ORIGINAL ARTICLE
ANIMAL STUDY

Huanghou Zhixie dropping pills alleviate ulcerative colitis via MAPK/ NF-κB signalling pathway

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Abstract

Objective: To investigate the effect and possible mechanism of Huanghou Zhixie dropping pills in ulcerative colitis treatment.

Method: The study was conducted at the Inner Mongolia Medical University, Hohhot, China, between March and November 2022, and comprised specific pathogen-free Bagg albino (C57BL6) adult female mice. Ulcerative colitis was induced using dextran sulfate sodium. The mice were divided randomly into control group, model group, low-dose Huanghou Zhixie dropping pills HZDP-L group, medium-dose Huanghou Zhixie dropping pills HZDP-M group, high-dose Huanghou Zhixie dropping pills HZDP-H group, and mesalazine group. The quality of the model was verified through Disease Activity Index, Colonic Mucosal Damage Index, and Histopathological) analysis. Enzyme-linked immunosorbent assay was used to measure inflammatory factors related to ulcerative colitis, including Interleukin-1 beta (IL-1β), Transforming Growth Factor-beta (TGF-β), Interleukin-4 (IL-4), Tumour Necrosis Factor-alpha (TNF-α), Interleukin-6 (IL-6) and Interleukin-10 (IL-10). Western blot analysis was conducted to examine the relevant factors, such as cyclooxygenase-2, inducible nitric oxide synthase, Protein 65 (P65), Mitogen-Activated Protein Kinase (MAPK), Phosphorylated Mitogen-Activated Protein Kinase (p-MAPK) and Phosphorylated Protein 65 (p-P65). Data was analysed using SPSS 20.

Results: Of the 36 female mice aged 8 weeks and having mean weight 18±2g, 6(16.66%) were in each of the 6 groups. Huanghou Zhixie dropping pills significantly reduced dextran sulfate sodium-induced ulcerative colitis symptoms, weight-loss and colon damage (p<0.05). Huanghou Zhixie dropping pills decreased the expression of pro-inflammatory TNF-α, IL-1β, TGF-β, and IL-6 factors, and increased the expression of anti-inflammatory IL-4 and IL-10 factors in the colon tissue of the mice (p<0.05). Additionally, Huanghou Zhixie dropping pills reduced the expression of cyclooxygenase-2, inducible nitric oxide synthase, P-MAPK and P-P65 in the colon tissue (p<0.05).

Conclusion: Huanghou Zhixie dropping pills alleviated colitis by modulating inflammatory factors through the Mitogen-Activated Protein Kinase/Nuclear Factor kappa-light-chain-enhancer of activated B cells (MAPK/NF-κB) signalling pathway. The Huanghou Zhixie dropping pills could be a potential therapeutic option for ulcerative colitis treatment, offering a novel approach to managing the disease by targeting specific inflammatory pathways.

Keywords: HZDP, Ulcerative colitis, MAPK/NF-κB signaling pathway, Inflammatory factors. (JPMA 75: S-18 [Suppl. 02]; 2025)

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Introduction

Ulcerative colitis (UC) is a type of inflammatory bowel disease. The incidence of the disease is high in Western countries, but there is also a rapid upward trend of incidence in some countries in Asia, the Middle East, Africa and South America.[1] Drugs, such as glucocorticoids, 5-aminosalicylic acid, and tumour necrosis factor (TNF) blockers, are commonly used for treatment.[2,3] These drugs have significant side effects, such as lupus-like symptoms, infections, autoimmunity, and lymphoma.[4] Accordingly, the development of a new drug is urgently needed.[5]

