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# Influence of puerarin, paeoniflorin, and menthol on structure and barrier function of tight junctions in MDCK and MDCK-MDR 1 Cells

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**Abstract**—

To find out how the drugs cross the blood-brain barrier (BBB), this study examined how puerarin, paeoniflorin, and menthol affected the structure and function of tight junctions (TJs) in MDCK and MDCK-multi-drug resistance 1 (MDR1) cells.

The cells were first treated with puerarin, paeoniflorin, and menthol. Then, they were stained with occludin, claudin-1, and F-actin using immunohistochemistry. Next, laser-scanning confocal microscopy was used to examine the cells. An epithelial voltage voltmeter was used to assess transepithelial electrical resistance (TEER), and ImageJ software was used to analyse the average optical density (AOD) of the protein immunofluorescence pictures. The results showed that tight junction proteins treated with puerarin and paeoniflorin were visible under confocal microscopy, but menthol reduced their expression. Similarly, the menthol group's AOD value was downregulated, while the control group's value was not different from the AOD values of cells treated with either puerarin or paeoniflorin, or both. After three hours, the TEER of cells that were not exposed to menthol were comparable to those of the control group, but treatment with

With a p-value less than.05, menthol considerably reduced the TEER score. And menthol's TEER-lowering effects on MDCK cells were noticed before those on MDCK-MDR1 cells. Ultimately, it seems that menthol, in contrast to puerarin and paeoniflorin, has the potential to decrease the barrier function of TJs, which in turn enhances paracellular transport and drug penetration of the blood-brain barrier.

## Introduction

Medications designed to target certain areas of the brain either do not reach those areas or do not reach enough concentrations once they do. The blood-brain barrier (BBB) governs and controls the molecular permeation between the brain's periphery and interior, and when the levels of drugs administered to the brain are inadequate, it is often due to the barrier function of the brain's capillary endothelial cells.1e4 One of the best cell models to mimic the BBB in vitro is MadineDarby canine kidney epithelial (MDCK) cells or MDCK cells transfected with the human multidrug resistance 1 (MDR1) gene (MDCK-MDR1).5, 6 The BBB is a barrier system that keeps the brain's internal environment steady and supports appropriate brain processes. It consists of a single layer of brain capillary endothelial cells joined by tight junctions (TJs). When it comes to molecules that go via the paracellular route, TJs are crucial for molecular permeability of the BBB. In addition to defining cell polarity by separating the apical and basolateral cell surface regions, they also serve as a barrier and fence.Upon examination under electron microscopy, transmembrane junctions (TMJs)—which include integral membrane proteins—are seen as a region of tightly connected

membranes at the apex of neighbouring cells that face the lumen.A variety of transient junction proteins, such as occludin, claudins, zona occuldens protein 1 (ZO-1), and F-actin, are expressed by MDCK and MDCK-MDR1 cells because their transient junction structures are similar to those of brain capillary endothelial cells. Through their hydrophobic barrier function and intramembranous fence, TJs limit medication delivery to underlying tissues and reduce drug permeability of the blood-brain barrier (BBB). Some medications may change the structure and characteristics of transmembrane junctions (TMJs) by acting on TJ proteins to make them more permeable to the brain or to other drugs, or both. This is because TJ tightness is dictated by the protein composition of the webs of the related proteinaceous filaments (called strands). In order to create medications that target the brain and effectively cure disorders, it is crucial to research how chemicals affect TJ proteins and how they may cross the blood-brain barrier (BBB). An essential aspect of the folkloric therapeutic practice is the prescription of tongqiaosanyu, a traditional medicinal formula from China. Kudzu root (Pueraria lobata), white peony root (Paeonia albiflora), and mint (Mentha hap-localyx Briq.) are among the plants that make up the mix. Tongqiaosanyu is a treatment for stroke in China. The primary active ingredients have been determined to be menthol, pseudoniflorin, and pierin by pharmacodynamic screening. “Puerarin (8-[beta-D-glucopyranosyl] “-7-hydroxy-3-[4-hydroxyphenyl]”Traditional Chinese Medicine (TCM) has made extensive use of -4H-1-benzopyran-4-one (C21H20O9), the main isoflavone glycoside extracted from kudzu root, for the treatment of ischemic stroke and cardiovascular diseases.17 with e19 5-Beta-[{Benzoyloxy} methyl] paeoniflorin5,4-dioxacyclobuta[cd] tetrahydro-5-hydroxy-2-methyl-2,5-methano-1H-[2H] pentalen-1alphaThe monoterpene glucoside iso-lated from white peony root is known as -yl-beta-D-glucopyranoside (C23H28O11). It enhances glucose uptake and possesses neuroprotective, anti-inflammatory, anti-allergy, and antihyperglycemic properties.20 The chemical formula for menthol is C10H20O, and it is a primary
component of peppermint oil, which increases blood-brain barrier (BBB) permeation and has excitatory effects on the central nervous system (CNS).21 Analytical methodologies for the primary active constituents were developed in our prior examination of Tongqiaosanyu. Furthermore, we investigated the in vivo pharmacokinetic behaviour of Tongqiaosanyu, its compatibility with other medications, and several methods of administration. The processes by which puerarin crosses the blood-brain barrier (BBB) have been documented, as have its cytotoxicity and transport in MDCK and MDCK-MDR1 cells. Nevertheless, it is still not known how these chemicals paracellularly cross the blood-brain barrier (BBB), particularly how they affect protein structure and barrier function. Hence, we aimed to find out how this formula TJs worked and what part each chemical played in transport by simulating the BBB using MDCK and MDCK-MDR1 cells. Researchers looked at how these medications entered the brain and how certain medicines improved their penetration.

