacy and mechanism of Zhenrenyangzang decoction in rats with experimental ulcerative colitis

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Abstract: Zhenrenyangzang Decoction (ZD) has been used as a classic formula in China for the treatment of gastrointestinal dysfunction such as chronic gastritis. However, there is less study on its application in ulcerative colitis (UC) and the effects are not yet clearly defined. To explore the effectiveness of ZD in trinitrobenzene sulfonic acid (TNBS)-induced UC rats, ZD was administered orally for 8 days at a dosage of 2, 4 or 8 g/kg/day. Following drug administration, the disease activity index (DAI) and tissue damage scores were recorded. In addition, mRNA and protein expression of nuclear factor kappa B (NF-κB), p38 mitogen activated protein kinase (p38MAPK) and Toll-like receptor 2 (TLR2) in colon tissues were examined by real time PCR and western blotting assay. As compared with the UC model group, ZD promoted the recovery of colitis and inhibited the colonic inflammation damage in UC rats by reducing the mRNA or protein expression of NF-κB and p38MAPK, as well as activating the production of TLR2 in colon tissues. And ZD significantly reduced the DAI and tissue damage scores. The therapeutic effect of ZD was found to be comparable to that of SASP. Our results suggested that ZD could improve colonic mucosa impairment and possesses favorable therapeutic action in TNBS-induced colitis, which provides direct pharmacological evidence for its clinical application.

Keywords: Zhenrenyangzang decoction, ulcerative colitis, therapy

Introduction

Ulcerative colitis (UC) is a chronic nonspecific inflammatory disease of the colon characterized clinically by diarrhea, abdominal pain and mucuspurulent stool. It is reported that factors such as immune disturbance, heredity, inflammatory cytokines and environment, etc. play a crucial role in the development of UC [1]. The incidence and prevalence of UC are now increasing worldwide. In recent years, the incidence rates appear to be increasing in developing countries in Europe and Asia with westernization of lifestyle and industrialization, including China, South Korea and India [2]. Available therapies for UC include conventional anti-inflammatory agents (such as 5-aminosalicylates and corticosteroids), immune modulators and biological therapy. Biological therapy also aims at antagonizing pro-inflammatory molecules. However, the current anti-inflammatory therapy (e.g., treatment with anti-tumor necrosis factor (TNF)-α antibody) does not cure the disease and results in long-term remission only in fewer than 34% of patients [3]. Hence, anti-inflammatory therapy is not completely effective in eliminating the disease, indicating that other pathogenic factors may play important roles in UC [4]. Furthermore, UC management requires long-term treatment that often leads to drug refractoriness or intolerance [5]. Patients who are unresponsive to the current therapy still suffer from this common disease. Therefore, it is necessary to develop novel therapeutic approaches.

Complementary and alternative medicine (CAM) is becoming increasingly popular and is used to treat patients with UC in China. Physicians and patients consider CAM as an effective adjunct.
Effects of *Zhenrenyangzang* decoction in UC rats

### Materials and methods

#### Preparation of modified *Zhenrenyangzang* decoction (ZD)

Herbal materials were obtained from Qiqihar Hospital of Traditional Chinese Medicine. ZD were prepared by grinding and mixing of the 9 component dried raw herbs (Ginseng, Angelica Sinensis, Rhizoma Atractylodis Macrocephalae, Nutmeg, Cinnamon, Radix Glycyrrhizae, Radices Paeoniae Alba, Radices Saussureae and Chebule) in proportions as shown in **Table 1**. Herbs were decocted with boiling water at 100 g/L for 60 min twice. Decoctions were then filtered and pooled. For the purpose of oral feeding to mice in a smaller volume, the pooled decoction was further concentrated and freeze-dried.

### Animals and ethical approval

Eight weeks old male Sprague Dawley (SD) rats weighting 200-240 g were received from Experimental Animal Care Center, Harbin Medical University, China (License No. SCXK 2013-001). Animals were housed under controlled environmental conditions (25°C and a 12 h light/dark cycle). All experimental procedures and protocols in this study including euthanasia were conducted in accordance with the Ethical Guidelines of the Experimental Animal Care Center, Qiqihar Medical University, China.