Clinical practice has proved that traditional Chinese medicine (TCM) has a specific effect on UC. TCM has fewer side effects and can significantly improve the quality of life of patients, and reduce the social and economic burden.[6,7] Originally, Huanghou Zhixie dropping pills (HZDP) were Magnolia officinalis pills, composed of Magnolia officinalis, Coptis, wood incense, and dried ginger, and processed by modern technology of Carbon Dioxide (CO_2) supercritical extraction.[8,9] Clinically, HZDP can be used to treat acute abdominal pain, diarrhoea and vomiting.[10,11] UC is characterised by several distressing symptoms, including abdominal pain, diarrhoea, purulent mucous membrane, bloody stool, internal urgency and weight-loss.[12-14] These symptoms significantly impact the quality of life of those affected.[15] While HZDPs have shown promise in treating

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UC, the exact mechanism by which they exert their therapeutic effects remains unclear.[16-18] Further research is needed to fully understand how HZDP works to alleviate the symptoms of UC. This will help in optimising its use and potentially improving treatment outcomes for patients suffering from this chronic inflammatory condition.

Due to the complex composition of the HZDP, most of the current studies focus on a specific chemical composition of each herb. Further research on TCM compound HZDP is needed. The current study was planned to explore the therapeutic mechanism of HZDP on UC.

Materials and Methods

The study was conducted at the Inner Mongolia Medical University (IMMU), Hohhot, China, between March and November 2022, and comprised specific pathogen-free Bagg Albino (C57BL/6) adult female mice procured from the market (SPF Beijing Biotechnology Co., Ltd., China). The mice were housed at room temperature (24±1°C) and fed freely. The study complied with the relevant regulations of the IMMU animal ethics committee. The mice were divided randomly into control group, model group, low-dose HZDP (HZDP-L) group, medium-dose HZDP (HZDP-M) group, high-dose HZDP (HZDP-H) group, and mesalazine group.

According to the relevant literature,[19] except for the control group, the mice drank 3% dextran sodium sulfate (DSS) solution freely after adaptive feeding. The mice were monitored daily. When the mice exhibited weight-loss, reduced movement, and bloody stool, the colons of the mice were analysed by haematoxylin-and-eosin (H&E) staining. When the colons were inflamed, the model was established successfully.

According to the conversion formula,[20] the dose for mice was 12.3-fold that of humans. The calculated equivalent dose was set as HZDP-M group 196.8mg/kg, the concentration of the HZDP-H group was twice that of the HZDP-M group (393.6mg/kg, and that of the HZDP-L group was half that of the HZDP-M group 98.4mg/kg). The dose of mesalazine-sustained release granules (adisa) was 0.73g/kg. The control and model groups were given the same dose of double-distilled water.

The primary conditions of the mice were judged according to the Disease Activity Index (DAI) scoring system: normal weight=0 point, loss 1-5%=1 point, loss 6-10%=2 points, loss 11-15%=3 points, loss >15%=4 points; normal stools=0 point, loose stools=2 points, diarrhoea=4 points; absence of blood in stool=1 point, slight blood=2 points and massive blood=4 points. The DAI score was calculated as the average of three specific scores, exploring whether or not HZDP, either alone or in combination with mesalazine, could effectively alleviate the symptoms of the disease.[21]

After two weeks of the treatment, the colon of the experimental subjects was anaesthetised using a 0.3% pentobarbital sodium solution to ensure accurate measurements without causing pain. The length of the colon was then measured to assess any changes or improvements. Following this, a portion of the colon was preserved in a 4% formalin solution for detailed pathological examination, which helped in identifying any cellular or tissue-level changes. The remaining part of the colon was placed in a cryopreservation tube and stored at -80°C to preserve it for further biochemical or molecular analyses. This process ensured that the samples remained viable for further examination, providing comprehensive data on the effects of the treatment.

