The permeability characteristics and interaction of the main components from Zhizi Bopi decoction in the MDCK cell model

Zhengyue Qian, Cheng Huang, Chenlin Shen, Xiaoming Meng, Zhaolin Chen, Tingting Hu, Yangyang Li, and Jun Li

School of Pharmacy, Anhui Key Laboratory of Bioactivity of Natural Products, Anhui Medical University, Hefei 230032, China

Abstract
1. Although emerging evidence indicates the therapeutic effects of Zhizi Bopi Decoction, the extent to which essential ingredients are absorbed and the possible synergistic actions are poorly understood.
2. In this study, MDCK cell model was used to determine the bi-directional permeability and interaction between the main components (geniposide, berberine and glycyrrhizic acid) of Zhizi Bopi Decoction.
3. The transport of the active ingredients was concentration-dependent in both directions. Moreover, the Papp (AP-BL) values of berberine and glycyrrhizic acid were significantly reduced when co-incubation with an ATP inhibitor. Additionally, uptake of berberine, glycyrrhizic acid were clearly inhibited by the inhibitors of P-glycoprotein and MRP2, indicating that P-gp and MRP2 may be involved in the transport of berberine and glycyrrhizic acid, respectively. However, it was found that geniposide may be purely passive diffusion. Furthermore, the combined incubation of geniposide with berberine and glycyrrhizic acid had a powerful sorbefulent effect than use of a single drug alone which may be regulated by tight junctions.
4. In summary, our study provides useful information for pharmacological applications of Zhizi Bopi Decoction and offers new insights into this ancient decoction for further researches, especially in drug synergism.

Keywords
MDCK, P-gp, MRP2, Zhizi Bopi Decoction, tight junctions

Introduction
The past decades have witnessed an ever increasing interest in traditional Chinese medicine (TCM) due to their moderate treatment effects, lower side effects and enhanced availability around the world (Huang et al., 2004). TCM potentially meets the demands of treating a number of complex diseases in a synergistic manner, since it has been used for thousands of years (Cheng et al., 2003; Huang et al., 2004). Formula is best reflected in TCM, which contains two significant fundamental concepts: the holistic treatment and synergy strategies, namely prescription compatibility. And it is normally administered to patients in the form of decoction, which is prepared by boiling the specific combination of different herbs, mainly with water (Qiu, 2007). In addition, according to the combination principle of “monarch, minister, assistant and messenger”, formula reveals the fact that each herbal ingredient exhibits its specific function when organized integrally and harmoniously (Guo et al., 2014).

Zhizi Bopi Decoction, derived from Zhang Zhongjing’s “Typhoid Theory” in the Han dynasty (200 BC), is composed of Gardenia jasminoides Ellis, Cortex phellodendri and Glycyrrhiza Radix et Rhizoma, having the efficacy of nourishing liver and treating damp heat jaundice (our unpublished data). Specifically, the weight ratio of 10:6:3 was considered to exhibit the best therapeutic action. Gardenia jasminoides Ellis is considered as “monarch” herb, and geniposide (G) is identified as the major bioactive ingredient of Gardenia jasminoides Ellis (Lee et al., 2014). Besides, the content of berberine (B) is highest among all effective components in Cortex phellodendri (Zhang et al., 2010). Accumulating evidence indicated the pharmacokinetics of berberine in rats, rabbits, dogs (Gong et al., 2014; Liu et al., 2011; Wang et al., 2013). Moreover, Glycyrrhiza Radix et Rhizoma is generally used as a unique “guide drug” in many Chinese multi-herb prescriptions and formulas, which accounts for its unique effect to enhance the effectiveness of other ingredients, to reduce toxicity or to improve taste in low dosages (Zhang et al., 2011). Glycyrrhizic acid (GA) is generally supposed to be the primary biologically active component (Asl & Hosseinzadeh, 2008; Yu et al., 2012). We also detected the principal compositions of Zhizi Bopi Decoction by Mass spectrum and HPLC, and found that the primary components were exactly the constituents.
mentioned above (our unpublished data). Therefore, glycyrrhizic acid was taken as the representative element, and geniposide, berberine as the other major components of Zhizi Bopi Decoction in our experiment (Figure 1).

