이학석사 학위논문

세포의 장기 계대 배양이 장 흡수 모델 Caco-2 세포의 integrity 와 에너지 대사에 미치는 영향

Effects of Long-Term Serial Passaging on the Integrity and Energy Metabolism of Caco-2 Model of Intestinal Absorption

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2022 년 8 월

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2022 년 8 월

Abstract

Intestinal absorption is an important property to determine bioavailability of oral drugs and a monolayer of human small intestinal epithelial cells differentiated from Caco-2 cell (human colon adenocarcinoma) is the most ideal in vitro assay for screening intestinal absorption of orally administered compounds during drug discovery. However, which passage number of Caco-2 should be used has not been standardized yet, even though drug efflux has been reported to be increased in late-passage cells like cancer cells. In this study, we evaluated drug efflux and energy metabolism in each early- and late-passage monolayers to check if over-passaging turns the monolayer of normal enterocytes into a cancer-like monolayer. Transepithelial electrical resistance (TEER) across the monolayer was higher in late-passage monolayers than in early-passage monolayers, which indicated that the tightness of the tight junctions in the late-passage monolayers was increased. Additionally, a higher gene expression of P-glycoprotein (P-gp), an efflux protein, was observed in late-passage monolayers. Consistently, the efflux ratio of P-gp substrates (digoxin, quinidine and saquinavir) in the late-passage monolayers were markedly higher than those of early-passage monolayers, whereas transcellularly or paracellularly transported drugs (metoprolol or atenolol) were equally permeated. LC-MS/MS analysis of tricarboxylic acid (TCA) cycle revealed the decreased metabolite pool size in glutamate, glutamine, lactate, serine and succinate in late-passage monolayers. Our study compared Caco-2 monolayers of different passages using a metabolomics approach and we conclude that late-passage Caco-2 monolayers are recommended for a better prediction of intestinal absorption of drugs designed to work in cancerous environments.

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Introduction

Drug discovery and development are vastly increasing in pharmaceutical industry. There are many possible routes of administration, but oral delivery is the most convenient, cost-effective and most commonly used medication administration route. For this reason, pharmaceutical companies are trying to develop highly absorptive oral drugs that can pass easily through the human intestinal wall and reaches to the site of action. For a chemical compound to become a marketable drug, it should have favorable absorption, distribution, metabolism and excretion (ADME) properties in addition to efficacy and safety [1]. Of these properties, absorption is a key property for orally administered drugs since it is quantified in terms of bioavailability, the extent to which absorption occurs and the fraction of the administered drug that reaches the systemic circulation in the unchanged form [1].

The intestinal epithelium is the first significant physical and biochemical barrier to limit drug absorption from the gastrointestinal tract. This barrier plays the important role as a defense mechanism against potentially harmful substances, but it also limits the bioavailability of orally administered drugs. In addition to passive permeation properties, active uptake and efflux systems as well as intestinal metabolic enzymes have been recognized to have a significant effect on the extent of absorption [2]. The multidrug resistance protein P-gp (P-glycoprotein) is probably the most studied efflux transporter and, although initially discovered in tumor tissues, is also present in normal human intestinal epithelium, blood-brain barrier and hepatic canalicular membranes [3]. Its primary physiological function in the small intestine is to protect tissues from harmful substances like toxins or xenobiotics by limiting their absorption. P-gp has also been observed to limit the oral bioavailability of several drugs including anti-cancer drugs by the same mechanism [4].

The human cell line Caco-2, when differentiated and grown as a monolayer, is often used as a model for the small intestine epithelium, and it is the most ideal in vitro assay for

screening uptake and efflux of orally administered compounds during drug discovery. These cells, originally isolated from colon carcinoma tissue [5], undergo spontaneous in vitro enterocytic differentiation [6] leading in about 21 days to the formation of a monolayer of highly polarized cells, connected by functional tight junctions, with well-developed and organized microvilli on the apical (AP) membrane. Differentiation of Caco-2 cells results in the polarized expression of brush border hydrolases and of several transport proteins, normally expressed in the absorptive enterocyte of the small intestine [7, 8] although some colonocyte characteristics are also present [9]. Maintenance of Caco-2 cells on permeable filter inserts has been shown to facilitate cell differentiation and polarity, and it allows transport studies to be carried out across the cell monolayer. Over the years Caco-2 cells have become the best-established in vitro model of intestinal absorption and have been extensively utilized to study the gastrointestinal permeability and identification of efflux substrates [10, 11]. Expression of P-gp has been demonstrated in Caco-2 cells, but several factors have been shown to influence its expression levels and functionality [12]. These factors include the age in culture and level of differentiation, passage number, and exposure to modulators, such as some P-gp substrates [13, 14]. Out of these factors, passage number has been reported as one of the important factors to be cautious about when performing a Caco-2 permeability assay, yet there is a lack of standardization of protocols regarding which passage number of Caco-2 cells should be used. It is known that P-gp expression is increased in the monolayer of late-passage Caco-2 cells and the outcome of P-gp mediated efflux transport is likely to be different by different passage number [15]. This creates a need for careful characterization of the late-passage Caco-2 cells in use in terms of efflux protein expression and function to develop a reliable in vitro model to study efflux substrates.

