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Evidence for the complementary and synergistic effects of the three-alkaloid combination regimen containing berberine, hypaconitine and skimmianine on the ulcerative colitis rats induced by trinitrobenzene-sulfonic acid

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ABSTRACT

Ulcerative colitis involves complicated etiology and presents diverse symptoms including intestine inflammation, bowel pain and diarrhea. Anti-inflammatory drugs are the mainstay in patient care, accompanied with antidiarrhea and analgesic agents used as symptomatic treatment. A classic traditional Chinese medicine formula, *Fructus Mume* pill (FMP), showed remarkable therapeutic efficacy in treating ulcerative colitis. However, since it contains many herbs and countless chemicals, the underlying mechanism is not clear. In this study, we selected three alkaloids from FMP, namely, berberine, hypaconitine and skimmianine to study the individual drug effect and compare these results with the BHS combination on: 1) The recovery of ulcerative colitis rats induced by trinitrobenzene-sulfonic acid. 2) Mice with xylene-induced acute exudative edema and acetic acid-induced writhing. 3) Gastrointestinal transit inhibition, and 4) the response of HT29 cells after treatment with lipopolysaccharide. We found that the compound hypaconitine showed a potent analgesic effect, while skimmianine acted as an antidiarrhea agent and the component berberine was the key agent exerting anti-inflammatory effect. However, since berberine killed the commensal bacteria and induced lipopolysaccharide release, it could at the same time aggravate colon inflammation. The three-alkaloid combination BHS produced complementary and synergistic effects in colon inflammation recovery, relieving acetic acid-induced bowel pain and xylene-induced acute exudative edema. BHS also decreased lipopolysaccharide production and enhanced the therapeutic efficacy. It is hoped that this study will lay the foundation to further dissect and understand the FMP formula to improve the treatment with simplified and well defined drug combinations for this dreadful disease.

1. Introduction

Inflammatory bowel disease, including ulcerative colitis and Crohn's disease, is an autoimmune disease associated with immunological disorder, genetic susceptibility, and microbial population disorder (Packey and Sartor, 2008). The main clinical manifestations include abdominal pain, diarrhea and purulent stools, recurrent attacks and relapses (Velayos and Sandborn, 2007). Anti-inflammatory and immunomodulatory drugs as well as antibiotics have been used to relieve the symptoms of patients with inflammatory bowel disease (Saw et al., 2010; Di Paola et al., 2009). Steroids and non-steroidal anti-inflammatory drugs are effective for temporary symptomatic relief. However, severe side effects have limited their use particularly for long-term therapy (Tung and Warner, 2002; Lakatos and Lakatos, 2008). Based on these diversified etiology and manifestations of inflammatory bowel disease, it is reasonable to believe that a combination of more than one type of drugs with different mechanisms is required to treat this disease. In the traditional Chinese medicine, combination therapy has been advocated for over 2000 years. It is a unique ancient Chinese medical science in treating various diseases (Wang et al., 2008).

The *Fructus Mume* pill (FMP) is a classic prescription which has been used to treat chronic diarrhea and lingering dysentery and has shown significant clinical efficacy (Yang and Zhang, 2007). We have also confirmed the significant therapeutic effects of FMP in trinitrobenzene-sulfonic acid (TNBS) induced experimental colitis in rats (Liu et al., 2009). FMP not only completely prevented diarrhea, reduced

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the area of ulceration, but also showed immunoregulatory effects and promoted the balance of the commensal flora. FMP consists of ten Chinese herbs (Table 1). Although FMP is a classic formula for anti-inflammation, analgesia and antidiarrhea (Gao and Guo, 2006; Xie et al., 2001; Zhang and Bai, 2007), the underlying mechanism in the treatment of colitis remains unknown.

FMP contains many alkaloids that include berberine, schinifoline, aconitine, hypaconitine, aconine, higenamine, skimmianine, etc. Berberine, the main alkaloid in *Rhizoma coptidis* and *Cortex phellodendri*, possesses significant bacteriostatical and anti-inflammationary effects. In both Western and the traditional Chinese medicine, berberine is known to be effective in treating colitis induced by TNBS (Zhou and Mineshita, 2000). Skimmianine, hypaconitine, aconine and higenamine from processed *Radix aconiti lateralis* or *Herba Asari* showed analgesic effect. And skimmianine from *Pericarpium zanthoxyli* showed intestinal relaxative effect. Based on these observations, it is reasonable to believe that the alkaloids from FMP are important to colitis therapy. In this study, we screened three alkaloids from FMP, namely, berberine (B), hypaconitine (H) and skimmianine (S) to study the individual drug effect and compare these effects with the three-drug (BHS) combination on: 1) The recovery of ulcerative colitis rats induced by TNBS, 2) Mice with xylene-induced acute exudative edema and acetic acid-induced writhing, 3) Gastrointestinal transit inhibition, and 4) The response of HT29 colon epithelial cells after treatment with lipopolysaccharide (LPS). It is hoped that this study will lay the foundation to further dissect and understand the classical FMP formula and to improve the treatment with simplified and well defined drug combinations for this dreadful disease.

