doi.org/10.36721/PJPS.2020.33.4.REG.1569-1575.1

Transport properties of paeoniflorin and amygdalin across caco-2 cell monolayer model and their modulation of cytochrome p450 metabolism

Huifen Zhou[1], Yu He[1], Jiehong Yang[1], Haofang Wan[1], Liqin Wang[2]* and Haitong Wan[1]**
[1]Zhejiang Chinese Medical University, Hangzhou, China
[2]The First Affiliated Hospital of Nanchang University, Nanchang, China

Abstract: Paeoniflorin and amygdalin are two major active saponins constituents in some Chinese herbal formulas used for cardio-cerebrovascular diseases. However, their intestinal absorption property and metabolic characteristics have not been clarified. The aim of this work was to study the absorption property of Paeoniflorin and Amygdalin across Caco-2 cell monolayer and their metabolic characteristics on the activity of cytochrome P450 (CYP450) enzyme. The results showed that the transport amount of Paeoniflorin and Amygdalin was positively correlated with the time and concentrations, and the transport amount from AP side to BL side was higher than that from BL to AP. The absorptions of Paeoniflorin and Amygdalin were reduced by P-glycoprotein, which provided the pharmacokinetic basis for their clinical application. Furthermore, we demonstrated that Paeoniflorin and Amygdalin had obvious inhibiting effects on CYP2C9 and CYP2E1. The transports of Paeoniflorin and Amygdalin across Caco-2 cell monolayer model were deduced as the passive transport, which indicated that the present bioassay system was appropriate and reliable for the evaluation of the transport characteristics and metabolic characteristics of active ingredient groups in Bu-yang-huan-wu decoction. Moreover, this research method may also be suitable for the appropriate bioactivity and metabolic characteristics analysis of other plant extracts.

Keywords: Paeoniflorin, amygdalin, bu-yang-huan-wu decoction, caco-2 cell, cytochrome P450.

INTRODUCTION

Caco-2 cell line is a widely used cell line which is derived from human colorectal carcinoma (Maggini *et al*., 1994). It is a quick screening tool for predicting intestinal absorption of new drugs, which can provide the absorption, distribution, metabolism, and toxicity information at the cellular levels (Wehe *et al*., 2013, Zhao *et al*., 2012, Hou *et al*., 2012). On the appropriate invitro culture condition, Caco-2 cells model is similar to the intestinal mucosal epithelial cells, and they all have the same enzymes system. P-glycoprotein (P-gp) is one of the main effluent proteins, and it is also an important factor for the low bioavailability and large fluctuations. Liver is the most important organ for drug metabolism of human body, and cytochrome P450 (CYP450) enzyme is one of main metabolic enzyme system in it. CYP450 enzymes are the most important enzymes involved in metabolic activation or detoxification of various drugs used in clinical practice, which are designed to explore the influence of metabolism on the cellular and molecular mechanisms of action of potential drugs and those used therapeutically. They can also be used to study the effect of tested compounds on activity and expression of metabolizing enzymes.

Paeoniflorin and Amygdalin are the major active saponins ingredients extracted from the Chinese herbal compound prescription Bu-yang-huan-wu decoction. As the previous studies showed Paeoniflorin performed its unique protection against ischemia-induced brain damages in rats via inhibiting inflammatory responses (Wu *et al*., 2013, Wang *et al*., 2012), and its anti-proliferative activity on cancer cells by blocking cell cycle progression and inducing apoptosis (Yang *et al*., 2008, Li *et al*., 2008). Amygdalin has its antitussive asthma, anti-tumor, inhibition of renal fibrosis in chronic kidney disease (Ito *et al*., 2003, Wu *et al*., 2013, Komatsu *et al*., 1998). However, the bioavailability of paeoniflorin is only 21.11±7.92% in rats (Kim *et al*., 2012), and the pharmacokinetics parameters of Amygdalin are lower as compared to Paeoniflorin in dogs (Qu *et al*., 2016). This experiment was dedicated to investigate the relationship between concentration of drugs and time of absorption and transport of Paeoniflorin and Amygdalin in human small intestine. We chose the caco-2 cells model as research methods to further explain the absorption and metabolism of active ingredients of Chinese traditional medicine in intestine, as well as drug interactions (Hamada *et al*., 2013). We used the method of rat liver microsome incubation containing the NADPH-generating in vitro, and studied the effects of drugs on CYP450 enzyme activity to avoid or lighten the occurrence of metabolic drug interactions.