So far the membrane permeability and compatibility mechanism of the main components from Zhizi Bopi Decoction has not been reported, therefore our current study focused on the mechanistic basis for the oral absorption of the active ingredients. In particular, Madin-Darby canine kidney (MDCK) cell monolayer model was used to study the transport mechanisms of geniposide, berberine and glycyrrhizic acid. MDCK cell monolayer model is a well-accepted model of human intestinal absorption, which is similar to Caco-2 cells due to the polarity and tight junctions (Guarino et al., 2011). Given the strong evidence implicating that P_app values and Spearman’s rank correlation coefficients correlated well between MDCK and Caco-2 cell lines (Guarino et al., 2011; Irvine et al., 1999). Furthermore, our previous results were in agreement with several other observations that MDCK cells also functionally express transporters for P-gp/MDR1 [ABCB1] and MRP2 [ABCC2] (Chen et al., 2014). Moreover, these transporters play crucial roles in protecting the organism against xenobiotic compounds which serve as the first protecting barrier for organisms (Chen et al., 2014; Kuteykin-Teplyakov et al., 2010; Yang et al., 2013). Thus, MDCK cells were used as an absorption model to clarify the transport mechanism of geniposide, berberine and glycyrrhizic acid.

Tight junctions (TJs) are localized at the most apical cell-cell connections and have high impact on the maintenance of tissue integrity which compose of various proteins including transmembrane proteins (occludin, claudin and JAM proteins), cytoplasmic attached ZO protein family (ZO-1, ZO-2, and ZO-3) and its relevant cytoskeletal proteins (actin) (Liang et al., 2015). As an integral part of TJs, transmembrane proteins claudin and the tight junction-associated protein occludin affect the structure and function of TJs thereby sealing the intercellular interspace and defining the permeability characteristics of epithelial cell layers (Shen et al., 2011). ZOs play a vital role in developing and stabilizing TJs as well by directly interacting with actin filaments (Lai et al., 2005). Many reports have proved that plentiful of drugs have the ability to disturb the expression of tight junctions and then the permeability values were increased (Liang et al., 2015). Therefore, it is assumed that the interaction of geniposide, berberine and glycyrrhizic acid may be positively related to TJs.

The present study was to discuss mechanisms governing the oral bioavailability of three key components of Zhizi Bopi Decoction and their possible synergistic actions using the MDCK cell model.

Materials and methods

Materials

MDCK cells were kindly donated by Professor Biwei Song (Zhejiang University of Technology, Zhejiang, China). Hanks balanced salt solution (HBSS), fetal bovine serum (FBS), modified Eagle’s medium (MEM), Fluorescein sodium, MK-571 and probenecid were all obtained from Sigma Inc. (St. Louis, MO). Verapamil, berberine hydrochloride and geniposide (purity > 99%) were purchased from NICPBP (National institution for Food and Drug Control) (Beijing, China). Glycyrrhizic acid (purity > 99%) was purchased from Yuanye Bio-Technology Co Ltd (Shanghai, China). Sodium azide was obtained from Red Crag Reagent Factory (Beijing, China). All other reagents employed in our study were of the highest commercially available purity.
Cell culture

MDCK cells were routinely grown in a medium containing MEM which was supplemented with 10% (v/v) FBS as well as 1% penicillin–streptomycin solution under a humidified atmosphere of 5% CO2 air at 37°C.

To carry out the transport experiment, the cells were seeded on Millicell Hanging cell culture inserts (13.0 mm diameter, 1.33 cm² area, 1 μm pore diameter; Millipore Corporation, Billerica, MA) in 12 Transwell plates at a density of 5 × 10⁵ cells per cm² filter. The culture medium was changed every 2–3 days. The monolayers were ready for further studies from 4 to 6 days after seeding.

