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Influence of puerarin, paeoniflorin, and menthol on structure and barrier function of tight junctions in MDCK and MDCK-MDR1 Cells

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KEYWORDS
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Abstract *Objective:* In this study, the influence of puerarin, paeoniflorin, and menthol on the structure and barrier function of tight junctions (TJs) in Madin–Darby canine kidney epithelial (MDCK) and MDCK-multi-drug resistance 1 (MDR1) cells was evaluated to determine the mechanisms by which the drugs cross the blood–brain barrier (BBB).
Method: Cells were treated with puerarin, paeoniflorin, and menthol followed by immunohistochemical staining with occludin, claudin-1, and F-actin. The cells were then observed using laser-scanning confocal microscopy. Average optical density (AOD) of the immunofluorescence images of the proteins were analyzed using ImageJ software while Transepithelial electrical resistance (TEER) was measured using an epithelial voltohmmeter.
Results: Confocal microscopy revealed that puerarin- and paeoniflorin-treated tight junction proteins were conspicuous while menthol suppressed their expression. Correspondingly, AOD values of cells treated with puerarin or paeoniflorin, or both showed no difference compared to the control group ($P > .05$) while the menthol group value was downregulated. In 3 h, TEER of cells not treated with menthol were similar to the control group, while treatment with menthol significantly decreased TEER value ($P < .05$). In addition, application of menthol decreased TEER in MDCK cells earlier than in MDCK-MDR1 cells.
Conclusion: Menthol but not puerarin and paeoniflorin may enhance paracellular transport and improve drug penetration of the BBB by disrupting the structure and, thereby, weakening the barrier function of TJs.

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Introduction

Brain-targeting drugs often fail to penetrate the brain or their levels are insufficient at their brain targets. Inadequate brain drug levels are usually because of the barrier function of the brain capillary endothelial cells, which form the blood—brain barrier (BBB) that controls and regulates the molecular permeation between the brain periphery and interior.[1–4] Madin—Darby canine kidney epithelial (MDCK) cells and MDCK cells transfected with the human multidrug resistance 1 (MDR1) gene (MDCK-MDR1) are widely regarded as an excellent cell model to simulate the BBB in vitro.[5,6] In the BBB, a single layer of the brain capillary endothelial cells connected by tight junctions (TJs) composes a barrier system that maintain a stable internal environment and ensures the normal functions of the brain. TJs play a vital role in molecular permeation of the BBB, especially for molecules that pass through the paracellular pathway. They distinguish the apical from the basolateral cell surface regions to define cell polarity, and possess a fence and barrier function.[7–10] Under electron microscopy, TJs are visualized as a domain of close membranes fused at the lumen-facing apex of adjacent cells, and consist of integral membrane proteins.[11–13] MDCK and MDCK-MDR1 cells have been shown to have analogous TJ structures consisting of brain capillary endothelial cells and therefore express diverse TJ proteins, including occludin, claudins, zona occuldens protein 1 (ZO-1) and F-actin.[14–16] The intramembranous fence and hydrophobic barrier function of TJs restrict drug delivery to underlying tissues and, thereby, decreases drug permeation of the BBB. In turn, since the tightness of TJs is determined by the protein composition of the webs of the associated proteinaceous filaments (called strands), some drugs can alter the TJ structure and properties by acting on TJ proteins to enhance their brain permeability or that of other drugs or both. Therefore, studying the effect of compounds on TJ proteins and mechanisms that can overcome the paracellular barrier of the BBB is critical for the development of brain-targeted medicines and efficacious treatment of diseases.

Tongqiaosanyu is a traditional Chinese medicinal formula that is prescribed as an integral part of the folkloric clinical experience. The formula is composed of a number of herbs, including kudzu root (*Pueraria lobata*), white peony root (*Paeonia albiflora*), and mint (*Mentha haplocalyx* Briq.). In China, Tongqiaosanyu is used to treat stroke. Pharmacodynamic screening has identified puerarin, paeoniflorin, and mentholas the main active constituents. Puerarin (8-[beta-D-glucopyranosyl]-7-hydroxy-3-[4-hydroxyphenyl]-4H-1-benzopyran-4-one, $C_{21}H_{20}O_9$), which is the major isoflavone glycoside isolated from kudzu root, has numerous clinical indications and has been widely used for the treatment of cardiovascular disorders and ischemic stroke in TCM.[17–19] Paeoniflorin (5beta-[{Benzoyloxy} methyl]tetrahydro-5-hydroxy-2-methyl-2,5-methano-1H-3,4-dioxacyclobuta[cd]pentalen-1alpha[2H]-yl-beta-D-glucopyranoside, $C_{23}H_{28}O_{11}$) is a monoterpene glucoside isolated from white peony root. It has antithrombotic, anti-inflammatory, anti-allergy, antihyperglycemic, glucose uptake enhancing, and neuroprotective effects.[20] Menthol (2-isopropyl-5-methylcyclohexanol, $C_{10}H_{20}O$) is a major constituent of peppermint oil and possesses central nervous system (CNS) excitatory effects and enhances BBB permeation.[21] Our previous investigation of Tongqiaosanyu established analytical methods for the main active constituents. In addition, we explored different administration routes of Tongqiaosanyu, its in vivo pharmacokinetic behavior, as well as its compatibility with other medicines. Cytotoxicity and transport of puerarin (alone or with other constituents in the prescription) in MDCK and MDCK-MDR1 cells have been reported, including the mechanisms by which puerarin penetrates the BBB. However, the mechanisms of action underlying the paracellular transport of these compounds through the BBB, especially their influence on protein structure and barrier function, remain unclear. Therefore, we sought to determine the effect of this formula TJs, as well as the transport role of each compound using MDCK and MDCK-MDR1 cells to simulate the BBB. Absorption mechanism mediating the penetration of these drugs into the brain and the penetration-enhancing effects of some drugs were studied.

Materials and methods

Materials

Puerarin, paeoniflorin, and menthol were purchased from the National Institute for Food and Drug Control (Beijing, China). Polyester (PET) cell culture inserts and 12-well plates (12-mm diameter, 0.4-μm pore size) were obtained from Corning Life Sciences (Corning, NY, USA). Rabbit anti-occludin antibody (ab31721) was obtained from Abcam Shanghai (Shanghai, China). Mouse anti-claudin-1 antibody (2H10D10) was purchased from Invitrogen (Camarillo, CA, USA). Anti-rabbit IgG -tetramethylrhodamineisothiocyanate (TRITC) conjugate was purchased from ZSG-BIO (Beijing, China). Anti-mouse-fluoresceinisothiocyanate (FITC) conjugated antibody was purchased from Kangwei Century Biotechnology (Beijing, China). Acti-stain 488 phalloidin staining for F-actin was obtained from Cytoskeleton (Denver, CO, USA).

Cell culture

MDCK and MDCK-MDR1 cells were generously provided by Dr. Zeng (Zhejiang University, China). Both cell lines were cultured with Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Fremont, CA, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. The medium was replaced with fresh medium every other day until the cells reached approximately 90% confluence.

Grouping and drug administration

In a previous study,[22] the cytotoxicity of puerarin, paeoniflorin, and menthol in MDCK and MDCK-MDR1 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetr-azolium bromide (MTT) assay. Results showed puerarin, paeoniflorin, and menthol were not cytotoxic at concentration ranges of