MATERIALS AND METHODS

Materials

Caco-2 the human colon adenocarcinoma cell lines (Chinese Academy of Sciences Shanghai Cell Bank);

**Corresponding author:* e-mail: wangliqin_0607@163.com, whtong@126.com

Trypan blue(Sigma, United States); Phenol red (Haotian Biology); alkaline phosphatase kit (ALP/AKP) (Nanjing Jiancheng Bioengineering Institute); DMEM (contain L-glutamine, Earle's BSS, sodium pyruvate) medium (Gibco U.S. company); Fetal bovine serum (FBS) (Gibco U.S. company); Non-essential amino acids (Gibco U.S. companies); D-Hank's (Haotian Biology); 0.25%-0.2% EDTA trypsin digestion (Gibco U.S. company); Paeoniflorin (NIFDC China); Amygdalin (NIFDC China); Potassium dihydrogen phosphate; Disodium hydrogen phosphate; Sodium chloride; Potassium chloride and other reagents are analytical grade.

Cells culture and model established

Caco-2 cells was inoculated in 25cm^2 flasks and incubated at 37^{o}C in 95% air -5% CO_2 in DMEM with L-glutamine containing 20% (v/v) defined FBS, 1% penicillin/ streptomycin solution and 1% minimal non-essential amino acids. Medium was changed every other day. Subcultured the cells until the growth area have reached about 80% area of the flasks, Cells counting and diluted to the density of 1×10^5/mL. Pipetting 400μL cells suspension was inoculated in Millicell polycarbonate membrane, then added 600μL medium in 24-well plates. The medium was changed every other day in the first week, after that changed every day till 21days later. Transepithelial electrical resistance (TEER) and alkaline phosphatase (ALP) activities were determined on the 21st day.

Cytotoxicity test

Caco-2 cells suspension were seeded at a density of 2×10^4/ml in 96-well plant, incubated 200μL to each well for 24h. DMEM containing different concentrations of Paeoniflorin and Amygdalin were added to 96-well plant, set several blank wells (only contain DMEM) and control wells (cells and DMEM) at the same time. After 4h, DMEM was removed, and MTT solution was prepared at 5g/L in PBS and was filtered through a 0.22μm filter. Then, 20μL MTT solution and 200ul DMEM were added into each well except blank wells. Cells were incubated for 4h at 37^{o}C in 95% air-5% CO_2, and MTT solution was removed and replaced with 150μL DMSO. The plate was further incubated for 10min at room temperature with a shake to make sure crystals dissolved fully, and the optical density (OD) of the wells was determined by using microplate reader at a test wavelength of 490 nm. It was the right drug concentration when Cells survival rate was more than 80%.

Transport experiments

Before the transport experiment, the cells monolayer was washed three times with the D-Hanks. After wash, the plates were incubated in D-Hanks for 30min at 37^{o}C and the TEER value was then measured. The D-Hanks on both sides of the cells monolayer was removed. In each transport experiment, each sample was repeated three times. Samples were prepared at 80, 160, 240, 400 μmol·L^{-1} in D-hanks. To measure the transport from the apical-to-basolateral (AP-to-BL) side, 0.5mL of the samples were placed to the apical side, and 1.5mL of D-Hanks without the drug was placed to the basal side. The plant was incubated at 37^{o}C in 95% air-5% CO_2, 50 r/min, a 200μL solution was moved from BL side to eppendorf tube at 30 min intervals for 2h and was assayed for Paeoniflorin and Amygdalin by high performance liquid chromatography (HPLC). To measurement of the basolateral-to-apical (BL-to-AP) transport of Paeoniflorin and Amygdalin, 1.5mL of sample was added to the basolatral side, and 0.5mL of D-Hanks without the drug was added to the apical side. A 200μL solution was removed from the AP side at appropriate time intervals for 2h and replaced with 200μL of D-Hanks. Each sample was repeated three times and assayed by HPLC. To investigate the influences for P-gp inhibitor of verapamil on the transport of Paeoniflorin and Amygdalin in caco-2 cells model, the cells was first incubated in D-Hanks contain 50μmol·L^{-1} verapamil for 30min at 37^{o}C. The D-Hanks on both sides of the cells monolayer was removed, and 0.5mL of samples were placed to the apical side, and 1.5mL of D-Hanks without the drug was placed to the basal side. The plant was incubated at 37^{o}C in 95% air-5% CO_2, 50r/min, a 200μL solution was moved from BL side to eppendorf tube at 30 min intervals for 2h. Each sample was repeated three times and assayed by HPLC.