2. Materials and methods

2.1. Animals

Eight week-old male Sprague–Dawley (SD) rats (weighing 220–250 g) and five week-old ICR mice (weight in 18–22 g) were obtained from the Animal Center of Fourth Military Medical University (Xian, China). The animals, four per cage, were given water and food ad libitum in a room with controlled temperature (22 ± 1 °C), humidity (50–70%), and 12 h light/12 h dark cycle in the Animal Center of the Fourth Military Medical University. And all animal experiments were conducted under the Institutional guidelines and approved by the Ethical Committee for Animal Care and Use of the Fourth Military Medical University according to an animal protocol.

Table 1
The formulation and dosage of each herb in *Fructus Mume* pill.

Name	Latin denomination	Dosage	Primary alkaloid
fructus mume	*Prunus mume (Sieb.) et Zucc.*	300 pieces	little
Rhizoma coptidis	*Coptis chinensis Franch.*	244 g	berberine
Cortex phellodendri	*Phellodendron chinense Schneid.*	64 g	berberine
processed Radix aconiti lateralis preparata	*aconitum carmichaeli Debx*	84 g	aconitine, hypaconitine, aconine, higenamine
Rhizoma zingiberis	*Zingiber officinale Rosc.*	140 g	little
Ramulus cinnamomi	*Cinnamomum cinnamom*	84 g	little
Pericarpium zanthoxyli	*Zanthoxylum bungeanum Maxim.*	56 g	skimmianine, higenamine
Herba Asar i	*Asarum heterotropoides Fr. Schmidt var. Mandshurium (Maxim.)*	84 g	skimmianine,
Radix ginseng	*Panax* Ginseng *C.A.Mey.*	84 g	little
Radix angelicae sinensis	*Angelica sinensis (Oliv.) Diels.*	84 g	little

2.2. Chemical regents

Berberine, hypaconitine and skimmianine (purity, 99%) were purchased from Shifang Longteng Botanical Products Co., Ltd (Chengdu, China). TNBS chloride (purity, 99%), and LPS (from *Escherichia coli*) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNF-α and PGE_2 ELISA kits were obtained from USCN LIFE Co., Ltd. (Wuhan, China). NF-κB/p65 and TLR4 antibodies were purchased from Abcam (Cambridge, UK and Santa Cruz USA). LBP ELISA kit was purchased from AMDL, Inc. (Tustin, CA, USA) and Protein Extraction Kit was from Bio-Rad (Hercules, CA, USA).

2.3. BHS combination preparation

FMP consists of ten Chinese herbs. The names and the dosage of each herb in FMP are listed in Table 1. According to the theory of FMP prescription, berberine, hypaconitine and skimmianine are considered as the major components of the formula, and the ratio of the three alkaloids is always berberine (6): hypaconitine (2): skimmianine (1) in each of the FMP formulations. In the present study, the selection of the dosage of each alkaloids is based on the ratio of these three alkaloids in FMP formulations.

Study from Zhou and Mineshita demonstrated that berberine showed a strong anti-inflammatory effect on TNBS-induced colitis in rats. And berberine at the dosage of 22.5 mg/kg showed the best therapeutic effect based on the report from Zhou et al. (Zhou and Mineshita, 2000) together with our preliminary studies.

For the in vitro studies, the dosage of berberine (30 μM) used to treat HT29 cells was based on the report by Hsu et al. (2007). Here, we used berberine as our positive control to define the dosage of the other two alkaloids. The dosage used for hypaconitine and skimmianine was basically according to the formulated ratio in FMP. The dosage ratio of BHS combination used in this study was 6:2:1.

2.4. Induction of experimental colitis in rats

The experimental colitis was induced in SD rats according to the well-established inflammatory bowel disease model described previously (Guo et al., 2001). In brief, the rat was lightly anesthetized with isoflurane and a polyethylene catheter (length 10 cm, diameter 2 mm) was inserted into the lumen of the colon via the anus with the tip positioned approximately 8 cm proximal to the anus. 0.25 ml of solution containing 30 mg of TNBS dissolved in 40% ethanol (vol/vol) was slowly infused into the colon. The same procedure was used with the control group but the rats were administered with normal saline instead of TNBS. All rats were fasted for 18 h before the induction of colitis.

2.5. Treatment: dose, schedule, and route of administration

The rats were randomly divided into six experimental groups: 1) rats were given saline enema and treated with saline as the same route as testing drug administration; all other rats (group 2–6) received a TNBS enema, then were randomly divided into 5 groups, and treated orally with 2) saline only (0.2 ml/day); 3) berberine [B] (22.5 mg/kg/day); 4) hypaconitine [H] (12 mg/kg/day); 5) skimmianine [S] (6 mg/kg/day); and 6) BHS (B: 18, H: 6 and S: 3 mg/kg/day). All treatments (saline, berberine, hypaconitine, skimmianine and BHS combination) were initiated 6 h after TNBS enema, once a day for seven consecutive days. The experiments were repeated three times with 3–4 rats (totally 10 rats) in each group.