### Induction of UC model and drug treatment

Experimental colitis was induced according to a modification of the procedure [8]. Prior to induction, all rats were fasted overnight but given free access to water. After anaesthetized with 30 mg/kg pentobarbital sodium, the rats were received a single rectal injection of TNBS/ethanol mixture (70 mg/kg TNBS diluted in 0.25 mL of 50% ethanol) slowly through a catheter with a 2 mm diameter at the depth of 8 cm from rectal sphincter, and then the rats were left for 15 min in a supine Trendelenburg position with the anus clipped. Seventy TNBS-induced rats were randomly divided into five groups: low, middle and high dosages of ZD groups (2, 4 and 8 g/kg, respectively), salazosulfapyridine (SASP) group (0.5 g/kg), and model group (an equal volume of saline). Additionally, fifteen rats used as normal control group were rectally injected with saline instead of TNBS. The animals were

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**Table 1.** Composition of herbs in the formulation ZD on a dry weight basis

<table>
<thead>
<tr>
<th>Chinese herbs</th>
<th>Alias</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginsnog</td>
<td>RenShen</td>
<td>11.36</td>
</tr>
<tr>
<td>Angelica Sinensis</td>
<td>Dang Gui</td>
<td>11.36</td>
</tr>
<tr>
<td>Rhizoma Atractyloides Macrocephalae</td>
<td>BaiShu</td>
<td>13.63</td>
</tr>
<tr>
<td>Nutmeg</td>
<td>Rou Doukui</td>
<td>13.63</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>Rou Gui</td>
<td>3.41</td>
</tr>
<tr>
<td>Radix Glycyrrhizae</td>
<td>ZhiGancao</td>
<td>6.82</td>
</tr>
<tr>
<td>Radices Paeoniae Alba</td>
<td>Bai Shao</td>
<td>17.04</td>
</tr>
<tr>
<td>Radices Saussureae</td>
<td>Mu Xiang</td>
<td>11.36</td>
</tr>
<tr>
<td>Chebule</td>
<td>He Zi</td>
<td>11.36</td>
</tr>
</tbody>
</table>

**Table 2.** Primers for real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-kB</td>
<td>5’ AGCACTGTGAGGAGCAGGATAC 3'</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>5’ CGTGAAGATATCCAGGTATGTC 3'</td>
<td></td>
</tr>
<tr>
<td>P38MAPK</td>
<td>5’ TAGAGCAATGGAAGCCTGAC 3'</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>5’ GGCCTTGAAATGTTGGAG 3'</td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>5’ AAGTAGAACGGTAACAATCCGAG 3'</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>5’ AGAACGAGGGAACACGAA 3'</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>5’ CGTAAAGACCTCTATCCCAAACAC 3'</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>5’ AGCCACCAATCCACACAGAG 3'</td>
<td></td>
</tr>
</tbody>
</table>
administered by intragastric gavage once a day, and continuously for 8 days.

**Histopathological investigations**

Colon sections were fixed 10% neutral buffered formalin then put for 24 h in decal. Samples were then cut into several sections and embedded into paraffin wax blocks. Tissues were stained with haematoxylin and eosin and were mounted and observed microscopically for histopathological changes by a pathologist in blinded fashion.

**Macroscopic and histological evaluation of colonic damage**

A 10-cm segment of the distal colon was removed for the morphological study. Colonic mucosa damage was assessed according to previously described macroscopic scoring system [9] as follows: 0= normal mucosa; 1= localized hyperemia but no erosions, ulcers, or scars; 2= linear ulcer or scar with inflammation at one site >2 mm but <5 mm; 3= two or more sites of ulceration and/or inflammation, each up to 5 mm; 4= two or more major sites of inflammation and ulcerations >5 mm each or one major site of inflammation extending >1 cm along the length of the mucosa. The tissue fragments (2×10 mm²) were excised from the central part of the lesion of each colon, fixed in 4% polyformaldehyde prior to wax embedding, sectioning, and staining with haematoxylin and eosin (HE). Histological scores were performed by a pathologist in a blind method using the criteria [10]. Each section was graded with a range from 0 to 4 as to depth of the lesion, extent of ulceration and with a range from 0 to 3 as to degree of inflammation. These changes were indicated according to the following scale: depth of the lesion, 0= none, 1= mucosa, 2= submucosa, 3= muscularispropria, 4= serosa; degree of inflammation, 0= none, 1= slight, 2= moderate, 3= severe; extent of ulceration, 0= none, 1= mild surface (0-25%), 2= moderate surface (25-50%), 3= severe surface (50-75%), 4= extensive-full thickness (more 75%).