Microsome incubations

Monitoring of metabolic stability by murine liver microsomes was performed according to Ackley *et al* (2004). In brief, total sample volume was 500μL (consisting of a newly thawed aqueous stock solution of probe substrate (PH/TOL/CHL, 10/100/20μM), 10mM magnesium chloride, 10mg/mL of the liver microsome solution, and different concentrations of Paeoniflorin and Amygdalin (10, 20, 40μM) in 100mM phosphate buffer, pH 7.4), transferred to a 37^{o}C water bath for 5 min, and 20μL of freshly made NADPH(1mM) regenerating system was added to each tube, then, vortexed and transferred to a 37^{o}C water bath for 20 min. The incubation tubes were quenched at the indicated time-points by adding 2mL ice-cold ethyl acetate and add 10μL Loratadine (10mM) as interior label, vortexing and centrifuged at 3000×g for 10 min at 4^{o}C, then take organic phase 1.5mL into vacuum drying oven. 200μL acetonitrile was added for dissolve the left after drying, vortexing and centrifuged at 16000×g for 4min at 4^{o}C, 20μL supernatant liquid was assayed by HPLC.

HPLC assays

The Paeoniflorin and Amygdalin after transport were analysis was performed using a Agilent 1200 separation module and Agilent Eclipse XDB-C_{18} column (4.6×150 mm, 5μm) and guard column (4.6×20mm, 5μm), and column temperature was 30^{o}C. The mobile phase

consisted two phase, methyl alcohol and water contain 0.04% phosphoric acid (27:73). The flow rate was 1 mL/min, detection wavelength was 230 nm and injection volume was 20μL. The probe drug analysis was performed using a agilent 1200 separation module and Agilent Eclipse XDB-C_{18} column (4.6×150mm, 5μm), the column heater was set to 30°C. The mobile phase consisted of two phases; A (100% acetonitrile), and B (0.1% phosphoric acid in water). The following gradient was selected for separation: 0 min: 20%A; 0-5 min: linear increase to 30% A; 5-15 min: linear increase 50% A. The detection wavelength was 280nm, flow rate was 1mL/min and injection volume was 20μL.

Standard solution was prepared by the standard substance of Paeoniflorin and Amygdalin with D-Hanks at the concentration of 0.2, 0.4, 0.8, 2, 4, 8, 16μmol·L^{-1}, and assayed by HPLC. With molar concentration as abscissa and peak area for as ordinate, the standard curves were established by linear regression analysis. The standard curve of Paeoniflorin was Y=34.413X -5.6208, r=0.9995; The standard curve of Amygdalin was Y=7.7298X +3.4481, r=0.999. Chromatogram was shown in (fig 1).

Prepare a series of probe drugs (PH, TOL, CHL) in different concentration, add 2mL ice-cold ethyl acetate to 500μL probe drug standard solution, vortexing and centrifuged at 3000×g for 10 min at 4°C, then take organic phase 1.5mL into vacuum drying oven. 200μL acetonitrile was added for dissolve the left after drying, vortexing and centrifuged at 16000×g for 4 min at 4°C, 20μL supernatant liquid was assayed by HPLC. With molar concentration as abscissa and peak area for as ordinate, the standard curves were established by linear regression analysis. The standard curve of PH, TOL and CHL were Y=6.0642X+0.5288, Y=0.133X+0.6441 and Y=5.6811X+0.6931, respectively.

STATISTICAL ANALYSIS

The values of apparent permeability coefficient (P_{app}) (cm/s) were calculated as the following equation:

P_{app}= dQ / (dt•A•C_0)

Where, dQ/dt is the rate at which the compound appears in the receiver chamber (nmol/s), A is the surface area of the filter membrane (cm^2) and C_0 is the initial concentration in the donor chamber (nmol/mL). The values of P_{app} in both AP-to-BL and BL-to-AP were calculated, and the ratios of the Papp in two directions were calculated from the following equation:

P_{ratio} = P_{app}(BL-to-AP) / P_{app}(AP-to-BL)

Data in the figures were analysed with the SPSS 19.0 software (SPSS Inc., USA). The results were expressed as the mean ± SD ($\bar{x}$±s) and were analysed by one-way analysis of variance (ANOVA). A value of P<0.05 was regarded to be statistically significant in the analyses.

RESULTS

The ranges of safe concentrations

According the result of Cytotoxicity test, when Cells survival rate was larger than 80%, the right drug concentration of Paeoniflorin was 0~400μmol·L^{-1}, and the Amygdalin was 0~400μmol·L^{-1}.

Caco-2 cells monolayer model validation

When the caco-2 cell monolayer was cultured in 6-well plate to 21st day, there was clear boundary between the cells (fig. 2A). TEER and ALP activities were determined on the 21st day. The ALP activities ratio of medium on both side was close to 3:1, the TEER value of each well was larger than 500 Ω·cm^2, and microvillus and desmosome could be observed by the electron microscopy (fig. 2B), it is means that the caco-2 cells monolayer model had been established and could be used for transport experiment.