Metabolomics is a discipline that measures the concentrations of a wide variety of metabolites in biological specimens allowing for real-time quantification of changes in cellular metabolism [16]. Although there are many metabolic pathways, the tricarboxylic acid (TCA)

cycle is one of the most important pathways for energy metabolism in aerobic organisms. It consists of series of successive reactions that oxidize citrate and are linked to other pathways such as amino acid metabolism and gluconeogenesis [17]. In recent years, the alterations in TCA cycle compounds have been investigated to correspond to various physiological conditions and the TCA cycle metabolites have been proposed as biomarkers for cellular states of organisms. For example, cancer cells differ from normal cells in that they metabolize the majority of the glucose into lactate, even in the presence of oxygen, and rely mostly on glutamine for carbon source for TCA cycle [18]. Since it is yet unknown if energy metabolism of Caco-2 cells changes as they passage over a long period of time, characterizing the metabolic fate of TCA intermediates in each early- and late-passage Caco-2 cells and comparing it between the two groups would be useful for understanding the effects of over-passaging on physiological properties of Caco-2 cells. Our approach toward characterizing over-passage Caco-2 cell was to use the technique on LC-MS/MS metabolite analysis to perform a preliminary study of the biochemistry for these cells as they are kept in in vitro culture system for an extended time.

Materials and Methods

1. Caco-2 cell Culture

Human Caco-2 cells were acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Minimum Essential Medium (MEM; Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 1% (v/v) nonessential amino acids, 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Rockford, IL, USA), 100 IU penicillin, and 100 µg/mL streptomycin in 5% CO_2 at 37°C. For transepithelial transport experiments, cells were grown as epithelial layers by seeding them (10 000 cells / 12 mm insert) on to Transwell™ polycarbonate membrane filter tissue culture inserts (Corning, Corning, NY, USA). Cell monolayers were maintained with medium changes every other day for 21-25 days after seeding. The formation of functional epithelial layers was evaluated by measuring the transepithelial electrical resistance (TEER) with an epithelial voltmeter (EVOM; World Precision Instrument, Sarasota, FL, USA).

2. Bidirectional transport studies

2.1 Caco-2 permeability assay

The transport experiments were performed in Hanks' balanced salt solution (HBSS, pH 7.3) containing 10 mM *N*'-(2-hydroxyethyl)piperazine-*N*-ethanesulfonic acid (HEPES; Thermo Fisher Scientific, Rockford, IL, USA). Prior to initiating the transport studies, culture inserts were preincubated from the apical and basolateral sides with 0.5 mL and 1.5 mL of prewarmed HBSS buffer per well, respectively and allowed to equilibrate for 30 min at 37 °C. The integrity of the cell monolayers was verified by measuring TEER values across each monolayer using Millipore Millicell-ERS device. Transport studies were allowed to progress if TEER values above 400 Ω x cm^2 were evident. TEER values were also taken at the completion of the study to ensure membrane integrity was

maintained during 2 to 3 h time course of the study. In the determination of apical to basolateral (AP-to-BL) directional transport, the medium in the apical (donor) compartment was replaced with 0.5 mL of 1 μM P-gp substrate in HBSS while the basolateral (receiver) compartment had 1.5 mL of fresh HBSS. The basolateral to apical (BL-to-AP) directional transport was determined by placing the drug in the basolateral compartment with the volumes as mentioned above (0.5 mL in the apical and 1.5 mL in the basolateral). Each of P-gp substrates in HBSS-HEPES was placed in the apical (1 μM) chamber. Transport of Atenolol (20 μM) and Metoprolol (10 μM) were also evaluated to determine the magnitude of passive diffusive fluxes across the epithelium and confirm maintenance of monolayer integrity during the flux measurements. The culture inserts were then incubated for 120 min at 37 ℃. Samples were taken from both the apical and basolateral compartment at the end of the incubation period, and the concentration of test compound were analyzed by a liquid chromatography with tandem mass spectrometry (LC-MS/MS).