**Disease activity index (DAI) analysis**

The DAI was determined at the end of the treatment period using the DAI scoring system described in the previous report [11]. The scores for stool consistency and occult blood for each rat were added and then given a DAI.
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Table 3. Evaluation of stool consistency after TNBS induction and ZD treatment in UC rats

<table>
<thead>
<tr>
<th>Stool Consistency</th>
<th>Day 2</th>
<th>Day 5</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Model</td>
<td>1.15 ± 0.45**</td>
<td>2.25 ± 0.25**</td>
<td>2.85 ± 0.36**</td>
</tr>
<tr>
<td>SASP</td>
<td>1.15 ± 0.5**</td>
<td>2.10 ± 0.35*</td>
<td>1.15 ± 0.15**</td>
</tr>
<tr>
<td>Low dose</td>
<td>1.15 ± 0.42*</td>
<td>2.20 ± 0.35*</td>
<td>2.55 ± 0.35*</td>
</tr>
<tr>
<td>Moderate dose</td>
<td>1.10 ± 0.35*</td>
<td>2.05 ± 0.25*</td>
<td>2.15 ± 0.25*</td>
</tr>
<tr>
<td>High dose</td>
<td>1.05 ± 0.25**</td>
<td>1.90 ± 0.15*</td>
<td>1.10 ± 0.25**</td>
</tr>
</tbody>
</table>

Scoring of stool consistency: 0= no diarrhea; 1= mild; 2= severe; 3= severe and bloody diarrhea. The scores of each group were the average of 6 animals. *P<0.05, **P<0.01 compared to model group. ZD, Zhenrenyangzang decoction; SASP, salazosulfapyridine.

Real time PCR analysis mRNA expression

mRNA was extracted from colonic tissue samples using Trizol according to the manufacturer’s protocols (Invitrogen) and RT-PCR was performed according to the instructions of the Takara RNA PCR kit 3.0 (AMV). An equal amount of cDNA from each sample was amplified using primers specific to each gene (Table 2). Real time quantitative PCR was performed in a 25 μl final volume containing the 2 X SYBR Green I master mix (Qiagen, Hilden, Germany). DNA amplification was done using a thermocycler under the following conditions: 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 90 s. The relative quantification of expression of the gene was normalized to the internal control gene actin and determined using the 2^ΔΔCt method as described [12]. The fold changes of the treated over the control group were calculated.

Western blotting assay

Proteins (50 μg) that were extracted from colonic mucosal scrapings were subjected to SDS-polyacrylamide gel electrophoresis after dissociation by boiling (5 min) in 2 X loading buffer (0.125 M Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.05% bromphenol blue). After electrophoresis proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were incubated with 1:200 rabbit polyclonal IkB-α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:500 mouse monoclonal phosphorylated p38MAPK antibody (Santa Cruz Biotechnology, Inc.), 1:200 mouse monoclonal TLR2 antibody (Santa Cruz Biotechnology, Inc.). Loading control was performed by using a mouse monoclonal antibody to GAPDH (1:2000; Santa Cruz Biotechnology, Inc.). The density of Western blotting bands was measured by using the Meta Morph 7.5 Video image Analysis System (Molecular Devices) and presented as relative density against density of GAPDH bands.

Statistical analysis

Data were presented as mean ± standard deviation. Differences among groups were analyzed using one-way ANOVA with SPSS 11.0 software. P<0.05 was considered statistically significant.

