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Pre-clinical toxicity of a combination of berberine and 5-aminosalicylic acid in mice

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ABSTRACT

Our previous study demonstrated that a combination of alternative medicine berberine and conventional 5-aminosalicylic acid (5-ASA) showed promise to be a novel therapeutic strategy for ulcerative colitis (UC). This present study aims to sketch the pre-clinical toxicity profile of this combination (1:10 dose ratio) on mice. In acute toxicity test, the determined median lethal dose (LD_{50}) was 278.7 mg/kg berberine plus 2787 mg/kg 5-ASA. The results from subacute toxicity test demonstrated that no toxic signs of clinical symptoms, no significant changes in hematological or biochemical parameters were detected in mice treated with 14 + 140, 28 + 280 or 56 + 560 mg/kg of berberine plus 5-ASA treatment. Histological examinations revealed that accompanied with an increase in spleen weight, frequently recorded enlargement and white pulp hyperplasia of spleen were detected in mice when exposed to three doses of combination treatments. Further *in vitro* assessment suggested that the spleen toxicity was originated from berberine by its inhibition in cell viability and cell proliferation of lymphocytes. The results of this study indicate that the combination of berberine and 5-ASA shows a slight toxic effect on spleen, suggesting that this combination should be used with caution for patients.

1. Introduction

In recent years, alternative medicine *Coptidis Rhizoma* (Huanglian) and its major component, berberine, have drawn extensive attention toward their anti-inflammation effects for various diseases (Ma et al., 2010; Singh and Mahajan, 2013; Tang et al., 2009; Tillhon et al., 2012). In China, berberine has been long and extensively used as an over-the-counter (OTC) drug for the treatment of acute diarrheal (Eaker et al., 1988; Khinmaungu et al., 1985; Rabbani et al., 1987). It is also treated as one of the major active components of herbal formulas which are being commonly applied as adjuvant therapy to classic anti-ulcerative colitis (UC) drugs in clinical practice of Chinese Medicine (CM) (Singh and Mahajan, 2013; Zhang et al., 2013). Importantly, the alone effects of berberine in UC have been extended by recent studies which provide evidence of its beneficial activity in experimental colitis models or UC patients (Chen et al., 2015; Li et al., 2015a; Minaiyan et al., 2011; Yan et al., 2012; Zhang et al., 2011a). Therefore, berberine represents a promising new CM-originated agent with marked anti-UC activity.

In the meanwhile, 5-aminosalicylic acid (5-ASA) is still the cornerstone of first-line therapy for UC even with lots of unresolved issues such as low response while high relapse rate and high incidence of side effects (Cottone et al., 2011; Katz et al., 2010; Zhao et al., 2014). Therefore, strategies which can overcome the shortages of 5-ASA are urgently required. Our recent study demonstrated that the addition of berberine to 5-ASA showed a better effect than a sub-clinical dosage of 5-ASA alone in experimental chronic UC (Li et al., 2015b). Our findings suggest that the combination of berberine and 5-ASA might become a new strategy in the therapy of UC and provide the rationale of being translated into clinic by reducing dose of standard therapy (Li et al., 2015b). In addition, because berberine or berberine-contained CM and 5-ASA are being clinically used together in Chinese UC patients, it is urgent to address their safety issue.

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In our previous study, although no obvious adverse interaction has been found with the berberine/5-ASA co-treatment in the UC model mice (Li et al., 2015b), systemic toxicity data are required to predict the safety outcome of much higher doses. The present study was thus aimed to evaluate the pre-clinical toxicity of the combination of berberine and 5-ASA to provide urgent information that could be useful in the current clinical uses as well as lucid evidence for future safe application.

2. Materials and methods

2.1. Chemicals and reagents

Berberine hydrochloride was purchased from Shenzhen Chem-Strong Scientific Co., Ltd (Shenzhen, China) at the highest available purity (≥95%). 5-ASA, phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Experimental animals

Adult healthy ICR mice (10–12 weeks old; 20–30 g) of both sexes were used for the acute and subacute toxicity tests. They were obtained from the Guangdong Medical Laboratory Animal Center and housed in plastic cages under normal laboratory conditions (12 h light/dark cycle: 25 ± 2 °C) for an acclimatization period of 7 days prior to experiments. All the animals were fed standard mice chow pellets and had access to filtered water supplied in bottles. All animal care and experimental procedures were in accordance with ARRIVE guidelines and were approved by the Animal Care and Use Committee of Hong Kong Baptist University (Kilkenny et al., 2010; McGrath et al., 2010). A total of 100 mice were used in the experiments described here. All efforts have been made to minimize the number of animals used and their suffering.