## 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-mm 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) con- jugated antibody was purchased from Kangwei Century Biotechnology (Beijing, China). Acti-stain 488 phalloidin staining for F-actin was obtained from Cytoskeleton (Den- ver, 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 me- dium every other day until the cells reached approximately
90% confluence.

### Grouping and drug administration

Chinese Traditional Medicine Journal
ISSN : 2693 6356
2024 | Vol 1 | Issue 1

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

0e0.3 mg/mL, 0e0.4 mg/mL, and 0e0.04 mg/mL, respectively. Furthermore, puerarin plus paeoniflorin (1:0.4 w/v, PP), puerarin plus menthol (1:0.5 w/v, PM), and puerarin plus paeoniflorin plus menthol (1:0.4:0.5, w/w/w, PPM) groups exhibited no cytotoxicity at concentration ranges of 0e0.2 mg/mL (paeoniflorin), 0e0.05 mg/mL (menthol), and 0e0.04 mg/mL (menthol), respectively. Based on the results of MTT assay, this study included the following six treatment groups: puerarin, paeoniflorin, and menthol (0.1 mg/mL, 0.04 mg/mL, and 0.05 mg/mL, respectively), as well as the PP group (0.1 mg/mL puerarin and 0.04 mg/mL paeoniflorin, respectively), PM group (0.1 mg/mL puerarin and 0.05 mg/mL menthol, respectively), and PPM group (0.1 mg/mL puerarin, 0.04 mg/mL paeoniflorin, and 0.05 mg/mL menthol, respectively) groups.

## Immunofluorescence microscopy

For immunocytochemical analysis, the cells were seeded on 15 mm coated glass coverslips. When cell fusion attained approximately 80e90%, the regular media were replaced with media containing the various compounds (puerarin, paeoniflorin, or menthol at 0.1 mg/mL, 0.04 mg/mL and the previously described drug combinations was measured at 30, 60, 90,120,150, and 180 min. TEER was measured immediately following drug treatment (0 min) and was set to 100%, with all the other values calculated and expressed relative to this value. Finally, TEER at each time point was compared to that of the control group and statistically analyzed.

## Data analysis and statistics

Immunofluorescence images were analyzed semi-quantitatively using ImageJ software based on the following equations:

$$\text{AOD} = \frac{\text{IntDen}}{\text{Area}} \quad (1)$$

where IntDen is the integrated optical density (IOD) of the image, Area is the region of fluorescence in the image, and AOD is the average optical density.