Transport studies of three compounds through MDCK cell culture model

Firstly, the monolayers were incubated with HBSS for 30 min at 37°C, then the incubation buffer was aspirated gently. Afterwards, the drug candidates were placed on donor side for the indicated times, while drug-free HBSS was loaded on the receiver side. The maximum concentration of essential ingredients was nontoxic to the cell viability which has been already proved before that the maximum final concentration of media was nontoxic to the cell viability which has been determined fluorocein sodium permeability. The permeability in AP-BL direction on days 0, 2, 4 and 7 was 16.8 × 10⁻⁶ cm/s, 10.23 × 10⁻⁶ cm/s, 1.56 × 10⁻⁶ cm/s and 0.67 × 10⁻⁶ cm/s, respectively, which showed significant change on days 4 and 7 (p < 0.01). Only monolayers which demonstrated a TEER value above 140 Ω×cm² and fluorocein sodium permeability <(0.2-2) × 10⁻⁶ cm/s were used in the transport experiment.

HPLC analysis of transport samples

The amounts of compounds in the media were analyzed by reversed phase chromatography on Agilent1200 HPLC instrument (Palo Alto, CA) which equipped with a Hypersil C18 column (4.6 mm × 250 mm, 5 μm). Analysis was performed at the flow rate of 1.0 ml/min with a gradient elution program and the injected volume was 10 μL. The ultraviolet detection was achieved at 250 nm. The mobile phase was methanol (solvent A) and water-phosphoric acid (998:2, v/v) (solvent B). The gradient elution program was: 90% B (0–12 min), 90–70% B (12–13 min), 70–40% B (13–20 min), 40–30% B (20–30 min), 30–90% B (30–35 min) and 90% B (35–50 min). This was followed by a 15 min equilibrium period before the next sample was injected. While the samples were analyzed alone, the programs were performed as follows: system and column were the same as mentioned, the mobile phase was composed of 10% A, 90% B for geniposide, 60% A, 40% B for berberine and 45% A, 55% B for glycyrrhizic acid. Representative chromatograms of the test analytes taken from apical to basolateral transport at 120 min in MDCK cell monolayer are shown in Supplementary Figure 1 and paeoniflorin was taken as the internal standard. The linearity of the method was evaluated over the range of 0.39–50 μM for geniposide and berberine, and 0.19–25 μM for glycyrrhizic acid, the limit of quantification (LOQ) was 0.28 μM for geniposide, 0.23 μM for berberine, and 0.12 μM for glycyrrhizic acid, which were sensitive enough to determine the low concentration of the analytes.

Western blot analysis

Cells were collected from the Transwells and proteins were extracted from MDCK cells with RIPA buffer (Beyotime, Shanghai, China), and quantified by a Bicin Choninic Acid (BCA) protein assay kit (Boster, Wuhan, China).

Proteins were separated by SDS gels and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp, Billerica, MA, USA). The membranes were blocked for 3 h at 5% skimmed milk then incubated overnight at 4°C with the following antibodies: anti-ZO-1, anti-occludin, anti-claudin-1 (Bioss Biosynthesis biotechnology co., LTD, Beijing, China) and anti-actin (ImmunoWay Biotechnology Company, Newark, DE). GAPDH (Santa Cruz Biotechnology Inc., Dallas, TX) was used as the internal control. On the second day, after incubation with the suitable secondary antibodies, the bands were developed.
with chemiluminescence reagents (Model No. ChemiQ 4600, Bioshine, Shanghai, China).

**Data analysis**

The apparent permeability coefficients, $P_{\text{app}}$ (cm/s), for both absorption and secretion studies were calculated by the following equation as described previously (Yunomae et al., 2003):

$$P_{\text{app}} = \frac{dQ}{dt}/A \times C_0$$

where $dQ/dt$ is the linear slope of the cumulative concentration of the compound in the receiving chamber with time (mmol/s), $A$ is the surface of the cell monolayer (cm$^2$) ($A = 1.33$ cm$^2$), and $C_0$ represents the initial concentration in the donor compartment at time 0 (mmol).