2.2 Sample analysis by LC-MS/MS

Permeability analysis for atenolol, metoprolol and P-gp substrates was performed by gradient elution LC-MS/MS using an Agilent HPLC system interfaced to a SCIEX QTRAP 5500 mass spectrometer. The column was an Atlantis dC18, 3 μm particle size, 2.1 x 50 mm (Waters, Milford, MA, USA) Eluent A = 0.1% formic acid in water; eluent B = 0.1% formic acid in acetonitrile (ACN); flow rate = 0.4 mL/min; column temperature 30 ℃. The injection volume was 2 ul. Full-scan tandem mass spectra were obtained under unit mass resolution conditions with N_2 as the collision gas and the most intense transitions were used for quantitation in the multiple reaction monitoring (MRM)

mode. Analyst Software was used to determine the peak area for all the MRM traces. All areas were normalized using an internal standard.

2.3 Determination of Permeability Coefficients and Efflux Ratio

The apparent permeability coefficient (P_{app}) of compounds was calculated according to the following equation:

$$P_{app}(\mathrm{cm}/s) = \frac{(dQ/dt)}{A \times C_0}$$

Where dQ/dt is the flux across the monolayer determined experimentally by measuring the amount of compounds transported as a function of time; A is the surface area of the insert; C_0 is the initial concentration in the donor side.

The efflux ratio (ER) for the BL-to-AP and AP-to-BL directions was determined by the following equation:

$$\mathrm{ER} = \frac{Papp_{BL\rightarrow AP}}{Papp_{AP\rightarrow BL}}$$

2.4 Statistical Analysis

All data were presented as mean ± SD in cm/s X 10^{-6}. A two-tailed Student's t-test for independent variables was performed for each compound to ascertain whether the permeability values were statistically different across different passages of Caco-2 cell monolayer.

3. Western blot

Passage 30 and 80 Caco-2 cells were grown for 21 days and subjected to hypotonic lysis to separate cytosolic proteins from membrane proteins (total membrane) using a commercially available membrane extraction kit (ab 65400, Abcam, Cambridge, UK).

Cell lysates were then centrifuged at 10,000 g for 30 min at 4 ℃ to isolate the pellet representing the total cellular membrane fraction. The pellet was subsequently solubilized in detergent-containing buffer (0.5% Triton X-100) for protein quantitation. Equal amounts of protein concentration were measured using a bicinchoninic acid (BCA) protein quantitative assay kit (Thermo Fisher Scientific, Rockford, IL, USA) and then 5X protein sample buffer was added to the sample (30 μg). Each sample was separated by polyacrylamide gels (8 %), and then transferred to PVDF membrane. After protein transfer, membranes were blocked with 5% BSA blocking buffer for 1 hour at room temperature and washed by 1X Tris-buffered saline/TWEEN 20 (1X TBST). Membranes were incubated with primary antibodies (anti-mouse α-tubulin, T9026, Sigma-Aldrich, ST. Louis, MO, USA; anti-mouse P Glycoprotein, GTX23364, GeneTex, Irvine, CA, USA) diluted in 1% BSA blocking buffer for overnight at 4 ℃. After washing 3 times for 10 min in 1X TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 1X TBST for 1 hour at room temperature. Membranes were washed 3 times for 10 min with 1X TBST. Immunoreactivity was detected with an enhanced chemiluminescence reagent (ECL).

4. Analysis of TCA energy metabolism in Caco-2 cell monolayer

4.1 Polar Metabolite Extraction from Caco-2 Cells

Caco-2 cells grown and differentiated for 21 days were removed from the incubator and the medium was aspirated. The plates were placed on dry ice and 2 mL of cold 80% (vol/vol) methanol were added. The plates were incubated at -80 ℃ for 20 min. The entirety of the contents of the plate were scraped and transferred to a 15-mL conical tube. The tube was vortexed for 1 min, and then it was incubated for 30 min at -80 ℃. It

was centrifuged at >4000g for 10 min at 4 ℃ to precipitate the insoluble material. The supernatant was transferred to a new 15-mL conical tube and the pellet was used for protein quantification. The supernatant was aliquoted in 1.5-mL microcentrifuge tubes. The tubes went under SpeedVac to a pellet, using no heat.

4.2 Total Protein Content Determination

The protein concentration was assessed from the precipitate dissolved in 1 mL 0.05 M NaOH. A BCA protein assay was performed to quantitate total protein concentration of the reconstituted cell pellet according to the manufacturer's protocol.

