Journal of Ethnopharmacology 192 (2016) 236–247

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Zhikang Capsule ameliorates dextran sodium sulfate-induced colitis by inhibition of inflammation, apoptosis, oxidative stress and MyD88-dependent TLR4 signaling pathway

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ARTICLE INFO

Article history:
Received 20 May 2016
Received in revised form
17 July 2016
Accepted 20 July 2016
Available online 21 July 2016

Keywords:
Inflammatory bowel disease
Inflammation
Apoptosis
Oxidative stress
Toll-like receptor 4

Chemical compounds studied in this article:
Dencichine (PubChem CID: 2360)
Glycyrrhizic acid (PubChem CID: 14982)
Imperatorin (PubChem CID: 10212)
Berberine (PubChem CID: 2353)
Isoimperatorin (PubChem CID: 68081)
Rhein (PubChem CID: 10168)
Aloe emodin (PubChem CID: 10207)
Emodin (PubChem CID: 3220)
Chrysophanol (PubChem CID: 10208)
Physcion (PubChem CID: 10639)

ABSTRACT

Ethnopharmacological relevance: Zhikang Capsule (ZKC) is a traditional Chinese medicine (TCM) modified from classic formulas Qi-Li-San (an ancient formula dating to Qing Dynasty) and Fu-Jin-Sheng-Ji-San (written into *The Golden Mirror of Medicine*). ZKC contains 14 kinds of materials and has been widely used for the clinical therapy of inflammatory bowel diseases (IBD) for a long time. However, the therapeutic mechanisms of ZKC are still unclear.

Aim of the study: To determine the protective effect of ZKC on dextran sodium sulfate (DSS)-induced colitis and explore the underlying mechanisms.

Materials and methods: C57BL/6 mice were fed with 3% DSS in drinking water for one week to induce experimental colitis. They were randomly assigned to six groups according to the treatment conditions. The histological changes of colon tissues were observed by hematoxylin and eosin (H&E) staining. The serum concentration of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, and IL-12) and anti-inflammatory mediators (IL-4 and IL-10) was detected by enzyme-linked immune sorbent assays (ELISAs). The production of MPO, SOD, MDA, NO, and caspase-3 was assessed by biochemical assay kits. The expression of iNOS, ICAM-1, and NF-KB was evaluated by immunohistochemistry staining. The levels of TLR4, MyD88, and TRAF6 were determined by western blot.

Results: Histologic analysis exhibited that ZKC alleviated the inflammation, loss of goblet cells, and submucosal edema induced by DSS. ZKC significantly suppressed the pro-inflammatory cytokines and promoted the anti-inflammatory mediators. The antioxidation of ZKC was indicated by increased activity of SOD and reduced production of MDA, NO, and iNOS in ZKC-treated mice. Furthermore, ZKC repressed the colonic expression of caspase-3 and the activity of the MyD88-dependent TLR4 signaling pathway.

Conclusions: This research demonstrated the protective effect of ZKC on DSS-induced colitis. For the first time, we identified four therapeutic mechanisms of ZKC, including effective inhibition of the inflammatory responses, significant alleviation of intestinal epithelium apoptosis, considerable prevention of oxidative stress, and selective down-regulation of the MyD88-dependent TLR4 signaling pathway. With high therapeutic effects and low toxic effects, ZKC exhibits great superiority over western medicines in IBD treatment.

Abbreviations: ZKC, Zhikang Capsule; TCM, Traditional Chinese Medicine; IBD, Inflammatory Bowel Diseases; DSS, Dextran Sodium Sulfate; H&E, Hematoxylin and Eosin; ELISA, Enzyme-Linked Immune Sorbent Assay; CD, Crohn's Disease; UC, Ulcerative Colitis; TNF-α, Tumor Necrosis Factor-alpha; IFN-γ, Interferon-gamma; IL-1β, Interleukin-1beta; IL-12, Interleukin-12; IL-4, Interleukin-4; IL-10, Interleukin-10; ROS/RNS, Reactive Oxygen and Nitrogen Species; MDA, Malondialdehyde; NO, Nitric Oxide; iNOS, Inducible Nitric Oxide Synthase; SOD, Superoxide Dismutase; MPO, Myeloperoxidase; ICAM-1, Intercellular Adhesion Molecule-1; TLR4, Toll-like Receptor 4; MyD88, Myeloid Differentiation Factor 88; TRAF6, Tumor Necrosis Factor Receptor-associated Factor 6; NF-KB, Nuclear Factor Kappa B; SASP, Sulfasalazine; HPLC, High Performance Liquid Chromatography; DAI, Disease Activity Index; HAI, Histological Activity Index; PBS, Phosphate Buffered Saline; IHC, Immunohistochemistry; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; PVDF, Polyvinylidene Difluoride