Percentage AOD (%) of the TJ proteins was calculated using the following equation:

$$\text{Relative AOD}(\%) = \frac{\text{AOD}_{\text{sample}}}{\text{AOD}_{\text{control}}} \times 100 \quad (2)$$

0.05 mg/mL, respectively) and the cells were further cultured for 3 h, and then fixed with cold 4% paraformaldehyde for 30 min. After washing thrice for 5 min each with phosphate-buffered saline (PBS), the coverslips were incubated for 1 h with goat serum as the blocking buffer. Then, after rinsing with PBS thrice, some coverslips were incubated with polyclonal anti-occludin or monoclonal anti-claudin-1primary antibodies (1:100, each) at 4◦Covernight followed by incubation with TRITC-conjugated anti-rabbit IgG or FITC-conjugated anti-mouse IgG. Other cells were only stained with Acti-stain 488 phalloidin stain (1:150) at 25◦C for 30 min. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Beijing Solarbio Science and Technology, Beijing, China). The cells were visualized using an inverted fluorescence microscope equipped with appropriate filters (Olympus, Tokyo, Japan). Images were acquired using a laser scanning confocal microscope and accompanying analysis software. Fluorescence intensity of the images was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

## Measurement of transepithelial electrical resistance

To investigate changes in the TJs barrier function of the MDCK and MDCK-MDR1 cells, transepithelial electrical resistance (TEER) of the monolayers was detected using the EVOM epithelial voltohmmeter (EMD Millipore, Billerica, MA, USA). Both cell types were cultured separately on0.4 mm pore size filters of 12 mm Transwellsand grown to confluence. Then, 0.5 mL and 1.5 mL of drug solution and Hank's basic salt solution (HBSS) were added to the apical (A) side and basolateral (B) side, respectively to simulate A ⁄ B transport while in a parallel experiment, B ⁄ A transport was simulated by reversing the order of the drug solution and HBSS. TEER of untreated or puerarin-, paeoniflorin-, and menthol-treated cells or those treated with where $\text{AOD}_{\text{sample}}$ and $\text{AOD}_{\text{control}}$ are the AODs of the sample and control, respectively.

Percentage TEER was calculated using the following equation:

$$\text{Relative TEER}(\%) = \frac{\text{TEER}_t}{\text{TEER}_{t0}} \times 100 \quad (3)$$

where $\text{TEER}_t$ is the TEER value at different times and $\text{TEER}_{t0}$ is the initial TEER value before the experiment was performed.

Results are presented as means (SD) of 3 separate par- allel experiments. Data were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's test to determine the difference between multiple groups compared to the control group using SPSS version 17.0 software. A $P < .05$ was considered statistically significant.

## Results

### Effect of compounds on expression of TJ proteins

To determine the effects of the compounds on TJ proteins of the MDCK and MDCK-MDR1 cells, we immunohis- tochemically analyzed occludin, claudin-1, and F-actin using laser scanning confocal microscopy (Fig. 1). All three TJ proteins showed positive staining in both cell types and occludin stained red, claudin-1and F-actin stained green, and the nuclei stained blue. Both cell types had similar TJ barrier function systems.

After treatment with puerarin and paeoniflorin, the visual morphology of occludin, claudin-1, and F-actin in both kinds of cells was not difference from that of the hardly observed (Fig. 1A, B, respectively). untreated cells. However, incubation with menthol, decreased the TJ proteins in both MDCK and MDCK-MDR1-cells and the morphology was