The score of colonic mucosal injury was evaluated according to the Cytomix Medium Depletion Index (CMDI): normal mucosa=0 point, local congestion without ulcers=1 point, mucosal ulcer without intestinal wall congestion and thickening=2 points, local inflammatory ulcers=3 points, two inflammatory ulcers=4 points, large ulcers extending ≥1cm along the colon=5 points, and ulcers ≥2 cm=6-10 points. For every 1cm of lesion, 1 point was added. No adhesion was scored 0, slight adhesion (still able to separate from the surrounding tissue) was scored 1, and severe adhesion was scored 2 points.[22]

The pathological damage to the tissue was scored using the standard pattern: normal and non-inflammatory=0, mucosa cup-shaped cells lost, mild inflammation, and inflammation=1, mucosa cup-shaped cells severely lost, and moderate inflammation=2, lack of mucosa hidden nests, extensive inflammation, and thickening of mucosal oedema=3, and large-scale hidden nest loss and low mucosal inflammatory inflammation=4.[23]

Data was analysed using SPSS 20. Data were presented as mean±standard deviation, providing a clear understanding of the average values and the variability within the dataset. To determine the statistical significance of the findings, one-way analysis of variance (ANOVA) and repeated measure ANOVA were employed. $P<0.05$ was considered statistically significant.

Results

Of the 36 female mice aged 8 weeks and having mean weight 18±2g, 6(16.66%) were in each of the 6 groups. The DAI score for the model group was significantly higher than that of the control group ($p<0.05$). However, after treatment with HZDPs and mesalazine, the DAI score was significantly reduced ($p< 0.05$). The effectiveness in the HDZP-M group was comparable to that of the mesalazine group alone and

was superior to the other treatment groups ($p>0.05$), indicating that HZDP, either alone or in combination with mesalazine, could effectively alleviate the symptoms of the disease (Figure 1).

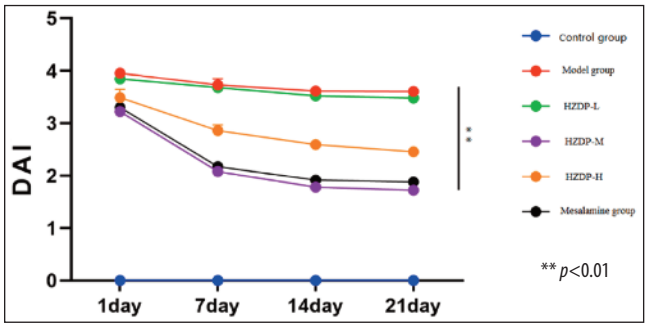

Figure-1: Figure 1: Disease Activity Index (DAI) score. (HZDP-L: low dose of Huanghou Zhixie dropping pills, HZDP-mM: medium dose of Huanghou Zhixie dropping pills, HZDP-H: high dose of Huanghou Zhixie dropping pills).