**Statistical analysis**

Experimentally derived *in vitro* data are presented as mean ± SD. Each measurement was run in triplicate. The results were subjected to statistical analysis by one factor analysis of variance (ANOVA) or Student’s $t$ test. All statistical analyses were determined by SPSS 12.0 software (Chicago, IL). Results were demonstrated significant at $p < 0.05$.

**Results**

**Absorptive and secretory transport of three tested chemicals across MDCK cell monolayers**

The transcellular transport of the three main chemicals (50, 100, 150 and 200 μM) from apical (AP) to basolateral (BL) side or from BL to AP side with incubation time from 0 to 2 h was determined using MDCK cell model. As shown in Figure 2, the transcellular transport amount of the three compounds increased gradually up to 2 h and was concentration-dependent in both directions. The transport amount (μmol) of B and GA at BL-AP direction was even larger than that in the negative direction, while no obvious difference was found in both directions for G. The $P_{\text{app}}$ values of the investigational compounds are summarized in Table 1. The data indicated that the $P_{\text{app}}$ values of B and GA from BL to AP tended to be greater than those from AP to BL at concentrations ranging from 50 to 200 μM ($p < 0.05$). However, the values of G in both directions did not differ significantly ($p > 0.05$) and the efflux ratio (ER) was less than 2.

![Figure 2](image-url)
Effect of temperature and ATP inhibitor on the three main compounds transport

The effect of ATP inhibitor and temperature on the bi-directional transcellular transport of G, B, and GA at concentration of 100 μM is shown in Figure 3. The efflux ratios of B and GA were decreased by 41% and 34% at 4°C compared to those at 37°C in MDCK cells, respectively (p < 0.05). Consistently, transport experiment co-treatment with ATP inhibitor suggested that transport rates of B and GA were reduced by almost 1.29–2.91-fold (p < 0.05). Besides, P_app values of G were not significantly variant in both directions, and no distinct difference was found when performed at 4°C or in the presence of ATP inhibitor.

Effect of pH on the three main ingredients transport

As shown in Figure 4(A), with pH increased from 7.4 to 9.0 in both chambers, the permeability of B distinctly increased (25%, p < 0.05) in AP-BL direction and slightly decreased in BL-AP direction at concentration of 100 μM. On the contrary, the P_app values of GA were significantly increased (p < 0.05) at pH 5.5 in AP-BL direction and slightly reduced in BL-AP direction. However, the P_app values of G showed little variation in both directions at pH 9.0 and 5.5 compared to those at pH 7.4.

Transport inhibition by verapamil, MK571 and probenecid

To determine whether an ABC transporter is responsible for the poor absorption of three active ingredients, the P-gp inhibitor verapamil and MRP2 inhibitors probenecid or MK571 were used in the experiment. Significant differences were discovered in the P_app value of B when co-incubation with verapamil, indicating that P-gp may be involved in the efflux of B (Figure 4B, p < 0.05), while no significant differences were found in the transport experiments of G and GA. On the other hand, co-incubation with MK571 or probenecid obviously decreased P_app (BL-AP) values of GA to about 60–72%, and increased the values of P_app (AP-BL) by 20% approximately (Figure 4B, p < 0.05), demonstrating a probably role for MRP2 in the transcellular efflux of GA. However, it is suggested that MRP2 might not be responsible for the poor absorption of G and B. Altogether, P-gp and MRP2 were involved in the efflux of B and GA, respectively. And the changes in ER values in the presence of the inhibitors were summarized in Table 2.

“Uphill” transport of the major ingredients

It is all known that the ability to transport drugs through cell monolayers against a concentration gradient is a symbol for the involvement of active transport. To further demonstrate the possible role of efflux transporters, we performed the “uphill” transport, and it was obvious that more accumulative drug on the apical side for B and GA after 2 h of experiment, suggesting that transport of B and GA may be mediated by transporters and regulated against a concentration gradient (apical 58.5 ± 3% versus basal 37.6 ± 1%, 56.9 ± 3% versus 41.9 ± 4%, respectively, n = 3–6, p < 0.05, percent of total amount of drug). In contrast, the cumulative amount of G was equal approximately in both sides (p > 0.05).

