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Original article

Studies on the intestinal absorption of the alkaloids in the Gancaofuzi decoction in a Caco-2 cell culture system by UPLC–MS/MS analysis

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ABSTRACT

In this study, the Caco-2 cell monolayer model was used to research the characteristic absorption and efflux of five diterpenoid alkaloids in Gancaofuzi decoction. An ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) method was developed for the determination of the simulated intestinal transport of five diterpenoid alkaloids with reserpine as internal standard. The use of the apparent permeability coefficient (Papp) and efflux rate (Er) was instituted to evaluate the intestinal absorption of the alkaloids. Transport of the five alkaloids in Caco-2 cell monolayer model was observed to better understand whether the intestinal absorption of alkaloids was influenced by the compatibility of four herbs in Gancaofuzi decoction. The results show that the Papp values of the five diterpenoid alkaloids were all more than 1×10^{-6} cm/s, confirming that the processes of permeability were valid. The flux of the alkaloids was time-dependent, and the intestinal absorption mechanism of the five alkaloids was mainly based on passive transport. The compatibility of Heishunpian, Baizhu, Guizhi and Gancao can reduce the intestinal absorption of alkaloids, especially the most toxic hypaconitine, and the attenuated effect of mixed herbal water extracts was better than that of different herbs' water extracts combination. The results prove that compatibility of four herbs in Gancaofuzi decoction is rational.

1. Introduction

Gancaofuzi decoction is composed of *Radix Aconiti Lateralis Preparata* (Heishunpian), *Atractylodes macrocephala Koidz* (Baizhu), *Cinnamomi Ramulus* (Guizhi) and *Glycyrrhizae Radix et Rhizoma preparata* (prepared Gancao), and has been widely used for the treatment of wind dampness over a long period of time in China [1]. The study found that various chemical ingredients in Heishunpian, Baizhu, Guizhi and Gancao cooperate with each other, *i.e.*, the chemical ingredients together play an important role in the efficacy of Gancaofuzi decoction [2]. Gancaofuzi decoction has beneficial effects of anti-inflammatory and analgesia, relating to Gancaofuzi decoction's anti-histamine effect or its regulation of serotonin and prostaglandins. The main chemical ingredients of Heishunpian are alkaloids [3], which has the effect as an anti-inflammatory, analgesia, immunomodulatory, antitumor, and so on [4]. Published studies show that oral Heishunpian decoction obviously decreased the foot swelling of the rat induced by formaldehyde or egg white [5,6], thus some researchers think that aconitine, hypaconitine and mesaconitine in Heishunpian decoction are the active ingredients for its anti-inflammatory effect, and its anti-inflammatory mechanism may be related to the excitatory effect of these three alkaloids on the hypothalamic–pituitary–adrenal system [2,7]. The main chemical ingredients of Gancao are flavonoids, which have the antagonizing effects on arrhythmia caused by alkaloids [8], and that the pharmacodynamic basis is that Gancao has a two-way adjustment function of NO and TNF-α [9].

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Long-term practice shows that the pesticide effect of Gancaofuzi decoction is better than that of any single herbal decoction alone, and the ratio of the four Chinese herbs contained in Gancaofuzi decoction is the best proportion for effectiveness. At present, research on the interaction of four herbs in Gancaofuzi decoction and its chemical ingredients mostly concentrated on the decoction process [2,10], and less on the interaction related to the absorption process. However, the interaction of drugs in the absorption process plays an important role in drug efficacy and therefore, modern pharmacy research, including theory and technology on the interaction mechanism of the four Chinese herbal medicines in Gancaofuzi decoction is the core content and key link of the research related to Gancaofuzi decoction.

Oral administration is the main route of administration of Chinese herbal compounds, and absorption is the key to exerting the effects of any oral medication in the body. The main route of drug absorption in the body is the small intestine [11]. Therefore, study of the absorption mechanism of drugs in the small intestine is a general trend at present. The Caco-2 cell monolayer model is one of the commonly used cell models to study the absorption mechanism of drugs *in vivo* as one of the effective tools to study drug absorption, transport and efflux in the small intestine, and also is the most widely used *in vitro* model in recent years in the world [12]. The Caco-2 cell line was originally proposed by Borchardt and WorkeM in 1989, and was first isolated from human colon cancer cells in the 1970s [13,14]. Cultured mature Caco-2 is able to form the same dense cell monolayer and cell polarity as small intestine epithelial cells, in this case its morphology and function are similar to those of human intestinal epithelial cells [15]. Therefore, the study of compatibility of traditional Chinese medicine and the interactions of components in the medicine with Caco-2 cells is beneficial to clarify bodily absorption of complex components, to understand the action mechanism of active constituents, and to prove the rationality of the compatibility of traditional Chinese medicine. The study of traditional Chinese medicine with Caco-2 cells plays an important role in the development and modernization of Chinese traditional medicine [16].