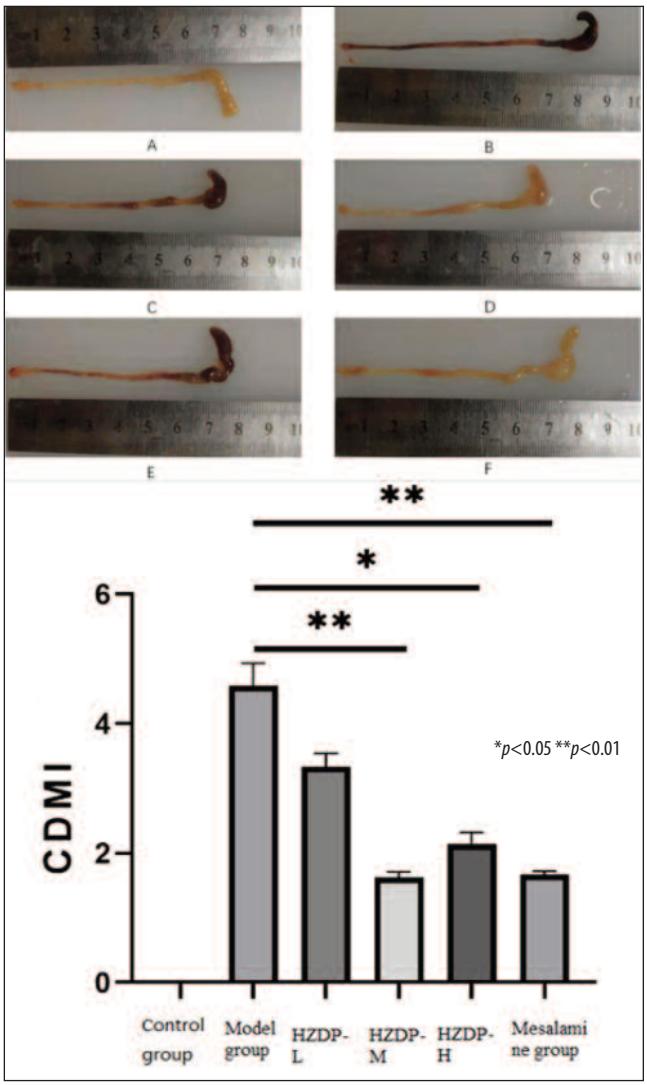

Figure-2: Macro comparison of colonic tissue samples in each group.

(CDMI: Colonic Mucosal Damage Index, HZDP-L: low dose of Huanghou Zhixie dropping pills, HZDP-mM: medium dose of Huanghou Zhixie dropping pills, HZDP-H: high dose of Huanghou Zhixie dropping pills.

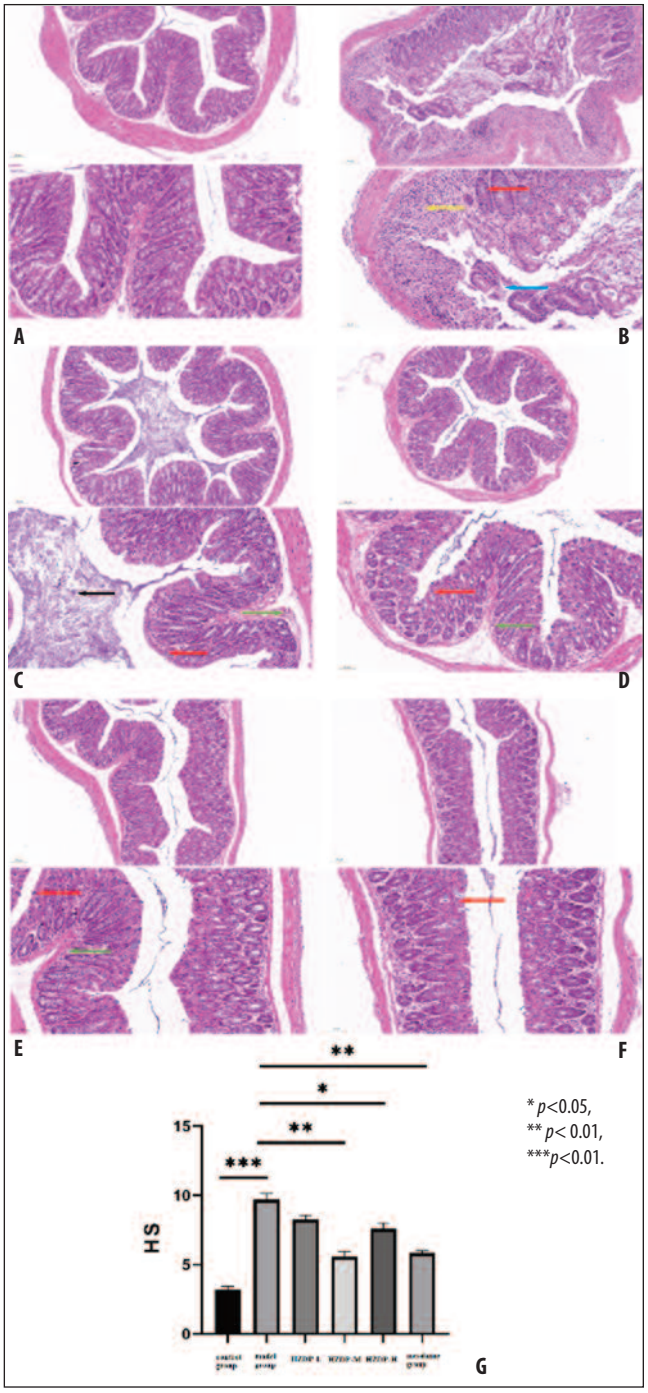

Figure-3: Macro comparison of colonic tissue samples in each group.

