Original Article

Effect of bioadhesive excipients on absorption of total flavonids from *Puerariae Lobatae Radix* transporting across Caco-2 cell monolayer

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ARTICLE INFO

ABSTRACT

Objective: Pueraria total flavonids (PTF) can treat cardiovascular and cerebrovascular diseases, but it has poor membrane permeability and oral bioavailability. Some excipients, such as carbomer, chitosan, and hydroxypropyl methylcellulose, can improve the oral bioavailability. Traditional *in vitro* evaluation techniques, including the rat intestinal perfusion and cell line models, cannot evaluate PTF absorption and holistic transporters.
Methods: This study evaluated excipients' adhesiveness and effect on PTF transport across Caco-2 cell monolayer. cDNA microarrays identified gene expression changes in Caco-2 cells exposed to PTF and PTF with excipients, and revealed the mechanism underlying the effect of excipients on PTF absorption.
Results: *In vitro* adhesion and transport experiments across Caco-2 showed that excipients had higher adhesiveness to gastric mucosa and transport efficiency across Caco-2 cells than PTF alone. The interaction of PTF with excipients significantly changed the expression of some genes, which might influence the absorption rate of PTF.
Conclusion: Different bioadhesive polymers can improve intestinal absorption of PTF, which was related to some genes affiliated to the ATP-binding cassette (ABC) and solute carrier transporter (SLC) to some extent.

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1. Introduction

Puerariae Lobatae Radix is the root of either *Pueraria lobata* (Willd.) Ohwi or *Pueraria thomsonii* Benth. As a member of the family Fabaceae, it is a kind of Chinese materia medica (CMM). *Puerariae Radix* can be beneficial to patients with cardiocerebral vascular disease, for example, high blood pressure, high cholesterol, elevated blood sugar levels (Shi, Deng, Liu, and Zhang, 2017) and migraines in menopausal women, commonly used tobacco easily irritated people (including pregnant women and infants), cosmetics and the daily diet of the elderly.

Eating *Puerariae Lobatae Radix* can regulate the body, boost health, increase the antiviral and antiaging properties of body, and prolong life by providing youthful vigour. The Pueraria total flavones (PTF) is the effective portion of *Puerariae Lobatae Radix* (more than 65% accounting for *Puerariae Lobatae Radix*, puerarin is the main constituent of PLF), PTF possess the function of anti-angina pectoris, reducing blood pressure, hyperlipidemic effect, decreasing cardiac oxygen consumption, improving myocardial is-

chaemia, promoting the heart and brain blood circulation, slowing atherosclerosis, antiarrhythmia, and limiting the scope of myocardial infarction, anti-oxidant and antitumor, which has been used clinically for the treatment of all kinds of cardiovascular and cerebrovascular diseases, such as high blood pressure, coronary heart disease, angina and arrhythmia, cardio-cerebrovascular disease (Li, 2008; Yu and Yin, 2003; Zhang and Fang, 1997). The main ingredients of PTF are isoflavonoids (mainly including puerarin, 3′–methoxy puerarin, and Daidzin) (Gu, Chen, and Feng, 1996). The matrix structure was shown in Fig. 1.

Several preparations made from *Puerariae Lobatae Radix* and its extracts are sold on the market in the form of tablets, pills, or capsules. However, PTF have low solubility, poor cell membrane permeability, and poor oral absorption and bioavailability. These flavonoids may take effect during the second or third week after treatment; Therefore, it is important to choose appropriate excipients to increase the solubility, cell membrane permeability, and bioavailability of PTF.

The integral animal model, the situ intestinal perfusion model, and *in vitro* cell model were used to predict the intestinal absorption of drugs. *In vitro* cell model can evaluate drug absorption rapidly, Caco-2 cells are human colonic cancer cells, they can form
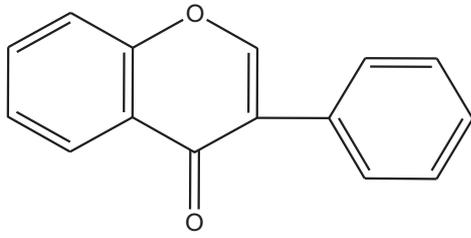
Fig. 1. Matrix structure of PTF.

polarity after culture for 21 d, and can express certain structural and functional characteristics of the matured intestinal epithelial cells, such as close connections, microvilli, landmark hydrolase, and some important transport carrier. Caco-2 cells are suitable for studying oral absorption mechanism of most drugs, such as passive transport, paracellular transport, carrier mediated endocytosis, and efflux mechanism. Its application in intestinal transport promotes the understanding of the drug absorption, biological transformation and bioavailability mechanisms from cellular and molecular level.

