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Blood-brain barrier permeability and neuroprotective effects of three main alkaloids from the fruits of *Euodia rutaecarpa* with MDCK-pHaMDR cell monolayer and PC12 cell line

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ABSTRACT

The fruits of *Euodia rutaecarpa* (Euodiae Fructus, EF), the widely used traditional Chinese medicine, have various central nervous system effects. Alkaloids following as evodiamine (EDM), rutaecarpine (RCP) and dehydroevodiamine (DEDM) are the major substances in EF. The MDCK-pHaMDR cell monolayer model was utilized as a blood-brain barrier (BBB) surrogate model to study their BBB permeability. The transport samples were analyzed by high performance liquid chromatography and the apparent permeability coefficients (P_{app}) were calculated. EDM and RCP showed high permeability through BBB by passive diffusion, while DEDM showed moderate permeability with efflux mechanism related to *P*-glycoprotein (*P*-gp). EDM and RCP could also reduce the efflux of DEDM probably by inhibiting *P*-gp. The neuroprotective effects of the three alkaloids were then studied on the PC12 cell line injured by 1-methyl-4-phenylpyridinium ion (MPP^+) or hydrogen peroxide (H_2O_2). EDM could significantly reduce MPP^+ or H_2O_2-induced cell injury dose-dependently. RCP could increase the cell viability in MPP^+ treated group while DEDM showed a protective effect against H_2O_2 injury. This study predicted the permeability of EDM, RCP and DEDM through BBB and discovered the neuroprotective substance basis of EF as a potential encephalopathy drug.

1. Introduction

Euodiae Fructus (EF), known as Wu-zhu-yu in China, is the dried and nearly ripe fruits of *Euodia rutaecarpa* (Juss.) Benth. (family Rutaceae). It is widely used as a famous traditional Chinese medicine (TCM) for the therapy of headache, abdominal pain, dysentery and cardiovascular diseases [1]. Various components including alkaloids, sterols, volatile oil and flavones have been isolated from EF [2]. Among them, alkaloids have been proved to be the main active constituents of EF, and the most highest ingredients in EF are evodiamine (EDM), rutaecarpine (RCP) and dehydroevodiamine (DEDM) (structures shown in Fig. 1) [3,4]. Previous studies have reported their therapeutic effects on encephalopathy, such as anti-inflammatory activities of EDM, RCP and DEDM on microglial cells [5], RCP-induced block of delayed rectifier K^+ current in neuronal cells [6], treatment of EDM and DEDM on Alzheimer's disease as a degenerative disorder [7] as well as regulatory effect of EDM on peroxisome proliferator-activated receptor gamma [8]. EDM and DEDM have been verified to be well-absorbed *in vitro* across Caco-2 cell monolayers in our previous study, while RCP was only moderately-absorbed [9,10]. The pharmacokinetic studies showed that EDM and DEDM could be absorbed into rat plasma after oral administration [11]. The blood-brain barrier (BBB) permeability of EDM, RCP and DEDM, however, have rarely been studied. Therefore, it is necessary to study the penetration abilities of the three alkaloids through the BBB for accessing their central nervous system (CNS) pharmacological activities.

BBB is the most important interface between the blood and brain interstitial fluid, providing CNS homeostasis to maintain its function and prevent circulating toxins [12]. Several *in vitro* cell models have been developed to study the drug permeability acrosse BBB, such as

Abbreviations: EF, Euodiae Fructus; EDM, evodiamine; RCP, rutaecarpine; DEDM, dehydroevodiamine; P_{app}, apparent permeability coefficients; *P*-gp, *P*-glycoprotein; MPP^+, 1-methyl-4-phenylpyridinium ion; H_2O_2, hydrogen peroxide; TCM, traditional Chinese medicine; BBB, blood-brain barrier; CNS, central nervous system; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HBSS, Hank's Balanced Salts Solution; MeOH, methanol; ACN, acetonitrile; OD, optical density; AP, apical side; BL, basolateral side; QC, quality control; RSD, relative standard deviation; *r*, coefficient correlations; TEER, transepithelial electrical resistance

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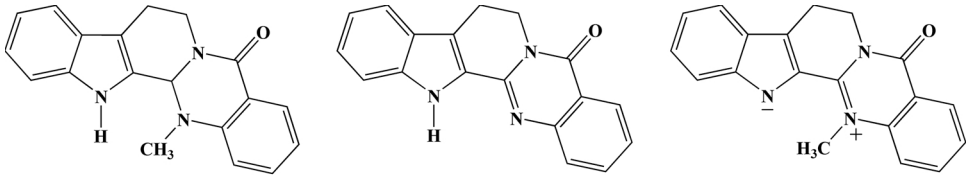

Fig. 1. Chemical structures of evodiamine (EDM), rutaecarpine (RCP) and dehydroevodiamine (DEDM).

cerebral microvascular endothelial cells [13] and endothelial-astrocytes coculture cells [14]. In this paper, the MDCK-pHaMDR cell line, derived from parental MDCK (Madin-Darby Canine kidney) cell line after infection with the MDR1 virus, was used to predict the BBB permeability of the above three alkaloids in EF. The characteristics of BBB, such as the microvilli and tight junction–desmosome, were demonstrated in the transfected MDCK-pHaMDR cells [15,16], while *P*-glycoprotein (*P*-gp) was proved to express in a polarized manner [15]. The MDCK-pHaMDR cell line can be operated conveniently, and has been demonstrated to correctly identify the passively and actively transported compounds across BBB [17]. It also has been used to investigate the BBB permeability of coumarins [18] and lignans [19] in our previous work.

Neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, are characterized by progressive losses of cognitive functions. Neuronal cell apoptosis has been reported to play an important role in the pathogenesis of neurodegenerative disorders [20–22]. Neurotoxins are often used to establish the neuronal cell apoptosis model for neuroprotective studies of different drugs. For example, 1-methyl-4-phenylpyridinium ion (MPP^+) can induce neuronal cell apoptosis by increasing proinflammatory cytokines in the nigrostriatal region [23,24], and hydrogen peroxide (H_2O_2) can increase the oxidative stress and neuronal cell apoptosis by inducing mitochondrial dysfunction [25]. PC12, the rat adrenal gland pheochromocytoma cell line, has been widely used to study the neuroprotective effects of drugs, especially for components in TCM with MPP^+ or H_2O_2 injured model [26,27].

In this paper, the *in vitro* BBB penetration of EDM, RCP and DEDM were examined in MDCK-pHaMDR cell monolayer model, and their cytoprotective effects on PC12 cells injured by MPP^+ and H_2O_2 were also investigated. The aim was to predict the BBB permeability and possible neuroprotective activities of EDM, RCP and DEDM, and discover the substance basis for the CNS activities of EF as a potential encephalopathy drug.

2. Materials and methods

2.1. Chemicals and reagents

EDM, RCP and DEDM were isolated from EF by our research group [3]. Their purities were above 98.0% by high performance liquid chromatography (HPLC) with diode array detector analysis.

Colchicine, caffeine, atenolol, verapamil, curcumin, hydrogen peroxide solution (H_2O_2, 3 wt.% in H_2O), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), MPP^+ iodide and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA, penicillin and streptomycin, phosphate buffered saline were obtained from Gibco (Life Science Technologies, Carlsbad, CA, USA). 12-Well Transwell® plates with polycarbonate inserts, 96-well plates and cell culture flasks were obtained from Corning Inc. (Cambridge, MA, USA).

Reagents for Hank's Balanced Salts Solution (HBSS) and other chemicals were of analytical grade from Beijing Chemical Works (Beijing, China). Methanol (MeOH), acetonitrile (ACN) and phosphoric acid were of HPLC grade (J. T. Backer, Center Valley, PA, USA). Milli-Q water (Millipore, Bedford, MA, USA) was used throughout the study.

2.2. Culture of MDCK-pHaMDR and PC12 cells

The MDCK-pHaMDR cell line was a gift from Dr. Michael M. Gottesman (National Institute of Health, Bethesda, MD, USA). The cells were cultivated in DMEM at 37 °C in 5% CO_2, supplemented with 10% FBS, 50 units/mL of penicillin, 50 μg/mL of streptomycin and 80 ng/mL of colchicine. They were seeded onto the transwell insert membranes at 1.0×10^5 cells/mL. After growing for 8 days, the cells reached confluence and full differentiation for transport studies. The cells in this study were between passages 15 and 25.

PC12 cell line was obtained from China Infrastructure of Cell Line Resources in the School of Basic Medicine in Peking Union Medical College (Beijing, China). The cells were cultured at 37 °C in 5% CO_2 in DMEM, supplemented with 5% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin.

2.3. Transport experiments on MDCK-pHaMDR cell monolayer

The stock solutions of the three alkaloids, caffeine, atenolol and verapamil were prepared with DMSO. They were further diluted to different concentrations by HBSS before experiments with the final concentrations of DMSO less than 1%.

The cytotoxicity of the three alkaloids on the MDCK-pHaMDR cells was assayed by the MTT method. The optical density (OD) values were read on a Thermo Multiskan MK3 Automated Microplate Reader (Thermo-Labsystems, Franklin, MA, USA).

2.3.1. Bidirectional transport experiments

The cell monolayers were washed twice with pre-warmed HBSS before the transport studies. The HBSS in apical (AP, 0.5 mL) side was replaced in absorption experiments and HBSS in basolateral (BL, 1.5 mL) side was replaced in efflux experiments by the test solutions. The transwell plates were shaken in a water bath for 90 min (55 rpm, 37 °C). Appropriate volumes of samples were collected from both sides of the cell monolayer, then frozen, lyophilized and preserved below −20 °C. The cell monolayers on the membrane were lyophilized after three freeze (−20 °C)–thaw (room temperature) cycles and preserved below −20 °C for intracellular accumulation analysis. The transports of caffeine and atenolol were conducted with the method described above to verify the cell monolayer integrity. The test concentrations of EDM, RCP and DEDM were 5, 5 and 20 μM, respectively.

2.3.2. Time- and concentration-dependent transport experiments

In the time-dependent transport experiments, test compounds with different concentrations were added to AP side in the absorption experiments (AP → BL) or BL side in efflux experiments (BL → AP). The incubation time was 30, 60, 90, 120, 150 and 180 min, respectively. To observe the concentration-dependence, test compounds with different concentrations were added to either AP or BL side, and the cells were incubated for 90 min with the method described above.

2.3.3. Verapamil inhibition experiment

The verapamil inhibition experiment was carried out for DEDM only. Verapamil (100 μM) was added to both sides of the inserts, and the cells were pre-incubated for 30 min. With the constant verapamil in both sides, 20 μM of DEDM was added to either AP or BL side. The cells