A: Control group, B: Model group, C: Huanghou Zhixie dropping pills (HZDP)-L group, D: HZDP-M group, E: HZDP- H group, F: Mesalazine group, G: Histopathology score.

the structure of the mucosal layer and mucosal epithelial cells →
inflammatory cells and fibrous tissue →
goblet cells →
inflammatory exudate →
inflammatory cell →

(HZDP-L: low dose of Huanghou Zhixie dropping pills, HZDP-mM: medium dose of Huanghou Zhixie dropping pills, HZDP-H: high dose of Huanghou Zhixie dropping pills)

The macro comparison of colonic tissue samples showed that the score of the model group was significantly higher than that of the control group, while the CDMI score of HZDP groups significantly decreased (p<0.05). HZDP-M and HZDP-H groups were significantly different from the model group (p<0.01 and p<0.05, respectively). HZDP-M had the same effect as mesalazine in reducing intestinal inflammation (p>0.05) (Figure 2A-G).

There was no obvious abnormality in the colonic tissue structure of the control group (Figure 3A). The standard structure of the mucosal layer in the model group disappeared, the mucosal epithelial cells eroded and fell, the number of cup-shaped cells in the tissue decreased, and a large number of inflammatory cells and fibre tissue hyperplasia were recorded (Figure 3B). The intestinal inflammation of the HZDP-L group was reduced, although the mucous cell infiltration and inflammatory cytokines oozed in the intestine. However, the number of goblet cells in the HDZP-L group was less than that in the model group (Figure 3C). Compared to the model group, the inflammation of the colon in the HDZP-M group was significantly reduced, with a small number of inflammatory cells and goblet cells (Figure 3D). In the HZDP-H group, intestinal inflammation was reduced, and a few inflammatory cells were found in the intestinal wall (Figure 3E). A small amount of inflammation was detected in the intestines in the mesalazine group, but there was no inflammatory cell infiltration, and goblet cells were slightly reduced (Figure 3F).

The severity of tissue damage, assesses using the histopathological score (HS), was significantly elevated after inducing UC using DSS. However, treatment with HZDPs and mesalazine led to a significant reduction in the HS score (p<0.05). The reduction in HS score was particularly notable in the HZDP-M group compared to the model group (p<0.01) (Figure 3G).