CMM contains many ingredients, the absorption site and absorptive mechanism of each component is widely divergent, however, existing technology cannot determine the absorptive properties of all ingredients, the absorption of some effective components cannot comprehensively reflect the absorptive properties of effective portion. Hence, we can study the genes expression change to reflect the changes of transporter proteins that participate in intestinal absorption, which can predict absorption mechanism of the whole ingredients of CMM. The cDNA microarrays can detect a large number of genes expression change simultaneously induced by complicated CMM ingredients, which can evaluate holistic absorption properties of CMM ingredients comprehensively (Press and Di Grandi, 2008; Khan et al., 2011). The distribution of drug absorption-related transporters in the intestine was illustrated in Fig. 2.

The ATP-binding cassette (ABC) and solute carrier transporter (SLC) play an important role in the absorptive process. Therefore, we investigated relevant genes change affiliated to ABC transporter superfamily and SLC transporter superfamily during the process of drugs reaction with cells, with aim to explore the mechanism of drug absorption from the integral level. The ABC transporter superfamily can transport substance outside the cell or into blood vessels from cytoplasm. Foreign material can produce tolerance through multidrug resistance (MDR) mechanism of cells,

such as absorption reduction, metabolism improvement, and targeted protein change, this mechanism limits the curative effect of chemotherapy drugs. ABC transporter superfamily plays an important effect in the process of MDR, such as ABCB1/PGP/MDR1, ABCC1/MRP1, and ABCG2/MXR/BCRP (Michael et al., 2008). The SLC transporter superfamily includes more than 300 genes, the human gene group divide it into 47 families, it can regulate the absorption and efflux of different solutes. The neutral and charged inorganic or organic ions can be transported by different members of the SLC transporter superfamily (Schlessinger et al., 2010).

This manuscript mainly studied the absorptive properties of PTF and investigated the effect of different excipients on intestinal absorption of PTF. In this study, cDNA microarrays were first employed to identify the genes expression change of Caco-2 cells exposed to PTF and study the permeability behaviour of PTF across Caco-2 cell monolayers. Furthermore, we tried to identify the interaction effects of PTF with the bioadhesive excipients and the mechanism of the bioadhesive excipients that improved bioavailability using cDNA microarray by investigating genes expression after the Caco-2 cells exposed to PTF with bioadhesive polymers.

2. Materials and methods

2.1. Materials

The reference standard for puerarin (part number: 110752-200511) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). PTF (puerarin accounts for above 17.93%) was lab-made (batch number: 040120). Transwell plates with six wells (1 mm, pore size: 0.4 μm) were purchased from Coning Costar (Cambridge, MA, USA). The alkaline phosphatase (AKP) assay kits were purchased from the Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Foetal bovine serum (FBS) was purchased from Lonza (Australia), and the nonessential amino acids (NEAA), penicillin and streptomycin, Hanks' balanced salt solution (HBSS) and Eagle's balanced salt solution were obtained from Gibco Laboratories (Life Technologies Inc., Grand Island, NY, USA). Minimum essential medium (MEM) was purchased from Hyclone (Logan, UT, USA). The human long oligonucleotide microarray containing 35 035 maize oligonucleotides was constructed by CapitalBio Corporation (Beijing, China). Caco-2 cells were provided by cell resource centre of Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). Carbomer (934PNF) was purchased from BF (Goodrich, USA). Chitosan was purchased from Zhejiang Jinke Marine Biochemistry Co., Ltd. (Zhejiang, China). Hydroxypropyl methylcellulose (HPMC) (K4M) was purchased from Shanghai Col-
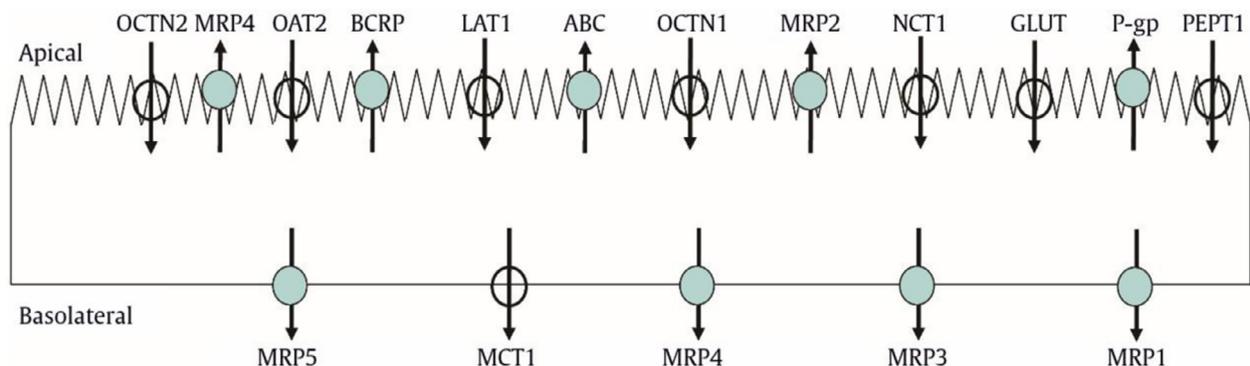