Results

ZD prevents TNBS-induced histopathological change in UC rats

In the normal group, the colonic mucosa was intact. The submucosal muscle and mucosal epithelium was continuous and integrated. In the model group, the colon lumen dilated and colonic wall thickened, and several irregular ulcers were accompanied by obviously peripheral mucosal hyperemia and edema. Tissue adherence and scar were also found in model
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Table 4. Effects of ZD on macroscopic and histological damage scores in UC rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g/kg)</th>
<th>Macroscopic score</th>
<th>Histological score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Saline (10 ml/kg)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Model</td>
<td>Saline (10 ml/kg)</td>
<td>6.15 ± 0.26**</td>
<td>9.17 ± 0.34**</td>
</tr>
<tr>
<td>SASP</td>
<td>0.5</td>
<td>2.12 ± 0.31*</td>
<td>3.15 ± 0.16*</td>
</tr>
<tr>
<td>High dose</td>
<td>8</td>
<td>2.64 ± 0.27**</td>
<td>3.42 ± 0.38*</td>
</tr>
<tr>
<td>Moderate dose</td>
<td>4</td>
<td>4.58 ± 0.39*</td>
<td>5.31 ± 0.22*</td>
</tr>
<tr>
<td>Low dose</td>
<td>2</td>
<td>6.02 ± 0.13*</td>
<td>8.92 ± 0.23*</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 compared to model group. ZD, Zhenrenyangzang decoction; SASP, salazosulfapyridine.

In UC rats, the tissue of model rats showed neutrophils, eosinophils and lymphocytes infiltration. In mucosa, part of glands missed, goblet cells reduced, irregular ulcer and inflammatory granuloma formed. In contrast, the ulcer area significantly reduced and edema disappeared in the SASP and ZD treated groups. And the high dose of ZD group more greatly relieved the lesions than the low dose group (Figure 1).

ZD improves clinical symptoms in UC rats

After administration of TNBS, animals developed colitis associated with soft stool and diarrhea (Table 3). All TNBS-treated rats (Model group) had profound weight loss compared with the weight obtained in normal group (P<0.01). Treatment with SASP or different dose of ZD gradually recovered the lost body weight beginning on day 3, accompanied by alleviating symptoms (Figure 2; Table 3).

ZD reduces colonic macroscopic damage and DAI scores in UC rats

Control normal animals showed no colonic damage and the colonic damage score was zero. Compared to the normal group, the macroscopic and histological damage scores of colon tissues in the model group significantly increased (P<0.01). Moreover, the scores were significantly lower in the SASP and ZD groups compared with the model group (P<0.05). The scores in the SASP group were lower than that in the ZD groups (P<0.05). And significant changes have been observed in the different doses of ZD groups (P<0.05, Table 4).

Also, we evaluated the severity of colitis based on the DAI scores. The higher the DAI score, the more severe the colitis. We found that the DAI scores of the different dose of ZD or SASP groups were significantly lower than those of the model group, and the high dose of ZD is the lowest. In contrast, the DAI score of the normal group was nil (Figure 3). These results suggest that oral administration of ZD or SASP clearly attenuated the impairment to the colon tissues.

mRNA expression of NF-κB, p38MAPK and TLR2

The expression levels of NF-κB and p38MAPK mRNA were significantly higher in the model group compared with those in the normal group. However, compared to the UC model group, NF-κB and p38MAPK mRNA expression in the ZD or SASP treatment groups were reduced, and the reduction by ZD was dose dependent. In contrast, a significant decrease of TLR2 mRNA expression in TNBS model group compared with the normal control group. But ZD or SASP treatment groups significantly activated the TLR2 mRNA expression comparing to the model group. And the activation of expression by ZD was in a dose dependent manner (Figure 4).

IκB-α, p38MAPK and TLR2 protein expression in colon tissues

As depicted in Figure 5, western blotting results showed that TLR2 protein level was obviously higher in ZD-treated rat tissues compared with
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that in TNBS induced model rats. And the ZD treatment followed a dose-dependent manner. In contrast, administration of ZD attenuated the protein expression of IκB-α subunit and phosphorylated p38MAPK (p-p38MAPK) in comparison with model group. The p-p38MAPK protein level was the lowest in the high dose ZD group. However, there were no obvious differences for the IκB-α protein expression among the low, moderate and high ZD groups (Figure 5).