Effects of the time and concentration in the transport

The permeability of Paeoniflorin in both AP-to-BL and BL-to-AP directions were investigated, the amounts of transport increased with increase in donor concentration and incubation time, which indicated that the transport volume was correlated positively with time and concentration of Paeoniflorin (fig. 3A); The permeability of Paeoniflorin in AP-to-BL directions was more than 10^{-6} cm·s^{-1}, which indicated that Paeoniflorin was well absorbed by caco-2 cells model.

The permeability of Amygdalin in both AP-to-BL and BL-to-AP directions were investigated, the amounts of transport increased with increase in donor concentration and incubation time, which indicated that the transport volume was correlated positively with time and concentration of Amygdalin (fig. 3B); The permeability of Amygdalin in AP-to-BL directions was close to 10^{-6} cm·s^{-1}, which indicated that Amygdalin was less absorbed by Caco-2 cells model. At the same donor concentration of Paeoniflorin, the transport volume of AP-to-BL was greater than BL-to-AP, and the P_{ratio} values kept 0.5^{-1}, which indicated that passive diffusion was the main transport way for Paeoniflorin. At same donor concentration of Amygdalin, the transport volume of AP-to-BL was far less than BL-to-AP, and the P_{ratio} values was more than 1.5. Namely the efflux volume of Amygdalin was greater than the absorption in Caco-2 cells model, which indicated that active transport was the main transport way for Amygdalin (table 1).

Effects of verapamil in the transports of paeoniflorin and amygdalin

There was lots of P-gp expression in Caco-2 cells monolayer, which could promote drugs efflux. Effect of verapamil on the transport of Paeoniflorin and Amygdalin was investigated, fig. 4A and fig. 4B showed that the

transport volume of Paeoniflorin and Amygdalin from AP to BL was significant improved under the verapamil pretreatment and the data indicated that transport of Paeoniflorin and Amygdalin was all effluxed by P-gp.