Fig. 2. Distribution of drug absorption-related transporters in intestine.

orcon (Shanghai, China). Trizol reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). NucleoSpin® RNA Cleanup Kits were purchased from Macherey-Nagel (Germany). Methanol and acetonitrile were purchased from Fisher (chromatographic grade).

2.2. Cell culture

Caco-2 cells were grown in MEM supplemented with 10% FBS, 1% NEAA, penicillin (100 U/mL), and streptomycin (100 mg/mL) in an atmosphere of 5% CO_2 at 90% relative humidity and 37 °C. When cells growth to 80 % convergence degree, cells were seeded onto transwell plates, changing culture every day, and AKP and transepithelial electrical resistance (TEER) were detected after 21 d. The cell monolayer which AKP ratio of apical (AP) and basolateral (BL) exceeds 2 and TEER exceeds 500 Ω was chosen for our further study.

2.3. High performance liquid chromatography

The exact content of puerarin was determined using a C_{18} reversed phase chromatographic column (150 × 4.6 mm; 5 μm particle size) by HPLC (LC-10AT, Japan) with a Shimadzu SPD-10AVP UV-detector, LC-10AVP controller and CTO-10ASVP column oven, the column temperature was 40 °C, the mobile phase consists of acetonitrile and 0.05% acetic acid in water (10:90), the flow rate was 1.0 mL/min, the detection wavelength was 254 nm and the injection volume was 20 μL.

2.4. Adhesiveness of bioadhesive excipients

The male SD rats fasted for 24 h [weight up to (250 ± 20) g]. The rats were then anaesthetized with sodium pentobarbital solution (40 mg/kg) by intraperitoneal injection, the stomachs were dissected, removed, and rinsed thoroughly with 0.1 mol/L hydrochloric acid (HCl), and the specimens were fixed on a glass slide. Bioadhesive materials (including carbomer, chitosan, and hydroxypropyl methylcellulose, 20 mg) were sprinkled on the mucosal tissue, respectively, and the sample was adequately hydrated and placed in a sealed container with a relative humidity of 92.5% (KNO_3 saturated solution). With the slide at a 45° angle, the flow rate was 100 rpm, rinsing with 100 mL 0.1 mol/L HCl solution. The eluent was collected in a beaker, dried at 70 °C and weighed.

$$\text{Adhesive percentage to the gastric tissue} = [M - (G - g - m)]/M \times 100\%$$

M represented the drug amount, g represented the weight of the empty beaker, G represented the total weight of the beaker and the dried residue, and m represented the amount of flushing fluid that contained solid matter.

2.5. Permeability across Caco-2 cell monolayer

2.5.1. Preparation of mixed solution of bioadhesive excipients and PTF
PTF and excipients were accurately weighed and dissolved in D-Hank's solution with the final pH 6. The PTF content in the solvent was 1.0 mg/mL, and the content of each excipient was 1.5 mg/mL, respectively. When the excipient was chitosan, chitosan was dissolved in D-Hank's solution supplemented with 1% acetic acid, the pH was adjusted to 6.0 with 0.1 mol/L NaOH, and PTF was then

added. When the excipient was carbomer or HPMC, PTF with carbomer or HPMC was accurately weighed and dissolved in D-Hank's solution (pH 6.0).

2.5.2. Transport experiments
After 21 d in culture, D-Hank's was used to wash cells three times, a certain concentration of sterile medicated D-Hank's of 0.5 mL was added to the AP side, the blank D-Hank's of 1.5 mL was added to the BL side, the culture plate was placed at water bath oscillator of 37 °C, shaking slowly, sample aliquots (200 μL) were taken from the BL side after 30, 60, 90, and 120 min, and adding the 200 μL blank D-Hank's to the BL side, all experiments were performed for three times parallelly. The sample aliquots were detected with HPLC. The lowest detection limit was 0.057 μg/mL. The apparent permeability coefficient Papp (mg/s) was calculated according to the equation:

$$Papp = (dQ/dt)/AC_0$$

Where Papp was the apparent permeability coefficient (cm/s), dQ/dt was the transport amount of drug at unit interval (mg/s), A was the area of the transport interface (cm^2), C_0 was the initial concentration of drugs (mg/L).