Effect of main ingredients on membrane permeability

In order to determine the synergistic action when the key ingredients were incubated together, different concentrations of G, B, and GA were used in the transport experiment which was based on the test results by HPLC. As shown in Figure 5(A), the P_app values of B were increased significantly when they were co-incubated with G and GA and it was higher than that of B alone, and similar results were observed in different compatibility proportion (p < 0.05). On the other hand, the absorption of G in AP to BL direction were reinforced while the P_app values in BL to AP direction were also improved when the concentrations of G, B, and GA were 220, 100, and 60 μM, respectively (p < 0.01).

To further investigate the probable mechanisms of synergistic action of the dominant components, the TEER was detected and it showed an obvious change in 2 hours after treatment with a certain concentration of three constituents compared with that of blank HBSS buffer (Figure 5B, p < 0.05), and it tended to get reversible recovery in 48 hours.

Table 1. Bi-directional apparent permeability coefficients (P_app) of the compounds across MDCK cell monolayers.

| Compound | Initial concentrations (μmol/L) | P_app (AP-BL) (×10⁻⁷ cm/s) | P_app (BL-AP) (×10⁻⁷ cm/s) | P_app (BL-AP)/P_app (AP-BL) |
|----------|---------------------------------|-----------------------------|-----------------------------|------------------------------|
| G        | 50.00                           | 5.62 ± 0.07                 | 6.18 ± 0.26                 | 1.10 ± 0.03                  |
|          | 100.00                          | 5.78 ± 0.18                 | 6.49 ± 0.26                 | 1.13 ± 0.01                  |
|          | 150.00                          | 5.78 ± 0.53                 | 6.17 ± 0.52                 | 1.07 ± 0.40                  |
|          | 200.00                          | 6.08 ± 0.27                 | 6.33 ± 0.11                 | 1.04 ± 0.06                  |
| B        | 50.00                           | 8.72 ± 0.45                 | 57.06 ± 0.66                | 6.56 ± 0.41                  |
|          | 100.00                          | 9.00 ± 0.17                 | 59.50 ± 0.70                | 6.90 ± 0.12                  |
|          | 150.00                          | 10.37 ± 0.07                | 62.04 ± 1.61                | 5.29 ± 0.31                  |
|          | 200.00                          | 10.45 ± 0.27                | 62.75 ± 0.02                | 6.00 ± 0.13                  |
| GA       | 50.00                           | 4.70 ± 0.58                 | 9.71 ± 0.49                 | 2.09 ± 0.28                  |
|          | 100.00                          | 4.59 ± 0.17                 | 10.22 ± 0.09                | 2.12 ± 0.14                  |
|          | 150.00                          | 4.62 ± 0.18                 | 9.95 ± 0.95                 | 2.16 ± 0.17                  |
|          | 200.00                          | 4.85 ± 0.45                 | 10.20 ± 0.89                | 2.11 ± 0.09                  |

Data are shown as the mean ± SD (n = 3–6).
In addition, treatment with G (220 μM), B (100 μM) and GA (60 μM) changed the most significantly in TEER (p < 0.01), and tight junction integrity of the cell monolayers has a tendency to get recovery, demonstrating that cell viability was not affected with the maximum concentration. The results showed that the three constituents could open cell tight junction possibly and trigger the increase of monolayer permeability, which might be one of the significant causes of reinforced absorption.

**Figure 3.** Effect of temperature and ATP inhibitor on the transepithelial transport of geniposide (G), berberine (B), glycyrrhizic acid (GA) from AP to BL side and BL to AP side across MDCK cell monolayers. The MDCK cell monolayers were incubated at 37 °C or 4 °C or 37 °C + NaN3 in HBSS (pH 7.4) with the test compounds (100 μM) for 2 h. Data are shown as the mean ± SD (n = 3–6). *p < 0.05, **p < 0.01, compared to those at 37 °C.