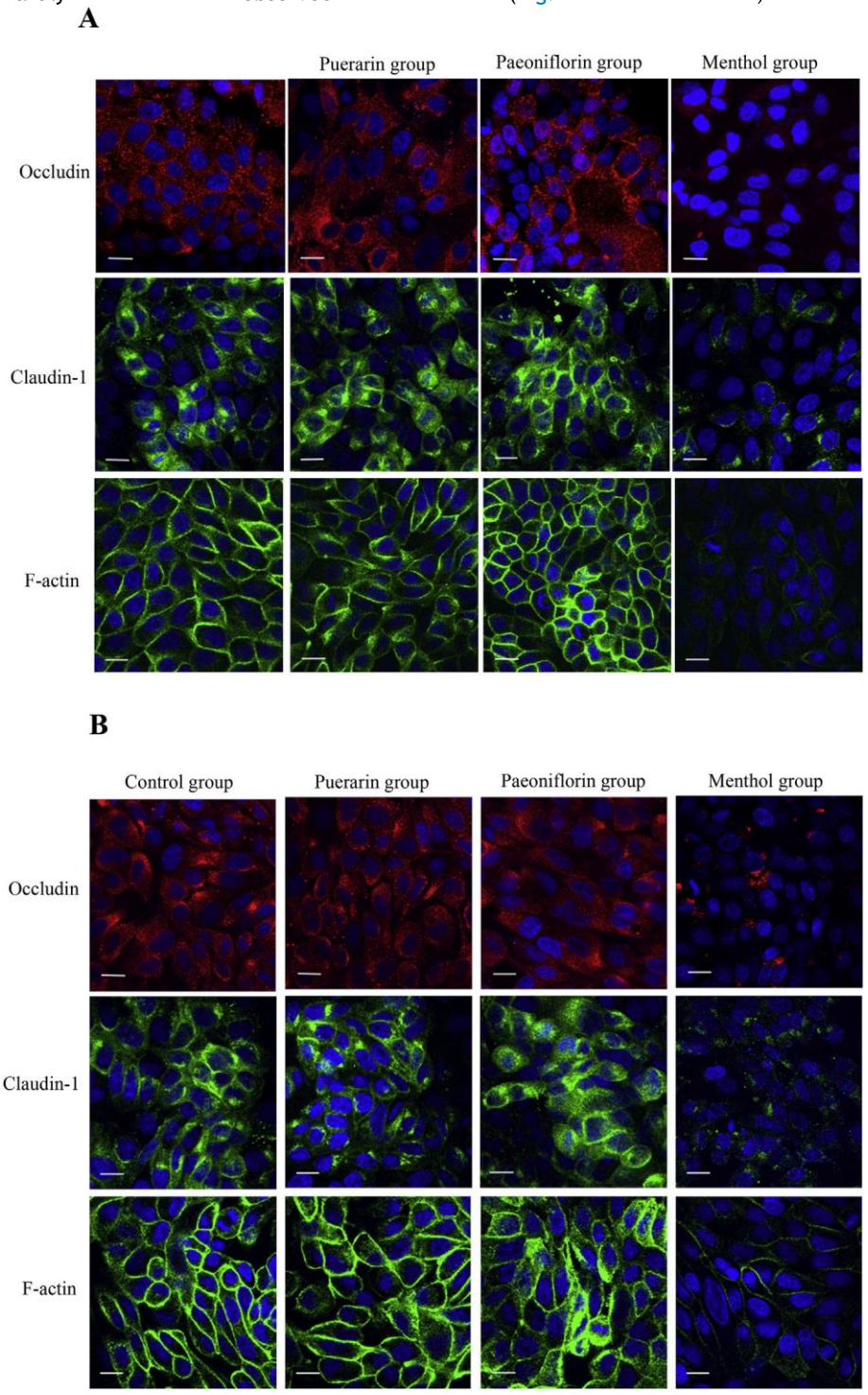


Fig. 1 Effect of puerarin, paeoniflorin, and menthol on tight junction (TJ) proteins in (A) MadineDarby canine kidney epithelial (MDCK) and (B) MDCK-multi-drug resistance 1 (MDR1) cells (×600). (A) Immunocytochemistry for occluding, claudin-1, and F-actin in MDCK cells with puerarin or paeoniflorin showed similar fluorescent morphology with the control group. All three proteins were deceased in MDCK cells by menthol. (B) Fluorescence intensity in MDCK-MDR1 cells with different compounds was similar to that in MDCK cells. Scale bar corresponds to 1.0 mm.

## Changes in AOD of TJ proteins after treatment with different compounds

Fluorescence intensity of the immunofluorescent images was semi-quantitatively analyzed. AOD values of each protein were calculated using ImageJ software and statistical analysis was conducted (Fig. 2). The control, puerarin, and paeoniflorin groups showed similar approximate relative AOD values in the MDCK and MDCK-MDR1 cells, while the menthol group was significantly different compared to

Chinese Traditional Medicine Journal

ISSN : 2693 6356
2024 | Vol 1 | Issue 1

the control group ($P < .05$).