2.6. Gene analysis with microarray

PTF and PTF with different bioadhesive excipients solution were added to the apical side of Caco-2 cell monolayer on transwell plate (1.5 mL/6 holes), 2 mL of D-hank solution was added on the basolateral side of transwell plate, blank D-hank were added to the apical side and basolateral side of control group. The transwell plates were put in cell incubator of 37 °C, after incubation for 2 h, the total RNA was extracted from the Caco-2 cells with Trizol reagents and purified with a NucleoSpin® RNA Cleanup Kit. The RNA concentration and purity were checked by electrophoresis on a 1.2% agarose/formaldehyde gel with index as A260 and A260/280 ratios, RNA was treated with reverse transcriptase to synthesize cDNA, the genes which concentration and purity meeting the test requirements was analyzed by microarray, the analysis process referred to the earlier report of our research group (Dang and Zhu, 2012). The ratio of the normalised intensity of a gene in experimental group and control group was higher or lower than two-fold, which was considered to be significantly differentially expressed (overexpressed or underexpressed).

2.7. Statistical analysis

The data were analyzed by one-way ANOVA test using SPSS 17.0 Software (SPSS, Inc., Chicago, IL, USA), all data have significant differences when $P < 0.05$.

3. Results and discussion

3.1. Validation of HPLC method

On the HPLC chromatograms, there was no interference peak at the retention time of puerarin. The linearity range of calibration curves for puerarin was the concentration range of 0.57−73.00 μg/mL. The correlation coefficient (r^2) was 0.9998, which demonstrated a good linearity between concentration and
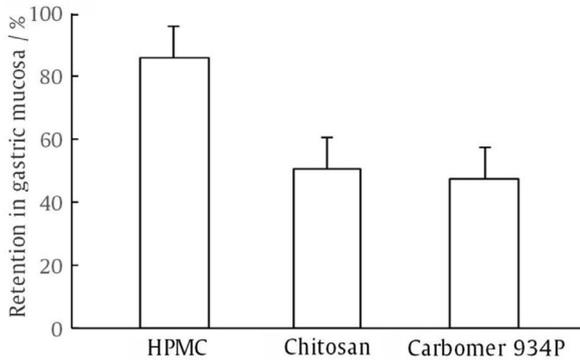
Fig. 3. Adhesiveness of different excipients ($n = 3$).

Fig. 5. Transport of PTF and PTF with excipients across Caco-2 monolayer ($n = 3$). $^*P < 0.05$ vs PTF.

chromatographic peak areas. The representative regression equation was as follows:

$$A = 13363C + 208.35$$

3.2. Adhesiveness of bioadhesive excipients

Adhesiveness of bioadhesive excipients was determined by the retention amount in the stomach method, the result demonstrated that carbomer, chitosan, and HPMC can successfully adhere to the gastric mucosa. The retention amount in gastric mucosa of carbomer and chitosan was approximately 50%, and the retention amount in gastric mucosa of HPMC K15M was approximately 90% (Fig. 3). These results demonstrated that carbomer, chitosan, and HPMC can extend the retention time in the gastric mucosa and promote absorption.

3.3. Transepithelial electric resistance of Caco-2 cell monolayer

The TEER changed in the Caco-2 cell monolayer before and after delivery of PTF and excipients and the TEER of the Caco-2 cell monolayer membrane with all excipients exceeded 800 Ω, which demonstrated that the Caco-2 cell monolayer membrane had good integrity. However, the TEER of the Caco-2 cell monolayer membrane did not change significantly with carbomer or HPMC (Fig. 4), these results demonstrated that all excipients increased the trans-
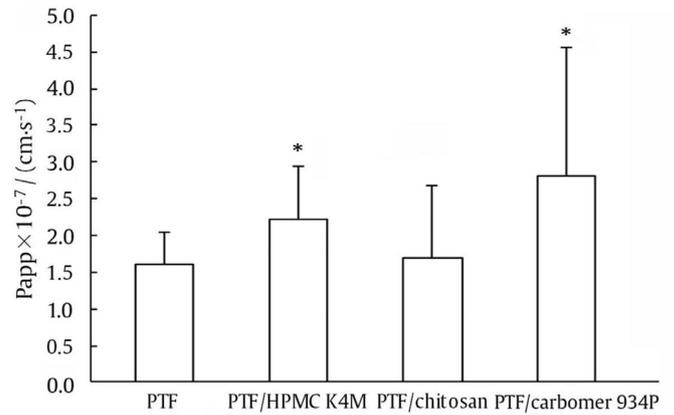
port of PTF across the Caco-2 cell monolayer and not through the paracellular route.