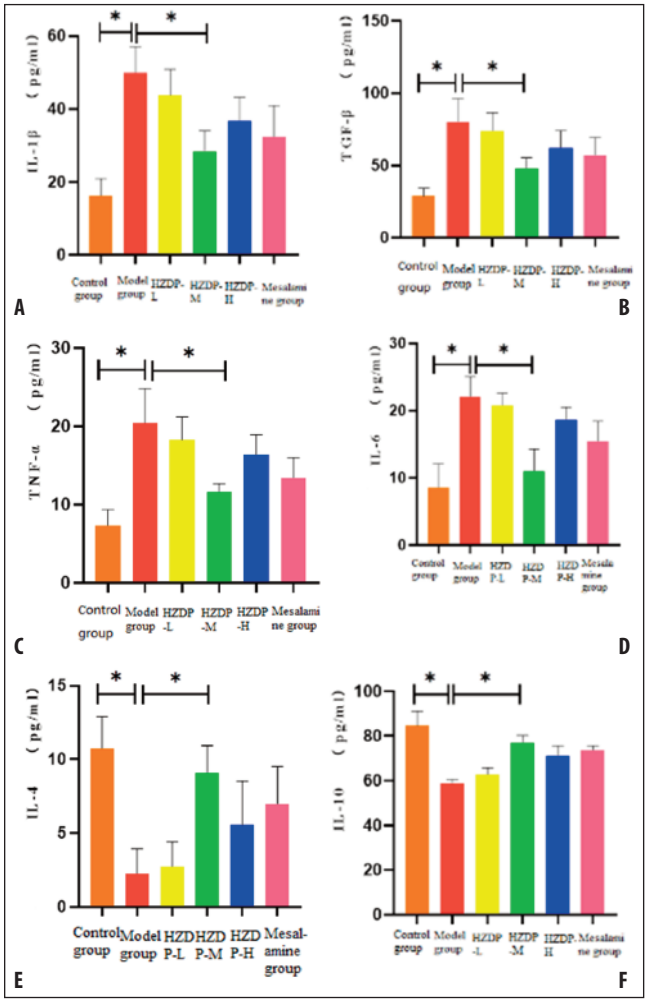

Figure-4: Comparison among groups; A: Control group, B: Model group, C: Huanghou Zhixie dropping pills (HZDP)-L group, D: HZDP-M group, E: HZDP-H group, F: Mesalazine group.
(HZDP-L: low dose of Huanghou Zhixie dropping pills, HZDP-mM: medium dose of Huanghou Zhixie dropping pills, HZDP-H: high dose of Huanghou Zhixie dropping pills).
* p<0.05 ** p<0.01.

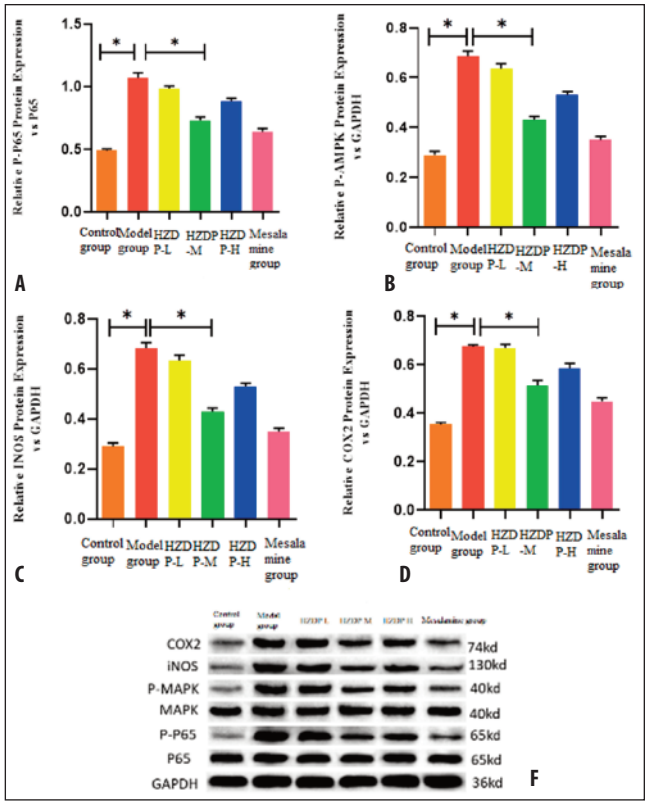

Figure-5: Expression of Cyclooxygenase-2 (COX-2), Inducible Nitric Oxide Synthase (iNOS), P65 (Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) P65), Mitogen-Activated Protein Kinase (MAPK), Phosphorylated Mitogen-Activated Protein Kinase (p-MAPK), and Phosphorylated P65 (p-p65) in colonic tissue.
**p<0.05. (GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, HZDP-L: low dose of Huanghou Zhixie dropping pills, HZDP-mM: medium dose of Huanghou Zhixie dropping pills, HZDP-H: high dose of Huanghou Zhixie dropping pills).*

Compared with the control group, the levels of inflammatory factors IL-1β, TNF-α, IL-6 and TGF-β in the model group were higher (p<0.05). After treatment, the above cytokine levels in the HZDP and mesalazine groups significantly reduced (p<0.05). Further comparison of each group of HZDP showed that the HZDP-M group had the best effect (p<0.05), which was similar to that of mesalazine (p>0.05) (Figure 4A-D). The levels of anti-inflammatory cytokines IL-4 and IL-10 decreased in the model group, and increased after treatment with HZDP and mesalazine (p<0.05). Similarly, the comparison of each group of HZDP showed that the HZDP-M group had the best effect (p<0.05). (Figure 4E-F).