## Changes in TEER of cells treated with various compounds

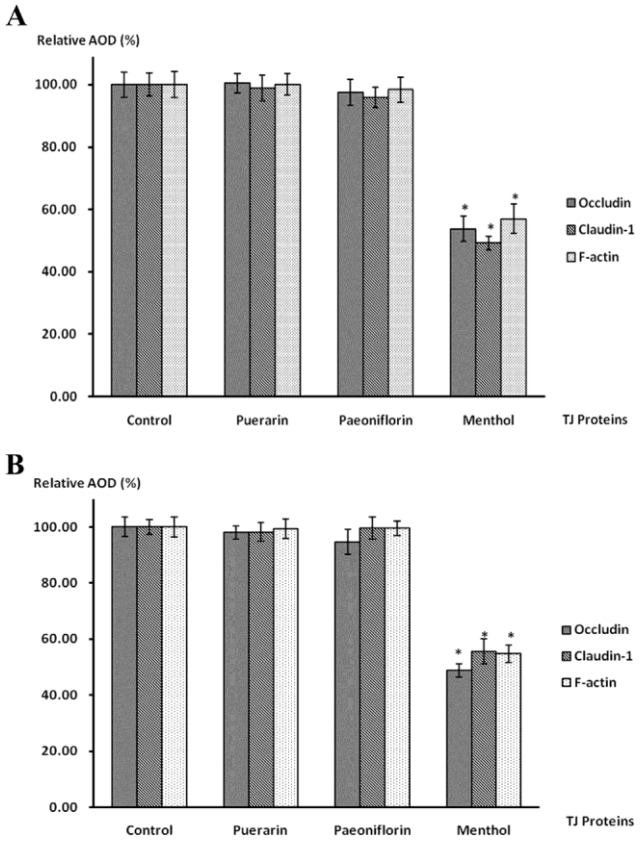


Fig. 2 Change in relative average optical density (AOD) of occludin, claudin-1, and F-actin in (A) MadineDarby canine kidney (MDCK) epithelial and (B) MDCK-multi-drug resistance 1 (MDR1) cells after treatment with different compounds. (A) Relative AOD values of tight junction (TJ) proteins in MDCK cells treated with puerarin or paeoniflorin showed no significant difference compared to the control group while AOD values of the menthol group were significantly reduced. (B) Relative AOD values in MDCK-MDR1 cells treated with different compounds were the same as in MDCK cells. Only menthol reduced AOD of all three proteins. Data presented as mean (SD) (n Z 3). )$P < .05$ compared to control group.

compounds for up to 3 h. TEER values of each group treated with the compounds for different times were statistically compared with those of the control group (Fig. 3). MDCK cells (Fig. 3A) treated with puerarin, paeoniflorin, and PP exhibited similar changes in TEER compared to the control group in the A ✓ B and B ✓ A transport processes. At 90 min, TEER of the menthol, PM, and PPM groups decreased significantly compared with that of the control group ($P < .05$). In MDCK-MDR1 cells (Fig. 3B), groups not treated with menthol exhibited a steady

To determine the effects of the compounds on the function of the TJ barrier, we measured the TEER values of MDCK and MDCK-MDR1 cells after exposure to the various

TEER change similar to the control group and menthol-treated groups showed a significant decrease at 120 min($P < .05$)in both the A ✓ B and B ✓ A transport processes compared to control.

## Discussion

Use of both in vivo and in vitro methods has led to the elucidation of certain aspects of the drug transport process across the BBB. In vivo studies of the BBB provide valuable observational information and direct analysis of drug permeation processes such as drug distribution in each brain region. However, there are some limitations and shortcomings to this method including the laborious nature of the experiments, individual differences, and compli- cated analytical methods. Accordingly, development of in vitro models is highly desired to facilitate the clarifica- tion of the BBB permeability mechanisms. Such desired models mainly include those that would predict the asso- ciated BBB processes using computer simulation technol- ogy, parallel artificial membrane permeability assay (PAMPA), and in vitro cell culture techniques.[6] Cell culture is currently the favored tool for simultaneously obtaining complex and comprehensive information of passive and active transport processes. Generally, there are two types of cell culture models that are used to simulate the BBB. One consists of the “real BBB model,” which is based on primary cultures of brain capillary endothelial cells or sin- gle cell lines that may be complemented or co-cultured with astrocytes/pericytes.[23,24] The other type is the “sur-rogate BBB model,” which uses similar epithelial cells such as MDCK, MDCK-MDR1,and human colon carcinoma cell lines (Caco-2).[5,25,26] Among these, MDCK and MDCK-MDR1 have been recognized as the ideal models for in vitro simulation of BBB drug penetration.[6,27e29] They have several advan- tages including (1) stable source with no batch-to-batch variability, (2) convenient for routine experimental appli- cation and thus reduce labor intensity, (3) adequately and closely mimic relevant in vivo properties of the BBB, (4) ensure adequate monolayer integrity and display a proper TEER value under conventional experimental conditions, and (5) show superior P-glycoprotein (P-gp) expression and activity, particularly in MDCK-MDR1 cells transfected with the human MDR1 gene, which is a very important efflux transporter in the BBB.[30e34] Therefore, we chose MDCK and MDR1-MDCK cells to establish our in vitro BBB model.