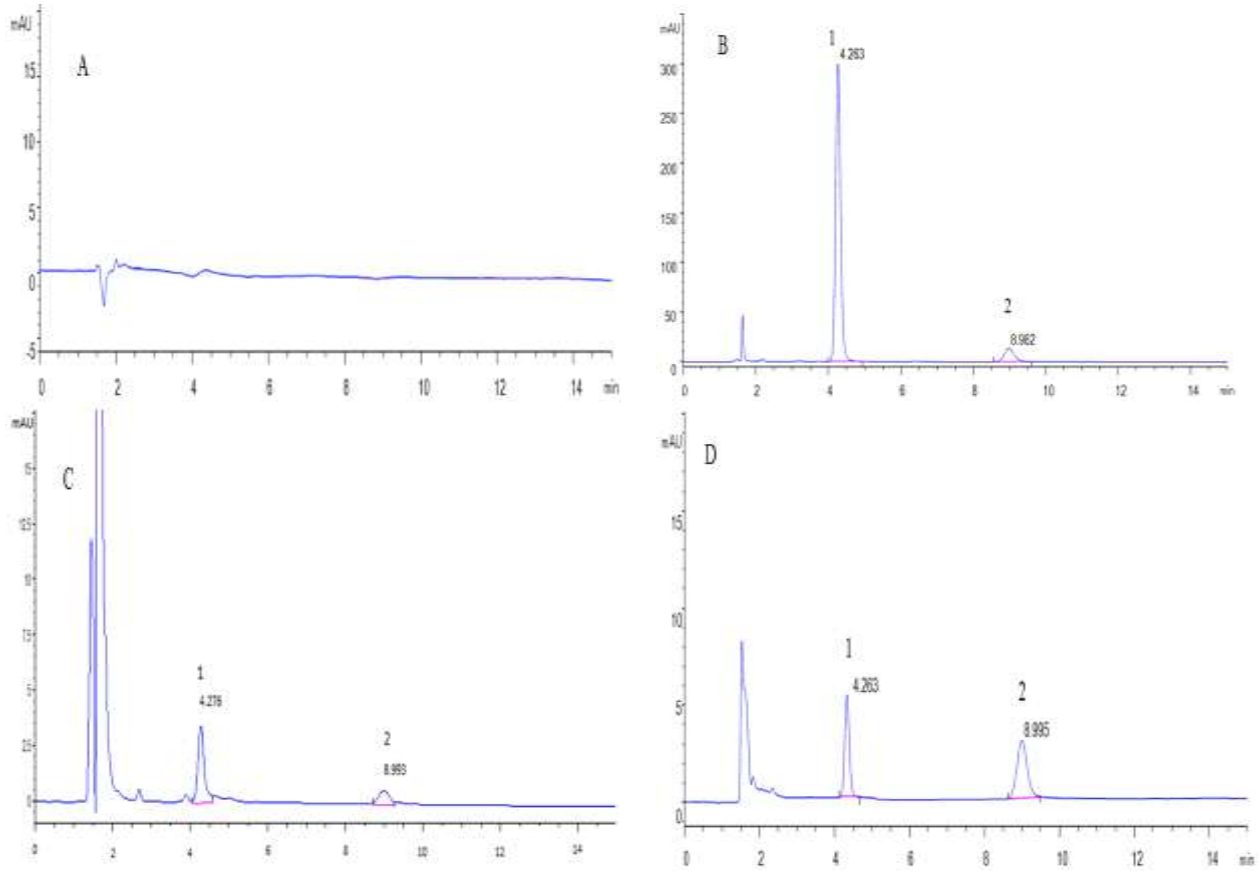

Fig. 1: HPLC chromatogram of blank sample (A), reference solution (B), sample solution after transport (C) and sample solution after metabolism (D) (1- Amygdalin; 2- Paeoniflorin).

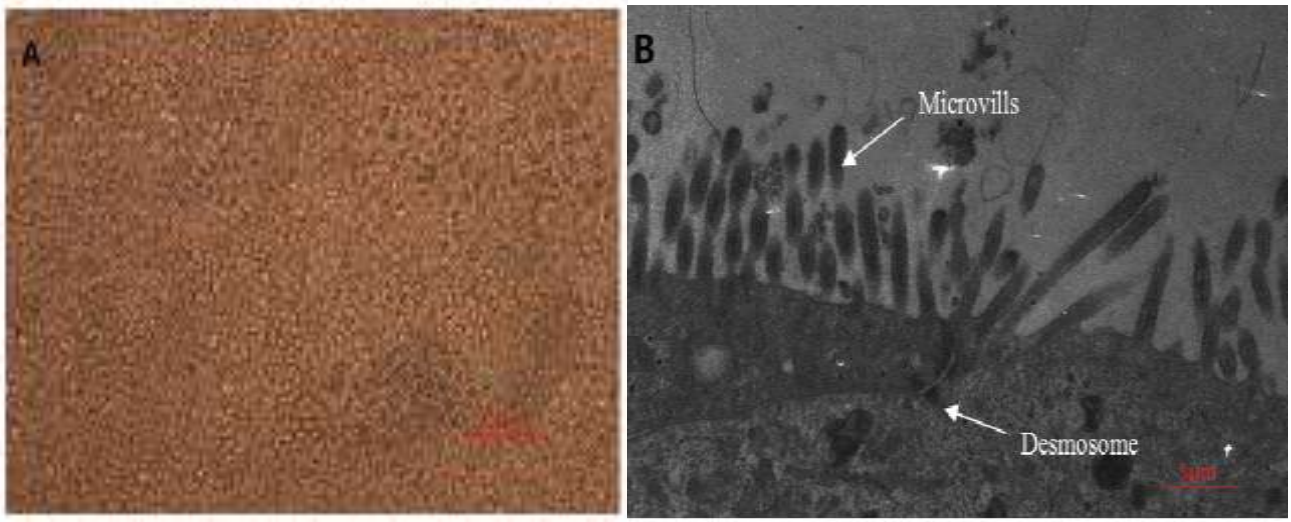

Fig. 2A: The morphology of Caco-2 cell model under the electron microscopy; **B**: The ultrastructure of Caco-2 cell model under the transmission electron microscopy (x 30000).

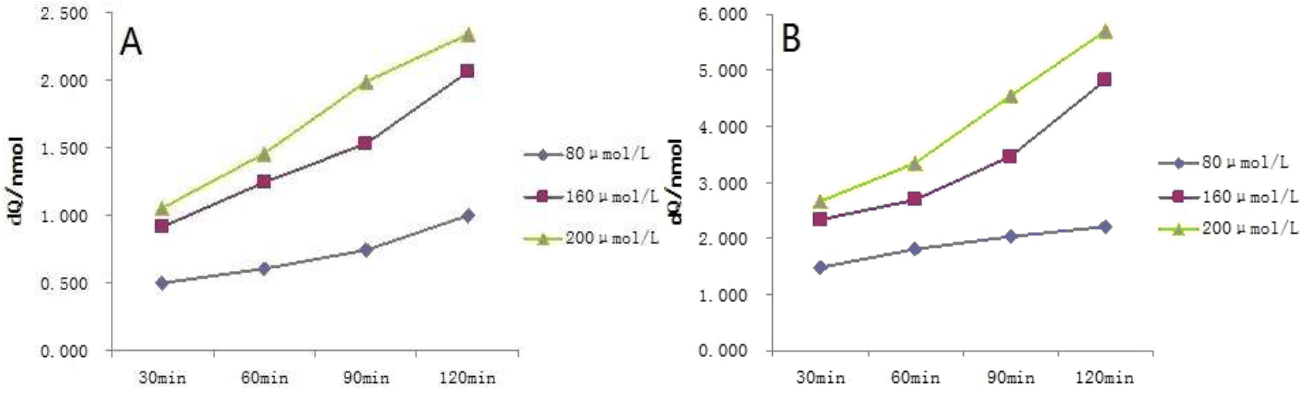

Fig. 3A: Effect of time and concentration on the transport volume of Paeoniflorin (AP-BL, n=3); **B**: Effect of time and concentration on the transport volume of Amygdalin (AP-BL, n=3)