Discussion

TNBS-induced UC model in present study was widely adopted to mimic acute phase in human UC and assess the effects of drugs [13]. It was characterized by extensive infiltration of inflammatory cells and colonic ulcers. Similar to acute phase, these major histological features also appeared in chronic phase of UC. In this study,
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Our results indicated that oral treatment with ZD significantly promoted the recovery of colitis and inhibited the inflammatory response, which were verified by macroscopic and histological examination, the decreased expression of NF-κB and p38MAPK, as well as the enhanced level of TLR2 in ulcerative colon tissues.

Several agents used in the management of UC, such as corticosteroids, sulfasalazine, and 5-aminosalicyclic acid, have been documented to regulate the NF-κB function [14]. NF-κB controls many important biological decisions, from formation of dorsal-ventral polarity in insects to activation of inflammatory and innate immune responses. The key event in NF-κB activation is IκB phosphorylation, the next logical step is to search for a stimulus-responsive IκB kinase (IκK) that could catalyze this event. The activated IκK complexes then phosphorylate IκB subunits in NF-κB: IκB complexes, triggering their ubiquitin-dependent degradation, and the activation of NF-κB [15]. In our study, we measured the mRNA and protein level of NF-κB or IκB-α. Rats in the TNBS model group exhibited higher mRNA levels of NF-κB than those in the normal control group. However, the NF-κB mRNA levels were reduced by ZD treated in a dose-dependent manner, compared with the TNBS model group, the NF-κB mRNA levels in the ZD high dose, moderate dose and SASP groups were obviously downregulated. Moreover, we did not observe obvious change of the IκB-α protein expression among these groups.

Our results also demonstrated that the mRNA and protein levels of (phosphorylated) p38MAPK were significantly decreased in ZD treatment groups comparing with TNBS model group. MAPK family of proteins is the principal regulator of gene expression, and critically controls transcription of a number of cytokine genes. P38MAPK is particularly involved in the inflammatory process, inflammatory stimuli being strong activators of this kinase, and its activation is required for inflammatory gene transcription in vitro [16]. P38MAPK has been reported to contribute to the hypercontractility and increase the Ca²⁺ sensitization in murine experimental colitis [17]. A specific p38MAPK inhibitor, SB203580, was found to have a dichotomal effect in TNBS induced mice. Treatment with SB203580 does not ameliorate TNBS colitis although it does prevent IFN-γ and IL-12p70 production [18]. This indicates that p38 MAPK may have a broader role in the mucosal immune response and is not only responsible for the production of proinflammatory cytokines but may also be involved in counter regulatory responses.

We also examined the mRNA and protein expression levels of TLR2 in ZD groups and TNBS model group. The Toll-like receptors (TLRs) are key regulators of the innate immune system in the gut through the induction of pro-inflammatory and immune modulatory responses in many cell types including immune and epithelial cells [19]. TLR1 to 9 have been reported detectable in human intestine at least in the mRNA levels both in healthy and disease conditions [20]. In particular TLR2 and TLR4 mRNA and protein have been reported to be upregulated in UC and in other intestinal inflammatory conditions [21-24]. Consistent with the previous findings, by qPCR and western blotting assay we showed that the level of TLR2 mRNA and protein in colonic tissues from ZD groups were significantly higher than that from UC model group. Increased TLR2 expression may enhance the recognition and presentation of antigens by inflammatory cells, rendering the normal bacterial flora to be identified, thereby breaking immune tolerance and leading to intestinal injury.

In conclusion, our results showed that treatment with ZD enema attenuates the clinical symptoms of TNBS-induced colitis, and ZD has a significant curative effect through activating TLR2 as well as inhibiting NF-κB and p38MAPK levels in UC rats. These results provide supporting evidence for the clinical application of ZD in UC. Protective factors will also be examined in the future in order to elucidate the therapeutic mechanism of ZD enema.

Acknowledgements

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Disclosure of conflict of interest

None.

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