2.3. Acute oral toxicity

Animals were randomly divided into five groups (n = 10, 5 males and 5 females) which received arithmetic doses (67% higher than the preceding one) of both drugs with 93.1 mg/kg berberine plus 931 mg/kg 5-ASA as the first dose via gavage oral administration. Those doses were chosen after several screenings on mice using an effective entity of 20 mg/kg berberine plus 200 mg/kg 5-ASA demonstrated in our previous study (Li et al., 2015b) as initial dose. The experimental animals were deprived of food for 18 h prior to drug administration. They were monitored continuously for 3 h thereafter for any signs of toxicity such as reduction in locomotion, aggressiveness, reaction to stimuli (tail pinch, noise), aspect of feces and mortality. After this period, the animals were supplied food and water ad libitum. Dead animals in each group were counted within 72 h after treatment and the median lethal dose (LD_{50}) were estimated by Bliss method. The surviving animals were monitored daily for 14 days and the water and food consumption were recorded per two days. No unexpected deaths were recorded in these surviving animals and they were sacrificed by cervical dislocation at the end of experiments.

2.4. Subacute oral toxicity

Forty mice of both sexes were randomly divided into four groups of 10 animals (5 males and 5 females) each. Three different doses of combination at 14 plus 140, 28 plus 280 and 56 plus 560 mg/kg of berberine and 5-ASA were administered per group, orally (gavage), daily for 28 consecutive days. The control group received only the vehicle (distilled water). The doses were chosen based on the Guideline 407 from OECD (Repeated Dose 28-Day Oral Toxicity Study in Rodents) (Buschmann, 2013). During treatment, body weight and possible signs of toxicity were observed and recorded and food and water consumption were monitored to be comparable between groups.

2.5. Hematological analysis

At the end of the observation period, all animals were placed into a metabolic cage to collect urine. After that, all animals were subjected to a 24 h fast, anaesthetized by intraperitoneal injection of thiopental. The terminal blood samples were collected and then dispensed into EDTA anticoagulant bottle for erythrocyte count, platelet count, hemoglobin concentration, platelet count and differential leukocyte counts by semi-automated hematology analyzer.

2.6. Biochemical analysis

The serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis. Alanine aminotransferase (ALT), glucose (GLU), total cholesterol (CHO), triglyceride (TG), blood urea nitrogen (BUN), and creatinine (CR) were automatically determined using auto analyzer.

2.7. Necropsy and histology analysis

All mice were sacrificed after the blood collection. The positions, shapes, sizes and colors of internal organs were evaluated. The vital organs (heart, liver, spleen, lung, kidney and brain), reproductive organs (testis or ovary) skeletal muscle, and colon were excised from all mice to visually detect gross lesions, and weighed, preserved in 10% neutral formalin for histopathological assessment. The tissues were embedded in paraffin, and then sectioned, stained with haematoxylin and eosin (H&E) and were examined microscopically.

2.8. Lymphocyte toxicity assay

Spleens were removed from male or female ICR mice. Viable single-cell suspensions were prepared from spleens according to procedures reported in our previous study (Li et al., 2015b) by using lympholyte-M (Cedarlane, Ontario, Canada) and cultured in RPMI 1640 containing 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. The obtained lymphocytes were seeded in 96-well plates (Nunc) at a density of 1×10^5 cells/ml in vehicle control (0.5% DMSO), berberine (0.3, 1, 3, 10, 30 and 100 μg/ml), 5-ASA (0.3, 1, 3, 10, 30 and 100 μg/ml) or berberine plus 5-ASA (0.3, 1, 3, 10, 30 and 100 μg/ml) for 24 h in the absence or presence of PMA (50 ng/ml) and ionomycin (500 ng/ml) before a MTT assay. Supernatants were collected and analyzed for TNF-α and IL-17 by enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (e-Bioscience) according to the manufacturer's instructions.

2.9. Confocal microscopy

The lymphocyte cells were cultured in coverglass bottom dish (SPL Lifescience Co. Ltd) and were then treated with vehicle control (0.5% DMSO), berberine, 5-ASA, or berberine plus 5-ASA at 1 and 10 μg/ml for 24 h before microscopy imaging. The fluorescence of intracellular berberine was observed with excitation at 405 nm and emission at 520 nm according to a previous report (Pang et al., 2014). Images were taken under a Leica TCS SP8 Confocal Microscope and further analyzed by Software LAS X.