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http://dx.doi.org/10.1016/j.jep.2016.07.055
0378-8741/

1. Introduction

Inflammatory bowel disease (IBD) is a complex chronic inflammation of digestive tract, characterized by crypt loss and inflammatory cell infiltration in intestinal epithelium. Crohn's disease (CD) and ulcerative colitis (UC) are principal forms of IBD (Baumgart and Sandborn, 2012; Danese and Fiocchi, 2011). Multiple factors seem to induce the pathogenic process of IBD, such as, autoimmune, microbial, hereditary, environmental, and dietary factors (Ananthakrishnan, 2013; Geremia et al., 2014; Jostins et al., 2012; Knights et al., 2013; Kostic et al., 2014).

Complicated pathogenesis is involved in the development of colitis. First of all, imbalances between pro-inflammatory cytokines and anti-inflammatory cytokines are observed to play an essential role in the pathogenic process of colitis (Das, 2016). Upon the activation of macrophages and mast cells in colitis conditions, pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1beta (IL-1β), and interleukin-12 (IL-12) are markedly produced (Coccia et al., 2012; Fais et al., 1991; Globig et al., 2014; Monteleone et al., 1997). On the contrary, anti-inflammatory cytokines, namely interleukin-4 (IL-4) and interleukin-10 (IL-10), are reduced (Kotlarz et al., 2012; Sattler et al., 2014). In addition, oxidative stress takes part in the progression of colitis (Martin-Subero et al., 2015). Infiltration of inflammatory cells, including neutrophils and macrophages, results in the overproduction of reactive oxygen and nitrogen species (ROS/RNS), which induce oxidative stress and subsequent intestinal epithelium damage (Zhu and Li, 2012). Oxidative stress products, in turn, aggravate inflammatory responses. Malondialdehyde (MDA) and nitric oxide (NO) are the primary ROS/NOS products in IBD. Upon noxious stimulation, inducible nitric oxide synthase (iNOS) produces a large amount of NO and plays a critical role in colitis progression (Hokari et al., 2001). Superoxide dismutase (SOD), an endogenous anti-peroxidase, can ameliorate the peroxidation reactions and inflammatory responses in colitis (Naito et al., 2007). Myeloperoxidase (MPO) is an extensively applied hallmark of oxidative stress and gastrointestinal inflammation in colitis (Klebanoff, 2005). Furthermore, previous study has reported that inflammatory cytokine-induced apoptosis exhibited a negative influence on the epithelial tight and adherent junction structures as well as mucosal barrier functions (Su et al., 2013). Inhibition of colonic apoptosis will be beneficial for treating IBD (Kearney et al., 2013). Finally, inflammatory cytokines and lipopolysaccharide stimulate the expression of toll-like receptor 4 (TLR4) in colitis (Cario and Podolsky, 2000). Increased TLR4 recruits the adapter molecule myeloid differentiation factor 88 (MyD88), activates the downstream tumor necrosis factor receptor-associated factor 6 (TRAF6), triggers the release of nuclear factor kappa B (NF-KB), and eventually accelerates the inflammatory responses in intestinal canal (Lucas and Maes, 2013).