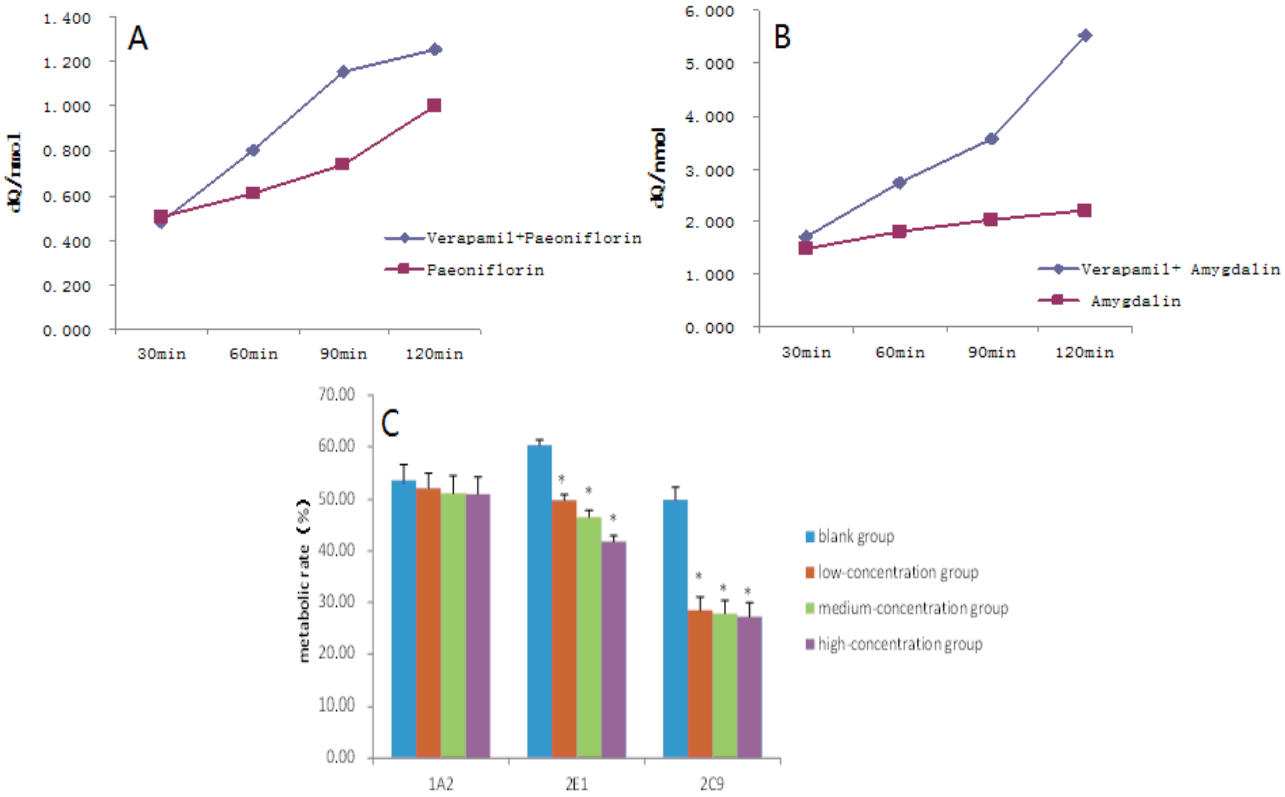

Fig. 4.A: Effect of Verapamil on the transport volume of Paeoniflorin; B: Effect of Verapamil on the transport volume of Amygdalin; C: Effect of Paeoniflorin and Amygdalin on CYP450 enzyme activity (*$P < 0.05$ *vs* blank group).