Drugs cross the blood brain barrier by two main methods: transcellular and paracellular transport. In addi- tion, numerous elements impede penetration of drugs and substances from the extra-brain region to the brain area, including the BBB morphologic characteristics (such as

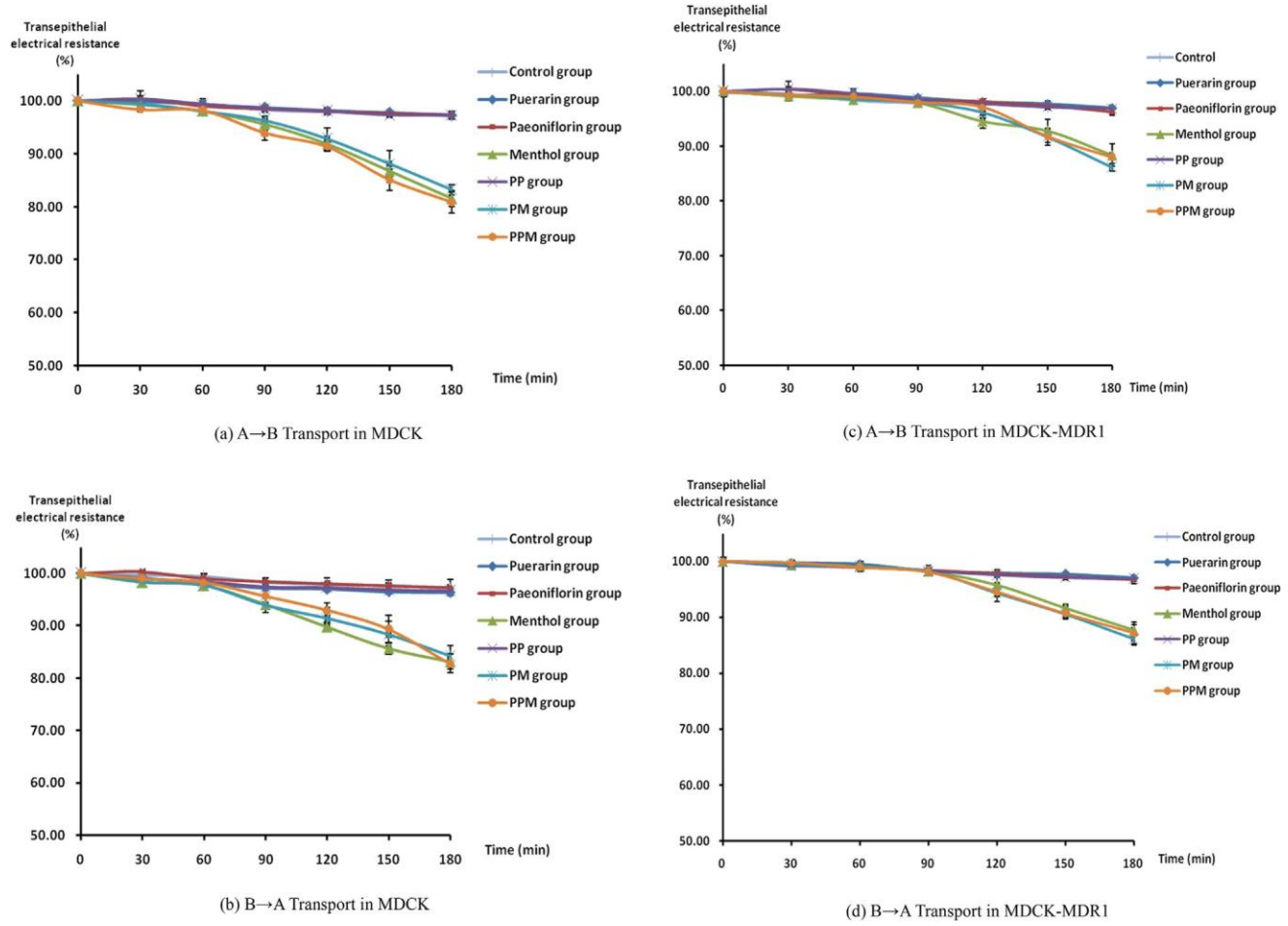


Fig. 3 Change in transepithelial electrical resistance (TEER) in (a, b) MadineDarby canine kidney (MDCK) epithelial and (c, d) MDCK multi-drug resistance 1 (MDCK-MDR1) cells after treatment with different compounds. (a, b) TEER of MDCK cells gradually decreased following treatment with compounds alone or in combination with menthol. (c, d) TEER in menthol-treated MDCK-MDR1 cells similarly declined. Data presented as mean (SD) (n Z 3). PP: puerarin and paeoniflorin; PM: puerarin and menthol; PPM: puerarin, paeoniflorin and menthol groups.

existence of TJs), enzymes in the cytosolor on the extra- cellular membrane, and P-glycoprotein (P-gp). For hydro- philous compounds, paracellular transport is a crucial pathway for penetrating the brain and exerting a thera- peutic effect. The physiologic function of the TJ is insep- arable from paracellular transport and its barrier and fence functions are regulated by various factors.[35e37] Using on the simulated BBB MDCK and MDCK-MDR1 cell model, we focused on studying the mechanism of the main active constituents in the Tongqiaosanyu prescription to deter- mine their effects on the structure and barrier function of TJs.

The TJ structure is composed of various types of proteins and occludin is regarded as the primary identifying mole- cule of the TJ strands. It is a tetraspan membrane protein of approximately 60-kD, with a short intracellular curve, two extracellular annuli, and N- and C-terminals in the cytoplasmic domain.