**Figure 4.** (A) Effect of pH on the transepithelial transport of geniposide (G), berberine (B), glycyrrhizic acid (GA) in both directions across MDCK cell monolayers. The MDCK cell monolayers were incubated at 37 °C in HBSS (pH 7.4, pH 5.5 or pH 9.0) with the tested chemicals (100 μM) for 2 h. Data are shown as the mean ± SD (n = 3–6). *p < 0.05, **p < 0.01, compared to those at pH 7.4. (B) Effect of transport inhibitors on geniposide (G), berberine (B) and glycyrrhizic acid (GA) from AP to BL side and BL to AP side across MDCK monolayers. The initial concentrations of compounds in the donor compartment were 100 μM. Data are shown as the mean ± SD (n = 3–6). *p < 0.05, **p < 0.01, compared to those at 37 °C.

**Effect of main ingredients pretreatment of MDCK cells on the expression of TJ-related proteins**

To investigate the effect of main components on TJs, TJ-related proteins expression levels was detected by western blot analysis. The results from Figure 6 suggested that the protein expression levels of ZO-1, occludin, actin and claudin-1 in the MDCK intestinal epithelial cells decreased after treated with G, B, and GA for 48 h. The rank order of TJ-related proteins expressions was about 1 ≈ 2 > 3 ≈ 4.
Although beneficial effects of Zhizi Bopi Decoction and active ingredients on all kinds of diseases have been identified, the absorptive mechanisms responsible for the pharmacological effects and probable synergistic effect of bioactive constituents are still unclear. Accordingly, the penetrative characteristics and transport mechanisms of the main ingredients isolated from Zhizi Bopi Decoction were studied with MDCK cell monolayers.

Increasing evidence has shown that cell monolayers, such as Caco-2 and MDCK, could serve as an excellent tool to determine drugs with potential absorption problems. They possibly also correlate well with observations in vivo due to its similarity to human intestinal epithelium cells (Artursson et al., 2001). Caco-2 cells have close relationship with human intestinal epithelial cells, and possess a great many of common epithelial cell characteristics, but this well-established cell culture model suffers from the shortcomings of 21-day-long culturing time (Hayeshi et al., 2008). Herein, it

### Table 2. The change of ER values for geniposide (G), berberine (B) and glycyrrhizic acid (GA) after exposure to different inhibitors.

| Compound | Inhibitors |\(P_{\text{app}} \, (\text{AP-BL})\) (\(\times 10^{-7}\) cm/s) |\(P_{\text{app}} \, (\text{BL-AP})\) (\(\times 10^{-7}\) cm/s) |\(\frac{P_{\text{app}} \, (\text{BL-AP})}{P_{\text{app}} \, (\text{AP-BL})}\) |
|---|---|---|---|---|
| G | Control | 5.78 ± 0.18 | 7.50 ± 0.52 | 1.30 ± 0.13 |
|  | Verapamil | 5.51 ± 0.45 | 7.41 ± 0.11 | 1.34 ± 0.09 |
|  | MK-571 | 5.80 ± 0.11 | 7.42 ± 0.33 | 1.33 ± 0.01 |
|  | Probenecid | 5.67 ± 0.57 | 7.21 ± 0.72 | 1.27 ± 0.02 |
| B | Control | 9.00 ± 0.17 | 62.04 ± 1.61 | 6.90 ± 0.12 |
|  | Verapamil | 11.57 ± 0.73 | 9.14 ± 0.59 | 0.79 ± 0.06 |
|  | MK-571 | 8.27 ± 0.54 | 60.04 ± 1.55 | 6.94 ± 0.61 |
|  | Probenecid | 8.84 ± 0.30 | 59.21 ± 1.16 | 6.70 ± 0.26 |
| GA | Control | 4.59 ± 0.17 | 9.76 ± 0.76 | 2.12 ± 0.10 |
|  | Verapamil | 4.53 ± 0.09 | 9.70 ± 0.90 | 2.14 ± 0.24 |
|  | MK-571 | 5.47 ± 0.25 | 5.92 ± 0.10 | 1.08 ± 0.07 |
|  | Probenecid | 5.51 ± 0.21 | 7.07 ± 0.55 | 1.28 ± 0.08 |

Data are shown as the mean ± SD (n = 3–6).