Table 1: Effects of Concentrations on the Papp Values of Paeoniflorin and Amygdalin (n=3)

	Concentration /μmol·L^{-1}	P_{app}/10^{-6}cm·s^{-1}		P_{ratio}
		AP→BL	BL→AP	
Paeoniflorin	80	6.86±0.66	6.25±1.51*	0.91
	160	6.30±0.66	5.91±1.02*	0.93
	200	6.73±0.59	5.68±1.23*	0.84
Amygdalin	80	1.93±0.46	2.18±0.39*	1.14
	160	1.72±0.58	1.96±0.49*	1.12
	200	1.59±0.46	1.94±0.48*	1.21

*$P < 0.05$ *vs* AP→BL

Effect of CYP450 enzyme

Paeoniflorin and Amygdalin have an obvious inhibiting effect on CYP2C9 and CYP2E1 (P<0.05) (fig. 4C).

DISCUSSION

Caco-2 cells derived from human colon cancer cells are similar to human intestinal epithelial cells in their structure and biochemical characteristics. These also contain related intestinal brush border enzymes, which can provide information about the drug absorption, metabolism and transport through small intestine (Zhao *et al*., 2012). The safe drug concentration is measured by MTT method, and it can avoid the illusion of absorption caused by cells death because of excessive high drug concentration (Ozdikicioglu *et al*., 2008). In Caco-2 cells model, P_{app} is determined in different concentration of drugs and confirm the transport mechanism (Artursson, 1990). According to the ratio of $P_{app\ (BL-AP)}$ and $P_{app\ (AP-BL)}$, transport mechanism can be further determined, when the ratio is larger than 1.5, it imply the transport way is active transport; and when the ratio is between 0.5 and 1, it imply the transport way is passive diffusion. On the other side, it is well-absorption for drugs when P_{app} is larger than 10^{-6}cm·s^{-1} and when P_{app} is less than 10^{-7}cm·s^{-1}, it mean drugs is poor absorption. In this study, the $P_{app\ (AP-BL)}$ of Paeoniflorin is larger than 10^{-6} cm·s^{-1}, which indicates that Paeoniflorin can be well-absorbed in human intestine, and drug bioavailability is better when given orally. Transport volume is correlated positively with time and concentration of Paeoniflorin, and the P_{ratio} is kept 0.5^{-1}, which indicates that passive diffusion is the main transport way for Paeoniflorin. The Papp of Amygdalin is close to 10^{-6}cm·s^{-1} and transport volume is correlated positively with time and concentration, the P_{ratio} values is more than 1.5, which indicate that active transport is the main transport way for Amygdalin.

P-gp is an efflux transporter protein that is located inside the human intestinal, kidney and blood-brain barrier, and most of compounds are the substrates of P-gp, which leads to the poor absorption of single compounds (Niederer *et al*., 1998). From the result the transport volume of Paeoniflorin and Amygdalin from AP to BL is significant improved under the verapamil pretreatment, which means that P-gp inhibit the absorption of Paeoniflorin and Amygdalin. However, Paeoniflorin still have a good absorption in caco-2 cells under the inhibition effect of P-gp, which suggest that the transport of Paeoniflorin is carrier-mediated.

Paeoniflorin and Amygdalin control the activity of CYP450 isoforms, which lead to the metabolism rate of other drugs slow down, increaseing the concentrations of drug in plasma, and prolonged duration of action, It means that in the clinical Paeoniflorin and Amygdalin can increase the drug concentration which mainly metabolized through CYP2C9 and CYP2E1, so we should pay more attention to the rational use of drugs, avoid other drugs accumulate in the body and produce adverse reactions.

CONCLUSION

Absorptions of most active compounds are poor by oral administration. Sometimes, during the pharmacokinetic study, only if the dose was given several times larger than on the normal people, the drug in the blood could be detected. Caco-2 cells model is a good method to study the drug absorption mechanism and predict the absorption *in vivo*. However, there must be some limitations in Caco-2 cells model. Therefore, the experimental results need to combine further experimental data of the integral animal experiment, which would provide theoretical basis for the development of the new formulation.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation of China (No.81904087, No. 81630105, No.81973560); Open Foundation of Scientific Research of Zhejiang Chinese Medical University (No. ZYX2018009); The National Key R & D Projects of China (No.2019YFC1708600, 2019YFC1708604); National Science Foundation of Jiangxi Province (No.20181BAB215036); Zhejiang Provincial Natural Science Foundation of China (No.LQ19H270001); The Health Commission Project of Jiangxi Province (No.2018B034, No.20195079).

REFERENCES

Ackley MC, Barry CG, Mounce AM, Farmer MC, Springer BE, Day CS, Wright MW, Berners-Price SJ, Hess SM and Bierbach U (2004). Structure-activity relationships in platinum-acridinylthiourea conjugates: effect of the thiourea nonleaving group on drug stability, nucleobase affinity and *in vitro* cytotoxicity. *J. Biol. Inorg. Chem*., **9**(4): 453-461.