Currently, the pharmacotherapy of IBD mainly focuses on three aspects: inhibition of inflammatory response, modulation of immune function, and application of biological agents (Fakhoury et al., 2014). Mesalazine, olsalazine, balsalazide and glucocorticoids are used as anti-inflammatory treatment. Immune regulation medicines include azathioprine, cyclosporine, methotrexate, and 6-mercaptopurine. Infliximab, a monoclonal antibody against TNF-α, is regarded as the mainstream biological agents in IBD (Paul et al., 2013). However, in most cases, these drugs display unsatisfactory therapeutic effects, fatal side effects, and high expenses. If pharmacotherapy fails, IBD patients have to face colectomy (Danese, 2012). Thus, a more effective way with greater safety and lower cost is urgently needed for treating IBD.

On account of high therapeutic effects and low side effects, numerous formulas of traditional Chinese medicines (TCMs) are widely applied to treat various diseases (Chen et al., 2016; Hsieh et al., 2016; Shen et al., 2014; Wu et al., 2016). A multi-component formula could bind various therapeutic targets through different pathways and show a synergistic effect on the disease. Qi-Li-San (QLS), a traditional Chinese herbal formula, was first described by Qing-Yuan Xie in 1842 in *Liang Fang Ji Ye*, a book assembling more than 400 classic formulas at the Qing Dynasty. Qi-Li-San has good effects on hemostasis and analgesia and is extensively applied to gastritis, peptic ulcer, and gastrointestinal hemorrhage. Fu-Jin-Sheng-Ji-San (FJSJS) was written into *The Golden Mirror of Medicine* (an authoritative medical textbook in ancient China) by Qian Wu in 1742. In this book, FJSJS has been claimed to possess certain therapeutic effects on varying degrees of ulcers. Zhikang Capsule (ZKC) is a classic TCM formula based on QLS and FJSJS, containing 14 kinds of materials.

ZKC was approved by the United Kingdom Medicines and Healthcare Products Regulatory Agency (MHRA) in 2005. In *Pharmacopoeia of the People's Republic of China* (version 2015), ZKC was documented to apply to hemostasis, analgesia, anti-inflammation, and tissue recovery. In recent decades, ZKC has contributed greatly to IBD, peptic ulcer, dysfunctional uterine bleeding, cervical erosion, wound hemorrhage and infection in clinical practice in China. ZKC, in particular, had marked clinical effects on IBD with less toxicity. Although ZKC has gained beneficial effects and growing application, the exact mechanisms of ZKC in treating IBD have not been discovered.

Therefore, in this study, we aimed to determine the dose-effects of ZKC on dextran sodium sulfate (DSS)-induced colitis in mice, evaluate the levels of inflammation, oxidative stress, as well as apoptosis, and investigate the underlying molecular mechanisms by detecting the activation of MyD88-dependent TLR-4 signaling pathway. These results may provide valuable insights into the therapeutic mechanisms of ZKC in IBD.

2. Materials and methods

2.1. Materials of ZKC

ZKC (drug approval number: Z20025043; product batch number: 20151101) was produced by Xi'an Chiho Pharmaceutical Co. Ltd. (Xi'an, China). ZKC is composed of 14 materials. The contents and voucher numbers are exhibited in Table 1. Their authenticity was identified by two experts from Xi'an Chiho Pharmaceutical Co. Ltd. The voucher specimens were deposited in Xi'an Chiho Pharmaceutical Co. Ltd. in China. The Latin names of plants in ZKC were checked and accepted by the website www.theplantlist.org.

2.2. HPLC analysis of ZKC

ZKC weighing 0.4 g was dissolved in 50 ml methanol. All solvents were treated by ultrasonic wave for 40 min and filtered twice through a 0.45 μm filter before analysis. To confirm the authenticities of all components in ZKC, a Dionex UltiMate 3000 high performance liquid chromatography (HPLC) System (Dionex, USA) equipped with a photodiode array detector was employed. The chromatographic separation was carried out at a flow rate of 1.0 ml/min on an Agilent TC-C18 column (4.6 × 150 mm, 5 μm) at 30 °C. The mobile phase consisted of Methanol (A) and water (B), using a gradient elution of 10–90% A at 0–60 min, then 90% A at 60–75 min. The sample injection volume was 10ul and the detection wave length was 270 nm.

2.3. Animals

All experimental procedures involving the use of animals in this study were reviewed and approved by the Ethics Review