Figure 5. (A) Synergy effects of geniposide (G) and berberine (B) from AP to BL side and BL to AP side across MDCK monolayers. *p < 0.05, **p < 0.01, compared to those at 37°C. (B) Effects of the dominant components on the MDCK cell monolayer transepithelial resistance. Data are shown as the mean ± SD (n = 3–6). 1: control groups with blank HBSS buffer, 2: G (110 μM) + B (50 μM) + GA (30 μM), 3: G (165 μM) + B (75 μM) + GA (45 μM), 4: G (220 μM) + B (100 μM) + GA (60 μM).
Figure 6. Expression of TJ-related proteins. MDCK cell monolayers were incubated with different concentrations of geniposide (G), berberine (B) and glycyrrhizic acid (GA) for 48 h. Data are expressed as the mean ± SD.

1: control groups, 2: G (110 μM) + B (50 μM) + GA (30 μM), 3: G (165 μM) + B (75 μM) + GA (45 μM), 4: G (220 μM) + B (100 μM) + GA (60 μM).

is necessary to choose a kind of “intestinal-like” cells to conquer the above disadvantages for option. MDCK cells, isolated from canine distal renal tissue, have lower TEER and shorter culture time than Caco-2 cells, but also have lots of conjunct epithelial cell characteristics exactly as Caco-2 cells (Volpe, 2011). Moreover, MDCK cells have been proved to express both efflux (Mdr1, Mrp1, Mrp2, etc.) and uptake (Oct2, etc.) transporters by lots of research teams (Brayden & Griffin, 2008; Chen et al., 2014; Volpe, 2011). So, in our present work, MDCK cell monolayer was used to determine the transcellular transport of the main components of Zhizi Bopi Decoction.

Our preliminary results demonstrated that only a fraction of these three test chemicals was transported across MDCK cells (Supplementary Figure 1), indicating the poor bioavailability of these three test chemicals was transported across MDCK cells (Supplementary Figure 1), indicating the poor bioavailability of these three test chemicals was transported across MDCK cells. So, in our present work, MDCK cell monolayer was used to determine the transcellular transport of the main components of Zhizi Bopi Decoction.

It is well-known that the pH gradient (acid in the stomach, acidic to neutral in the small intestine, basic in
the colon and pH 7.4 is the physiological pH) in gastrointestinal tract throughout its length exerts essential roles in the absorption of drugs (Pugsley et al., 2008). The absorption of drugs could be reinforced by considering charge state at biologically correlative pH values. Therefore, to determine whether a drug’s permeability is changed in different pH conditions, the importance of gastrointestinal pH in modeling absorption should be highlighted. Results of the drug candidates at different pH in AP-BL direction, while glycyrrhizic acid exhibited better absorption at pH 5.5 oppositely. However, no significant difference was observed for geniposide transport at different pH in both directions. This may be caused by the possibility that many physicochemical and biological factors would influence the bioavailability of drugs (Figure 4A).