3.4. Permeability across Caco-2 cell monolayer

Chitosan can interact with the cell membrane with negatively charged and induced the redistribution of F-actin, and ZO-1 protein closely related to intercellular tight junctions, to increase the permeability of hydrophilic drug by the paracellular route. Chitosan is one of the widely used cationic polysaccharides due to its non-toxicity, biocompatibility, and biodegradability with permeation-enhancing properties (Agnihotri, Mallikarjuna, and Aminabhavi, 2004; Ravikumar, 2000). The inherent positive charge of chitosan can stimulate cell interactions, and functional groups can allow for protein binding (Duceppe and Tabrizian, 2010; Riva et al., 2011; Sezer and Cevher, 2012). By far, chitosan-based hydrogels have proven to be very efficient for the delivery of biologically active molecules like insulin or growth factors and for providing organisation in cells and tissues because of the ability to create multi-layered systems (Teixeira, Feijen, van Blitterswijk, Dijkstra, and Karperien, 2012).

Carbomer can inhibit the activity of some enzymes combining with Ca^{2+} and Zn^{2+}, and then increase the permeability of the cell membrane and promote absorption (Borchard et al., 1996). Car-
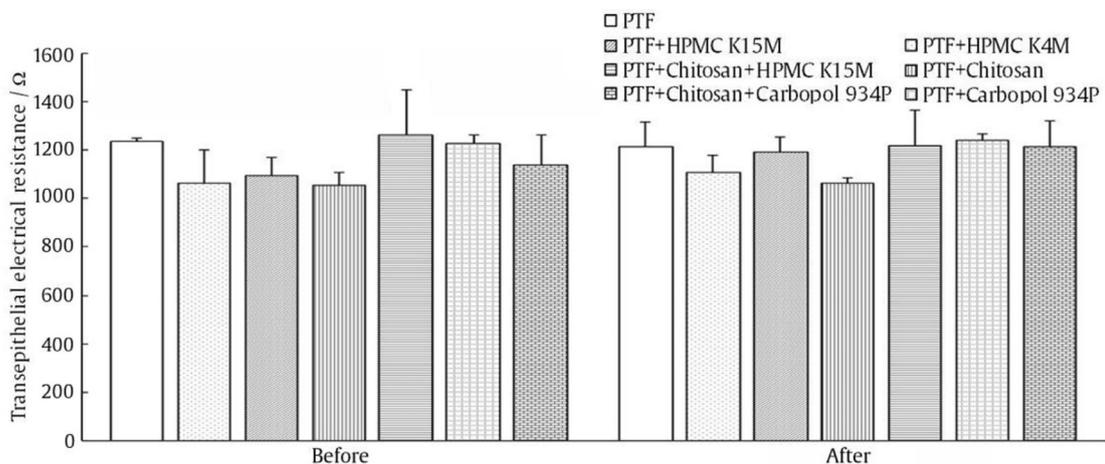