After inducing UC in the mice using DSS, the levels of cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS) were significantly elevated in the model group (p<0.05). However, treatment with HZDPs significantly reduced the levels of COX2 and iNOS (p<0.05). Additionally, HZDP inhibited the phosphorylation of p65 and MAPK. Among the different HZDP treatment groups, the HZDP-M group showed the most significant improvement (p<0.05), with effects comparable to those of the mesalazine group (p>0.05) (Figure 5).

Discussion

Some studies have shown that berberine, the main component of Coptis, reduces the colonic injury in mice with UC induced by DSS, and regulates the intestinal flora by increasing lactic acid bacteria, carbohydrate-hydrolyzing bacteria, and reducing conditional pathogenic bacteria.[24] Magnolol, the main component of Magnolia officinalis, has an anti-inflammatory effect and can promote the integrity of the mouse intestine, thereby alleviating colitis caused by DSS. The treatment mechanism might be the activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signal pathways.[25] Costunolide can pass through NF-kB, signal transducer and activator of transcription 1/3 (STAT1/3) and protein kinase B (Akt) signalling pathways to reduce acute colitis in mice caused by DSS, and several studies have reported the compatibility of Coptis chinensis and Aucklandia in being effective on UC.[26] One of the main active components of dried ginger, 6-gingerol, can inhibit the over-activation of the Notch pathway and repair damaged mucosal tissues by regulating the differentiation balance of colon epithelial secretory cell lines and absorption cell lines.[27] However, the therapeutic mechanism of pills formulated by the combination of these 4 TCMs on UC has not been proven.

In the current study, the DAI and CDMI scores of the model group were higher than those of the control group. Histopathological examination showed that the typical structure of colonic mucosa in the model group disappeared, the number of goblet cells decreased, and a large number of inflammatory cells infiltrated. Abundant intestinal inflammation proved that the UC model was established successfully. This study closely mimics the acute-phase manifestations of UC without the chronic-phase manifestations of UC, i.e., no significant colon shortening. After the mice were grouped, the model mice were treated with HZDP and mesalazine, respectively. The comparative analysis showed that HZDP improved weight-loss, diarrhoea, blood in the stool, and colon damage in the UC model mice. The HZDP-M group showed therapeutic effects similar to mesalazine.

Another study showed that Interleukin-1 beta (IL-1β) was highly expressed in the colon mucosa of UC cases.[28] Studies have confirmed that IL-6 is associated with the development of UC and UC-associated colorectal cancer[29] because it can activate signal transducer and activator of transcription 3 (STAT3), which plays a vital role in the inflammatory response. Also, IL-6 and its receptors are elevated in patients with inflammatory bowel disease, including UC and Crohn's disease.[30] A study pointed out that TNF-α was very critical in the pathogenesis of inflammatory bowel disease, and anti-TNF drugs could effectively treat UC.[31] Moreover, activated TNF-α can increase the secretion of pro-inflammatory cytokines IL-1, IL-6, IL-8 and Interferon-gamma (IFN-γ), and inhibit the secretion of anti-inflammatory factor IL-10, thus aggravating the inflammatory response.[32] Several studies have reported that specific cytokines, such as IL-1β， IL-6, IL-21, IL-23 and Transforming Growth Factor-beta (TGF-β), can induce T-helper 17 (Th17) polarisation and then aggravate UC.[33] IL-10 downregulates TNF-α and iNOS, thereby inhibiting colitis and cancer.[34] IL-4 inhibits the production of IL-1β and TNF-α by mononuclear macrophages, and downregulates the ability of activated mononuclear macrophages to secrete oxygen-free radicals in order to maintain normal intestinal immunity. TGF-β is a multifunctional polypeptide produced by a variety of lymphocytes and non-lymphocytes.[35] It has immuno-suppressive and anti-inflammatory effects and is closely related to UC. Previous studies have shown that the regulation of TGF-β signalling can be used as a potential therapy for inflammatory bowel disease.[36] Thus, it can be inferred that the above-mentioned inflammatory factors are involved in the occurrence and development of UC, and these can interact with each other to aggravate or reduce intestinal inflammation. Pro-inflammatory factors were significantly increased, and anti-inflammatory factors were decreased in the intestinal tissue of the mice. The results showed that HZDP could promote the dynamic balance of