[38] Assembly of TJs and concomitant increase in TEER have been demonstrated to be associated with tyrosine phosphorylation of occludin, which is a phe- nomenon commonly observed after recovery from adeno- sine triphosphate (ATP) depletion or during calcium repletion.[39,40] Claudins have different sequences from occluding and are 18- to 27-kDtetraspan proteins with two extracellular annuli, a short N-terminus, and a C-terminal in the cytoplasmic domain.[41] Claudin-1 is also considered an important strand or even the back bone of TJs.[42] Recent studies have shown that claudin-1 is the decisive compo- nent of TJs and possesses cell adhesion activity.[43] It can directly affect the regulation of paracellular transport and the selectivity of solute size. F-actin, which is one of the peripheral membrane proteins of TJs, is related to organ- elle movement, protoplasmic streaming, and intercellular junctionregulation.[7,44e47] Importantly, the F-actin belt can control TJ function and limit the material intake.[48]

Although we did not investigate all TJ protein types in MDCK and MDCK-MDR1 cells, the proteins we chose were excellent representations. We individually analyzed the immunofluorescence staining of occludin, claudin-1, and F- actin in MDCK and MDCK-MDR1 cells after treatment with puerarin, paeoniflorin, or menthol. The puerarin and paeoniflorin group did not affect the staining properties of TJ proteins. Only menthol inhibited the expression and reduced the fluorescence intensity (AOD value) of all three kinds of TJ proteins. This characteristic of menthol indicated that it directly disrupted the structure and integrity of TJs to open the BBB barrier. These results combined with previous reports led us to infer that disruption of the configuration and integrity of TJ by menthol might be

Chinese Traditional Medicine Journal
ISSN : 2693 6356
2024 | Vol 1 | Issue 1

caused by phosphorylation of TJ components, activation of protein kinases, or calcium depletion.[49e52]