Fig. 4. Transepithelial electric resistance (TEER) change of Caco-2 cells before and after delivery of PTF with excipients.
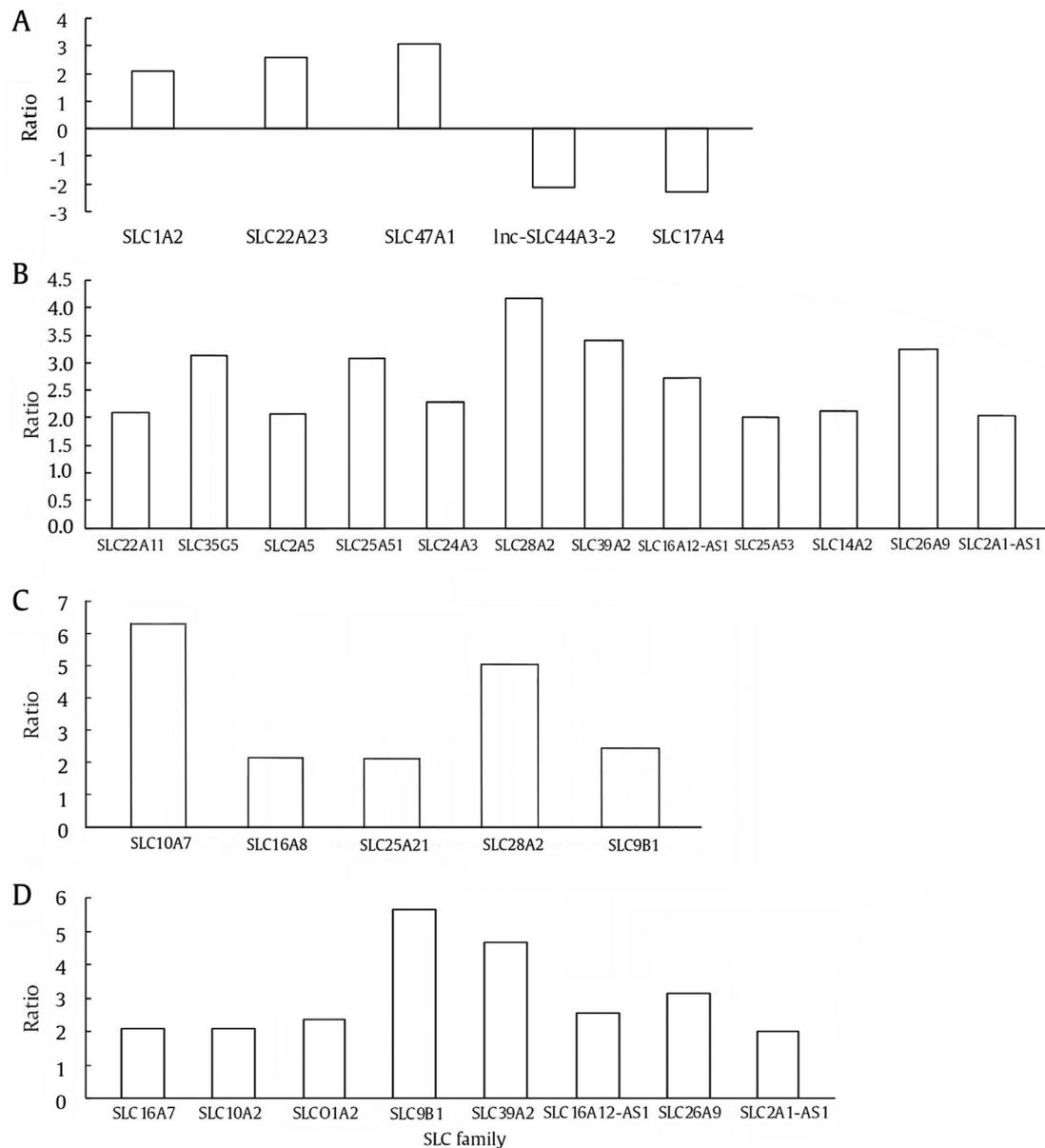
Fig. 6. Gene expression of SLC transporters on Caco-2 cells.
A: PTF *vs* blank group; B: PTF with carbomer *vs* PTF; C: PTF with chitosan *vs* PTF; D: PTF with HPMC *vs* PTF.

bomer has a unique network structure with covalent cross-linking between the chains. It expands and forms a gel after hydration with excellent compressibility. It also has good biocompatibility, making the drug release in zero-order or approximate zero-order model (Meshali, El-Sayed, El-Said, and Abd El-Aleem, 2008; Zheng and Shen, 2002).

HPMC, a semisynthetic polymer, has a hydrophobic alkyl chain as well as hydrophilic hydroxyl groups in its side chain. It is a GRAS (generally recognised as safe)-listed ingredient and included in the FDA's inactive ingredient database used for the manufacturing of a variety of commercially-available dosages. It is an expandable gel-forming polymer in an aqueous medium, and the documented mechanism for the hydrophobic drug release from its formulations is the erosion of its hydrated outer layers (Nelloreb, Rekhia, Hussainc, Tillmand, and Augsburgera, 1998). It is available in a wide range of viscosity grades (3–100 000 MPa) (Nafee, Ismail, Boraie, and Mortada, 2004) and has biocompatible and biodegradable nature (Narendra, Srinath, and Prakash Rao, 2005). The viscosity of HPMC K4M is 4000 MPa, and it has sustained-release properties. It has been extensively employed

for the development of gastroretentive formulations (Khan et al., 2010).

The results were illustrated in Fig. 5. In comparison with PTF, HPMC K4M and carbomer 934p can significantly increase the transport of PTF across the Caco-2 cell monolayer.

3.5. Gene analysis with microarray

We mainly analyzed the expression changes in the ATP-binding cassette (ABC) and solute carrier transporter (SLC), which were two important drug absorption transporter protein families. In the comparison of two groups, when the ratio was larger than 2, which demonstrated that genes were up-regulated, when the ratio was larger than the absolute value of −2, which demonstrated that genes were down-regulated.

3.5.1. Gene expression of SLC transporters

The SLC protein family plays an important role in the transport of biological materials, such as nutrients, metabolites, and neuro-

transmitters. The SLC transporter has a wide range of selectivity, and they are sensitive to different drugs with different structures.