the two factors by upregulating the content of anti-inflammatory factors and down-regulating the content of pro-inflammatory factors so as to achieve the therapeutic effect of UC. However, the current study observed that HZDP reduced the secretion of the anti-inflammatory factor TGF-β, which is different from other drugs for the treatment of UC.[37]

In 1996, Professor M.F. Neurath found that blocking NF-κB reduced or completely eradicated intestinal inflammation. Hence, he proposed that NF-κB is related to intestinal inflammation.[38] Current studies have identified five proteins in the NF-κB family: RelA (p65), RelB, c-Rel, p50/p105, and p52/p100 (all subunits of NF-κB family) , which mainly exist in the form of dimers, and p50/p65 (heterodimers of NF-κB) is the most common dimer.[39] NF-κB/p65 is a significant factor that can regulate protein-coding genes related to inflammatory signalling pathways. Its expression level was low in normal intestinal mucosa, but was significantly upregulated in UC patients and colitis mice.[40] During the development of colitis, the upregulated expression of NF-κB/p65 results in the excessive release of inflammatory factors.[41] The intestinal environment is disrupted due to increased secretion of pro-inflammatory factors or insufficient secretion of anti-inflammatory mediators in the intestinal epithelial barrier, which is an essential factor in the development of colitis.[42]

Professor Dongqiu Wang pointed out that in the pathogenesis of UC, oxidative stress (OS) is the main trigger of colon tissue damage, and NF-κB can also induce the secretion of iNOS and COX2 by intestinal mucosa.[43] The activation of iNOS and COX2 can induce the production of Reactive Oxygen Species (ROS) and active nitrogen, and inhibit the antioxidant system, thus causing damage to colorectal mucosa.[44] However, Professor Monica Guma found that continuous activation of the NF-κB pathway in epithelial cells does not cause tissue damage, and requires a cytokine-activated MAPK signalling pathway.[45] Professor Ma Xiaobin pointed out that the MAPK signalling pathway is a major upstream component of the NF-κB signalling pathway. Activation of MAPK can induce phosphorylation of NF-κB/p65.[46] Some studies have found that silent information regulator 1 (SIRT1) can inhibit intestinal inflammation through the MAPK/NF-κB signalling pathway, suggesting that MAPK/NF-κB plays a vital role in the development of UC.[47]

The current study showed that after successful DSS model establishment, the contents of P65, MAPK, Phosphorylated P65 (P-P65), and Phosphorylated Mitogen-Activated Protein Kinase (P-MAPK) and their downstream inflammatory factors COX2 and iNOS in the colon tissue of the mice increased. This suggested that the MAPK/NF-κB signalling pathway in the intestinal tissue is overstimulated during the occurrence of UC, which leads to the overproduction of pro-inflammatory factors, COX2 and iNOS, thereby causing UC. The levels of each factor were corrected after applying HZDP. Thus, it could be deduced that HZDP inhibits the excessive activation of MAPK/NF-κB signalling pathway, thereby reducing the content of downstream pro-inflammatory factors, such as COX2 and iNOS, and exerting an anti-inflammatory effect in UC.

Conclusion

HZDP helped treat UC by influencing the balance between pro-inflammatory and anti-inflammatory cytokines. This balance was achieved through the MAPK/NF-κB signalling pathway, which played a crucial role in regulating inflammation. By modulating this pathway, HZDP reduced inflammation, and promoted healing in UC subjects. This mechanism highlighted the potential of HZDP as an effective treatment for UC.

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Conflict of Interest: None.

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