Anderle P, Niederer E, Rubas W, Hilgendorf C, Spahn-Langguth H, Wunderli-Allenspach H, Merkle HP and Langguth P (1998). P-Glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: The influence of culturing conditions and drug exposure on P-gp expression levels. *J. Pharm. Sci*., **87**(6): 757-762.

Artursson P (1990). Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. *J. Pharm. Sci*., **79**(6): 476-482.

Chen YF, Wu KJ and Wood WG (2013). Paeonia lactiflora Extract Attenuating Cerebral Ischemia and Arterial Intimal Hyperplasia Is Mediated by Paeoniflorin via Modulation of VSMC Migration and Ras/MEK/ERK Signaling Pathway. *Evid Based Complement Alternat Med*., 482428.

Fukuda T, Ito H, Mukainaka T, Tokuda H, Nishino H and Yoshida T (2003). Anti-tumor promoting effect of glycosides from Prunus persica seeds. *Biol. Pharm. Bull*, **26**(2): 271-273.

Guo J, Wu W, Sheng M, Yang S and Tan J (2013). Amygdalin inhibits renal fibrosis in chronic kidney disease. *Mol. Med. Rep*., **7**(5): 1453-1457.

Guo RB, Wang GF, Zhao AP, Gu J, Sun XL and Hu G (2012). Paeoniflorin protects against ischemia-induced brain damages in rats via inhibiting MAPKs/NF-kappaB-mediated inflammatory responses. *PLoS One*, **7**(11): e49701.

Hashiguchi Y, Hamada A, Shinohara T, Tsuchiya K, Jono H and Saito H (2013). Role of P-glycoprotein in the efflux of raltegravir from human intestinal cells and CD4+ T-cells as an interaction target for anti-HIV agents. *Biochem. Biophys. Res. Commun*., **439**(2): 221-227.

Hung JY, Yang CJ, Tsai YM, Huang HW and Huang MS (2008). Antiproliferative activity of paeoniflorin is through cell cycle arrest and the Fas/Fas ligand-mediated apoptotic pathway in human non-small cell lung cancer A549 cells. *Clin. Exp. Pharmacol. Physiol*., **35**(2): 141-147.

Hwang YH, Kim T, Cho WK, Jang D, Ha JH and Ma JY (2012). Food- and gender-dependent pharmacokinetics of paeoniflorin after oral administration with Samul-tang in rats. *J. Ethnopharmacol*., **142**(1): 161-167.

Leffers L, Wehe CA, Huwel S, Bartel M, Ebert F, Taleshi MS, Galla HJ, Karst U, Francesconi KA and Schwerdtle T (2013). *In vitro* intestinal bioavailability of arsenosugar metabolites and presystemic metabolism of thio-dimethylarsinic acid in Caco-2 cells. *Metallomics*, **5**(8): 1031-1042.

Liang XL, Zhao LJ, Liao ZG, Zhao GW, Zhang J, Chao YC, Yang M and Yin RL (2012). Transport properties of puerarin and effect of Radix Angelicae Dahuricae extract on the transport of puerarin in Caco-2 cell model. *J. Ethnopharmacol.*, **144**(3): 677-682.

Kong H, Qu H, Qu B, Zeng W, Zhao Y, Wang X and Wang Q (2016). Correlation between the transdermal characteristics of pseudoephedrine and amygdalin in majiepingchuan *in vitro*. *J. Tradit. Chin. Med*., **36**(2): 238-242.

Pignata S, Maggini L, Zarrilli R, Rea A and Acquaviva AM (1994). The enterocyte-like differentiation of the Caco-2 tumor cell line strongly correlates with responsiveness to cAMP and activation of kinase A pathway. *Cell Growth Differ*, **5**(9): 967-973.

Qiu F, Hou XL, Takahashi K, Chen LX, Azuma J and Kang N (2012). Andrographolide inhibits the expression and metabolic activity of cytochrome P450 3A4 in the modified Caco-2 cells. *J. Ethnopharmacol*, **141**(2): 709-713.

Ulukaya E, Ozdikicioglu F, Oral AY and Demirci M (2008). The MTT assay yields a relatively lower result of growth inhibition than the ATP assay depending on the chemotherapeutic drugs tested. *Toxicol In Vitro*, **22**(1): 232-239.

Wu H, Li W, Wang T, Shu Y and Liu P (2008). Paeoniflorin suppress NF-kappaB activation through modulation of I kappaB alpha and enhances 5-fluorouracil-induced apoptosis in human gastric carcinoma cells. *Biomed. Pharmacother*, **62**(9): 659-666.

Yuan D, Komatsu K, Tani H, Cui Z and Kano Y (1998). Pharmacological properties of traditional medicines. XXIV. Classification of antiasthmatics based on constitutional predispositions. *Biol. Pharm. Bull*, **21**(11): 1169-1173.