Based on these data, conclusion was drawn that all the main components of Zhizi Bopi Decoction were considered to be poorly absorbed because $P_{app}$ was nearly lower than $1.0 \times 10^{-6} \text{cm/s}$ (Li et al., 2008). Just as lisinopril and ranitidine, which were classified as having low permeability for BCS permeability classification, and the permeability values were $6.7 \pm 2.1 \times 10^{-7} \text{cm/s}$, $7.5 \pm 3.3 \times 10^{-7} \text{cm/s}$ at pH 7.4 in MDCKII-MDR1 cell monolayers (Thiel-Demby et al., 2009). However, the synergistic action was found that the permeability of berberine and geniposide were increased apparently when they were incubated together (Figure 5A). But, it still requires validation to make them applicable for in vivo predictions. On the other hand, the change of TEER has been investigated in the experiment of each point over time which has been used as an index for tight junction integrity of the cell monolayers (Bromberg & Alakhov, 2003). It was noteworthy that TEER of MDCK cell membranes changed significantly when geniposide, berberine and glycyrrhizic acid were co-incubated in a certain range of concentration. It showed obviously different from untreated monolayers over the same time period. Furthermore, 2-hour incubations were also conducted with each ingredient alone and TEER values decreased marginally (data not shown). Results indicated that the tight junctions of cell monolayers were opened when ingredients were co-incubated together which might lead to an increased absorption of geniposide and berberine (Figure 5B).

Considering of the obvious decrease of TEER during treatment with geniposide, berberine and glycyrrhizic acid, the altered intercellular space, namely TJs, may need to be taken into account. Various reports have proved the role of TJs in the control of endothelial barrier integrity and vascular permeability (Garcia-Ponce et al., 2015; Watari et al., 2015). Therefore, regulating the TJ seal is a promising option for increasing the transdermal absorption of agents. From our results (Figure 6), it could be concluded that the three ingredients, like plentiful of other drugs, have the ability to disturb the expression of TJ components (Liang et al., 2015; Watari et al., 2015), thus reinforce the absorption of berberine and increased the permeability of geniposide in vitro. In addition, the absorption of glycyrrhizic acid might also be enhanced on account of the open of TJs. Considering that every herb in Zhizi Bopi Decoction is a chemically complex component except the main ingredients described in the manuscript, and Traditional Chinese Medicine generally supports a multiple components combination of herbs to keep overall healthful balance in a holistic way, so Zhizi Bopi Decoction may perform better absorption and therapeutic effect in vivo.

More importantly, Glycyrrhiza Radix et Rhizoma, which arises in more than half of TCM prescriptions, is widely used as a “guide drug” to enhance the effectiveness of other ingredients and to reduce toxicity including Zhizi Bopi Decoction (Xing et al., 2011). It should be emphasized that the therapeutic action of many compounds exhibits a marked enhancement. Moreover, the mechanism of their activity may even alter on account of glycyrrhizic acid in some cases (Polyakov et al., 2008). In particular, glycyrrhizic acid could enhance the permeability (about 60%) and decrease the elasticity modulus of cell membranes (Selyutina et al., 2015), this might one of the mechanisms by which the absorption of geniposide and berberine was promoted. Additionally, the decreased expression and function of efflux transporters (Wang et al., 2014) or the inhibited activity of metabolic enzyme (Potter et al., 2015) could also reinforce absorption of drugs candidates, the underlying molecular mechanisms responsible for the pharmacological effects still need to be further investigated.

Conclusions

In conclusion, the absorption and interaction of the essential ingredients in the Zhizi Bopi Decoction were studied for the first time by using the MDCK cell model. It was found that the absorption of all the active components in Zhizi Bopi Decoction were poor, however, when they were co-incubated, the absorption of geniposide and berberine could be dramatically reinforced through triggering the TJs, indicating that these three compounds have a synergistic reaction in the MDCK cell model, which may suggest the compatibility mechanism of TCMs for the first time.

Acknowledgements

The authors thank Pro. Biwei Song for offering MDCK cell line.

Declaration of interest

This project was supported by the National Science Foundation of China (nos:81273526, and 81473268), Anhui provincial key Scientific and technological project (1301042212), Anhui Provincial Natural Science Foundation (1308085MH145), Specialized Research Fund for the Doctoral Program of Higher Education (20123420120001).

References

Artursson P, Palm K, Luthman K. (2001). Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev 46:27–43.

Asl MN, Hosseinazadeh H. (2008). Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds. Phytother Res 22: 709–24.

Brayden DJ, Griffin J. (2008). Avermectin transepithelial transport in MDRI- and MRP-transfected canine kidney monolayers. Vet Res Commun 32:93–106.