The results of genes change in the SLC family are illustrated in Fig. 6. The results demonstrated that many genes in the SLC family significantly changed. During the transport process of PTF with carbomer, 12 genes affiliated with the SLC family significantly increased (Fig. 6B). For PTF with chitosan, five genes affiliated with the SLC family significantly increased (Fig. 6C). For the PTF with HPMC, eight genes affiliated with the SLC family significantly increased (Fig. 6D).

SLC26A9 is a sulphate/anion transporter. SLC2A1-AS1 and SLC39A2 encodes a member of the ZIP family of metal ion transporters. SLC28A2 encodes a sodium/nucleoside cotransporter. SLC9B1 encodes the cation proton antiporter.

Organic anion transport systems could be expressed and functioned as drug absorptive transporters in the small intestine (Jacquemin, Hagenbuch, Stieger, Wolkoff, and Meier, 1994; Tamai, 2012). It exhibits significantly broader substrate selectivity and could be inclined to be transporters for oral delivery. In comparison with the PTF group, carbomer can increase the expression of SLC22A11, HPMC can increase the expression of SLCO1A2. Carbomer could also increase the expression of SLC2A5, which encodes the fructose transporter responsible for fructose uptake by the small intestine.

Compared to PTF control group, PTF with excipients pass through Caco-2 mediated by monocarboxylate transporters (MCTs). The MCT protein family contains 14 genes, and only MCT1-MCT4 can catalyse the absorption *in vivo* of proton coupling of single carboxylic acid substances, which encode the SLC16A family. MCT1 (SLC16A1) widely exists in the human body, and in comparison with the PTF group, carbomer and HPMC can increase the expression of SLC16A12-AS1, which is a monocarboxylate transporter. Chitosan could increase the expression of SLC16A8. The content of MCT1 in PTF with excipients exceeded that of the PTF group, which suggested that the increase of transporter promoted PTF absorption by the reaction of bioadhesive excipients with the Caco-2 cells. The Papp across the Caco-2 cells of PTF with different excipients obviously exceeded that of the PTF group. In comparison with the PTF group, PTF with excipients had a significant influence on the SLC protein family, which suggested that these proteins played an important role in the absorption of PTF.

3.5.2. Gene expression of ABC transporters

The ABC family is the main protein responsible for the body resistance, and the ABC proteins transport various molecules across extra- and intracellular membranes. The ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, and White). MRP2 has the closest relationship with the drug transport and can efflux phase II metabolites and some nonconjugate organic anions for glutathione conjugate, sulphate conjugates, and glucuronic acid conjugates. The flavonoids are known for their antitumor effect and treatment of cardiovascular diseases. Most flavonoids are susceptible to efflux mediated by MRP2 or can be metabolised into sulfuric acid combination or glucuronic acid combination after intestinal absorption, then these metabolites are recognised by MRP2 and transported out of the lumen, which induced the poor bioavailability of these drugs.

The results of genes change in the ABC family were illustrated in Fig. 7. After the effects of carbomer on the Caco-2 cells,
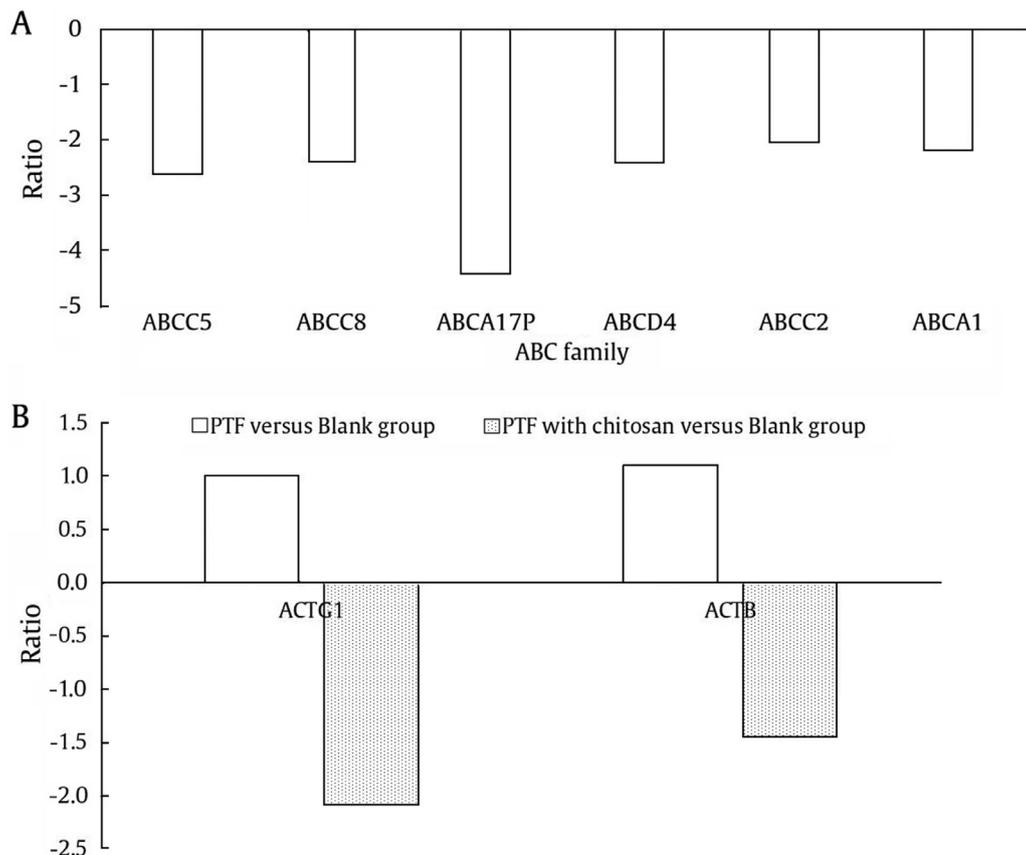