2. Experimental

2.1. Materials

The Caco-2 human colon carcinoma cell line was taken from the Shanghai Cell Bank of Chinese Academy of Sciences. Heishunpian was purchased from the Sichuan Jiangyou Zhongba Aconiti Technology Development Co., Ltd. Baizhu, Guizhi and Gancao were purchased from Changchun Tongrentang Chinese Medicine-Since. Dulbecco's modified Eagle's medium (DMEM) with high glucose medium, heat-inactivated fetal bovine serum (FBS), HEPES were obtained from Sigma (USA). Penicillin, streptomycin, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Dingguo Corporation (Beijing, China). DMSO was provided by Xilong Chemical Corporation (Guangdong, China). Acetate, methyl alcohol and acetonitrile were obtained from Fisher Scientific (USA). All other chemicals were of analytical grade or better. The water used in the study was from the Milli-Q water purification system (Millipore Inc., USA). Transwell transport board (1.12 cm^2 surface, 0.4 μm pore size, 12 mm diameter) was provided by the Corning Costar Corporation (USA); 96-well culture plates were obtained from Thermo (USA).

2.2. Sample preparation

Extraction of water-soluble portion of herbs: Crude powders of Heishunpian, Gancao, Baizhu and Guizhi, each 15 g, were added to 10 times total weight of distilled water to soak for 0.5 h and then heated to reflux for 1 h. The solutions (the first extracts) were filtered, and the residue was heated to reflux for 40 min in 8 times total weight of distilled water (the second extracts). The two extracts were individually filtered, and centrifuged, and then the supernatants were mixed as well as concentrated to 0.5 g crude drug/mL. After that, the supernatant was precipitated by 95% alcohol to remove the polysaccharide. At 24 h. later, the solutions were centrifuged and the supernatant was freeze-dried into powders. Crude powders of Gancao, Guizhi and Baizhu were mixed respectively with crude powders of Heishunpian in the ratio of 1:2, 1:1 and 1:2. Crude powders of Heishunpian, Gancao, Guizhi and Baizhu were mixed in the ratio of 2:1:2:1. Then, the mixtures were extracted and freeze-dried by the same method mentioned above.

2.2.1. Preparation of sample solution for the study of MTT

In the study of MTT, dried extracts of Heishunpian were dissolved in DMEM, and further diluted with DMEM to 10 different concentrations (the concentrations of Heishunpian extracts were 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3 and 1.4 mg/mL, respectively), the solutions so generated were used as the working standard solutions in the study.

2.2.2. Preparation of sample solution for the study of transport

In the study on transport of alkaloids in Heishunpian, the single Heishunpian extract was dissolved in HBSS to obtaining the working solutions. The final concentrations of Heishunpian extracts in the samples for the transport study were 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL and 1.0 mg/mL, respectively. To observe the mechanisms of absorption and transport of alkaloids, Baizhu, Guizhi and Gancao were each respectively mixed with Heishunpian base on the ratios, 1:2, 1:1 and 1:2. The mixtures were also individually dissolved in HBSS. And the concentration for each mixture was 1.0 mg/mL. The extracts of decoction herbs were further processed using the same method mentioned above.

2.3. Cell culture

The Caco-2 cells were cultured in DMEM high-glucose medium containing 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. The cells were grown in a CO_2 incubator at 37 °C temperature, containing 5% CO_2 and 90% relative humidity. When the cells covered 80% of the bottle bottom, 0.25% trypsin was added, 10–15 min. later, 0.5 mL fetal bovine serum was added in order to stop the digest. After that the cells were centrifuged, complete medium was added to suspend the cells, and then the cells were counted with cell counting plate.

2.3.1. MTT cytotoxicity assay

The Caco-2 cells in logarithmic growth phase were seeded in 96-well culture plates at a density of 2×10^4 cells per well and incubated for 48 h in a CO_2 incubator. After the supernatants were discarded, different concentrations of the test liquids were added to 96-well culture plates, in which, each concentration was set to five parallel holes and the negative control group was set which was only added DMEM. The cells were incubated in CO_2 incubator for 2.5 h, after that, the supernatants were discarded, and then 100 μL of MTT (1 mg/mL) was added. Continually, these cells were incubated in CO_2 incubator for 4 h, the supernatants were discarded, and then 100 μL of DMSO was added to dissolve the formazan crystals. The absorbance of the 96-well culture plates were measured at 490 nm by a microplate reader.

2.3.2. Transport

The Caco-2 cells in logarithmic growth phase were seeded in Transwell polycarbonate insert filters at a density of