TEER measurement has been widely applied in evaluating the integrity of the monolayer and studying the permeability of transport processes. Changes in TEER values have been directly linked to the function of the paracellular occluding barrier[53,54] TEER is caused by cell-substrate contact, and if the distance of the cell- substrate is short, a high TEER value is observed. Based on this physiologic characteristic, paracellular resistance and the average cell-substrate distance can be estimated by the TEER. Paracellular resistance consists of the serial resistance of the TJs as well as lateral intercellular resistence.[55] Therefore, to establish the relationship between the change in TEER and the state of TJ barrier function, we used EVOM instrumentation to determine the TEER of two kinds of cells at different time points after treatment with the compounds. The results were consistent with those of immunocytochemistry, and revealed that TEER value gradually declined following treatment of cells with menthol, and values of the other groups were relatively stable during the test period. Therefore, this experiment showed that menthol is capable of weakening the BBB and enhancing paracellular transport. The mechanism of the decrease in TEER also involves $Ca^{2+}$ influx and the $Ca^{2+}$ chelator EGTA blocked cofilin dephosphorylation. Furthermore, the alteration may have been caused by activity variation of intrinsic membrane proteins.[56] For example, removal and addition of $Ca^{2+}$ to the filter where MDCK cell layers form a monolayer may reversibly lead to their TJs opening and resealing, and the cell-substrate separation may correspondingly increase or decrease.[57] In addition, we were surprised to find that reduction in TEER of the MDCK cells occurred earlier than it did in MDCK-MDR1 cells, which suggests that the TJ barrier in the MDCK-MDR1 was stronger than it was in MDCK, and menthol might have a rapid onset of action in MDCK cells.

Our previous research in rats and mice on the pharmacokinetics of puerarin in Tongqiaosanyu prescription used different methods of administration, including caudal vein injection as well as nasal and oral administration. The study employed reversed-phase high-performance liquid chromatography(RP-HPLC) to determine the concentration of the puerarin in blood and brain tissue samples, which were collected at different time points. Pharmacokinetics in rats showed that $AUC_{0eN}$ following nasal administration was 376.56  93.93 mg/min/L and absolute bioavailability of puerarin was 47.78% by nasal administration, which was significantly higher than oral administration. These results were the basis for our choosing nasal administration of Tongqiaosanyu prescription. Plasma and brain pharmacokinetics in mice indicated that the brain targeting coefficient (Re) was 132.25% following intranasal administration and the brain drug targeting index (DTI) was 2.70, both of which were significantly higher than Re and DTI obtained following injection administration. These results showed that intranasal administration enhanced the medicine's penetration and absorption into the brain.[58] Furthermore, we used the abdominal skin of the American bullfrog *Rana catesbeiana* as an in vitro model to study the effect of different concentrations of puerarin and the effect of different proportions of menthol on the apparent permeability coefficient ($P_{app}$) using the Franz diffusion cell system and HPLC. The results showed there was no significant difference in the effects of different concentrations of puerarin on $P_{app}$, indicating that the permeation of puerarin is via passive diffusion. Furthermore, when the mass concentration of menthol was 5 mg/L, there was a significant difference compared with the puerarin group, which proved that menthol enhanced the permeation of puerarin at a certain concentrationrange.[59]

The main limitation of this study is that we only explored a single drug dose. Future studies will further investigate the dose-effect relationship. Therefore, the mechanism underlying the effect of drugs on the TJ and transport through the BBB needs further elucidation.

## Conclusion

In conclusion, this preliminary investigation demonstrated the mechanism of puerarin, paeoniflorin, and menthol on TJ proteins and TEER. Compared with puerarin and paeo- niflorin, menthol in Tongqiaosanyu prescription affected the TJ structure and function in MDCK and MDCK-MDR1 cells, which were used as in vitro BBB models. Menthol inhibited occludin, claudin-1, and F-actin, which suggests that it may be a potential drug for enhancing the transport of other drugs though the BBB and, thereby, improving their permeation in the brain. Moreover, menthol downregulated the TEER, which verified the relationship between TJ and TEER value, and contributed to opening the barrier to increase paracellular transport. This study was carried out to advance our understanding of the effect of puerarin, paeoniflorin, and menthol on the BBB and provide insight into how Tongqiaosanyu affects TJ structure and function. The study also helps explain the interaction of drugs in a multicomponent herbal prescription.

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ISSN : 2693 6356
2024 | Vol 1 | Issue 1

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