Fig. 7. Gene expression of ABC transporters on Caco-2 cells.
A: PTF with carbomer *vs* PTF; B: PTF or PTF with chitosan *vs* blank group.

eight genes affiliated with the ABC family underwent significant changes, and the expression of seven genes affiliated with the ABC family was significantly decreased, which showed that carbomer could inhibit efflux transporter (MRP/P-gp) activity in the Caco-2 cell monolayer by changing the microenvironment of P-gp. These genes are ABCC5, ABCC2, ABCC8, ABCD4, and ABCA1, which are all affiliated to ABC transporters (Fig. 7A). Thereinto, ABCC5, ABCC2, and ABCC8 are affiliated to the MRP subfamily which is involved in multi-drug resistance. ABCC5 participates in the cellular export of its substrate and cyclic nucleotides, which is caused by the degradation of phosphodiesterases. ABCC8 participates in ATP-sensitive potassium channels modulating and insulin release. ABCC2 is expressed in the canalicular (apical) part of the hepatocyte and functions in biliary transport (Torre, Bocci, Focardi, and Corsi, 2014). ABCD4 is a member of the ALD subfamily which is involved in peroxisomal import of fatty acids and/or fatty acyl-CoAs in the organelle (Gray et al., 2014). ABCA1 is a member of the ABC1 subfamily which is found exclusively in multicellular eukaryotes (Korhonen, Olkkonen, Lahesmaa, and Puolakkainen, 2013).

Chitosan can influence F-actin's function between cells, open the tight junctions between the cells, and promote the transport capacity of biological macromolecules through transcellular or paracellular pathways to improve the instant permeability of drugs through cells (Plapied, Vandermeulen, Vroman, Préat, and des Rieux, 2010). F-actin includes three subtypes: α, β, and γ. β and γ exist on the cell membranes, and the corresponding coding genes are ACTB and ACTG1. Our results demonstrated that in the transport process of PTF with chitosan and PTF alone, the ratios of ACTG1 were −2.08 and 1.01, respectively; the ratios of ACTB were −1.44 and 1.11, respectively (Fig. 7B). Chitosan caused a decrease in ACTB and ACTG1, which suggested that chitosan can influence the quantity and function of F-actin, promoting PTF transport through intercellular spaces and intestinal absorption.

We analyzed two transport protein coding genes for ABC and SLC. Based on the results for PTF, the ABC genes did not change significantly, which suggested that PTF resistance does not easily occur, but carbomer can significantly inhibit the expression of ABC genes which indicated that carbomer increased the absorption of PTF through partly inhibiting excretion. Some genes affiliated with the SLC family significantly changed after a reaction with PTF and excipients, which demonstrated that the transporters encoded by genes participated in the transport process.

4. Conclusion

The polymers, chitosan, carbomer, and HPMC are commonly used as bioadhesive excipients to improve drug retention time in the stomach and improve intestinal absorption. The excipients in this study significantly improved the transport of PTF across the Caco-2 cell monolayer. In addition, for the reason that these excipients increased the retention time in the stomach, the carrier materials may also affect the expression of transporter proteins, thereby changing the drug absorption. Microarray technology is used to evaluate the change in transporter proteins overall after the drug transit; It is a fast and simple operation, which could provide a large amount of information as an effective means of evaluating drug absorption mechanisms.

Conflicts of interest

The authors have no conflicts of interest to declare.

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