4.3 LC-MS/MS Analytical Method Parameters.

LC-MS/MS analyses were conducted on a 5500 QTRAP mass spectrometer coupled with High Performance Liquid Chromatography system (Sciex, Concord, Ontario, Canada). The liquid chromatography column used for analyte separation and elution was a Hypersil Gold C8 column, 5 μm particle size, 2.1 x 150 mm (Thermo Fisher Scientific, Rockford, IL, USA). Mobile phase A was water with 1 mM ammonium acetate, and mobile phase B was methanol with 1 mM ammonium acetate. The chromatographic separation was achieved by a linear gradient starting from 10% B and progressing to 90% B over a period of 6.5 minutes. A sample volume of 2 uL was injected onto the LC column at a flow rate of 0.25 mL/min. Electrospray ionization (ESI) was used in the positive mode for acetyl CoA, alanine, aspartate, glutamate, glutamine, and serine and in the negative mode for the rest of other TCA cycle intermediates. TCA cycle intermediates were monitored by tandem MS using multiple reaction monitoring (MRM) mode. Identification was achieved based on retention time and product ions.

4.4 Data Processing

The LC-MS/MS results were processed using Analyst® Data Processing software (AB Sciex). Peak areas of each metabolite were normalized to those of the internal standard. A calibration curve was drawn for each metabolite and the concentration of the metabolite in the sample was calculated from the peak area ratio. The concentration values were further normalized by total protein content for each sample to calculate metabolite pool size (nmol/mg). Fold change and *p*-values for the metabolites in the late-passage Caco-2 cells were calculated relative to those in the early-passage Caco-2 cells. Metabolites with *p*-value less than 0.05 were considered as significantly different.

Results

1. Integrity of Caco-2 Cell Monolayers

Transepithelial electrical resistance (TEER) values are measurements of integrity of Caco-2 cell monolayer since they epresent how tight the monolayer has formed [19]. TEER values were measured in the beginning of the transport study for the monolayers from each passage Caco-2 cells. As a result, the TEER values of Caco-2 cell monolayer formed from passage 30, 60, 80 and 100 parental Caco-2 cells were 870 ± 53 Ω x cm^2, 1313 ± 162 Ω x cm^2, 1437 ± 25 Ω x cm^2 and 1363 ± 35 Ω x cm^2, respectively (Fig. 1). TEER values of the monolayers from passage 60 or higher Caco-2 cells were significantly higher than the values measured in the monolayer from passage 30 Caco-2 cells. This demonstrated that over-passaging of Caco-2 cells caused changes in integrity of Caco-2 cell monolayers by increasing the tightness of the monolayer.

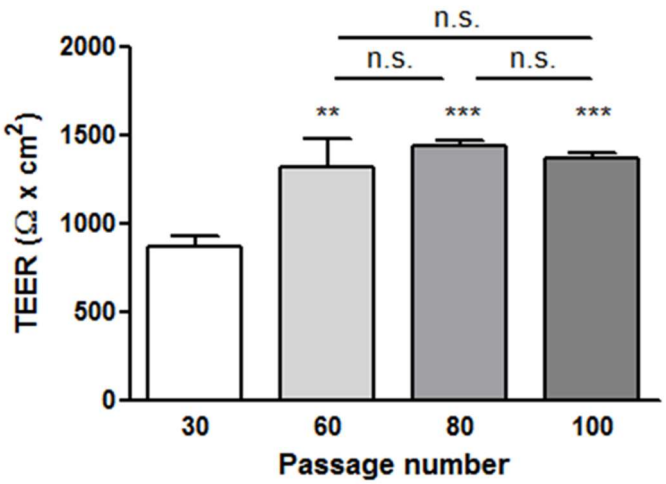


Fig. 1. TEER values versus passage number of Caco-2 cells.

TEER measurements were conducted in the HBSS buffer. The data were analyzed by a one-way ANOVA test followed by a Tukey post hoc test. Values are indicated as mean ± S.D. (n = 3); *P < 0.05, ** < 0.01, ***P < 0.00,. n.s., not significant.

2. Permeability of test compounds across Caco-2 Cell Monolayers

To examine whether changes in the integrity of Caco-2 monolayers leads to changes in the permeability of compounds, we conducted transport studies to calculate the apparent permeability coefficients for diffusive marker compounds and efflux ratio (ER) for P-gp substances in Caco-2 cell monolayers. For simple diffusion marker compounds, we used atenolol for the paracellular route and metoprolol for the transcellular marker [20]. Since these compounds simply diffuse from an area of low concentration to an area of high concentration, we conducted the unidirectional (AP-to-BL) transport studies to evaluate their permeability across Caco-2 monolayers. The AP-to-BL apparent permeability coefficients in the monolayers from each passage 30, 60, 80 and 100 parental Caco-2 cells were 0.3 x 10^{-6} cm/s , 0.3 x 10^{-6} cm/s, 0.3 x 10^{-6} cm/s and 0.3 x 10^{-6} for atenolol and 27.3 x 10^{-6} cm/s, 24.9 x 10^{-6} cm/s, 25.0 x 10^{-6} cm/s and 27.8 x 10^{-6} cm/s for metoprolol (Fig. 2). Interestingly, the permeability of simple diffusive drugs was not changed even though the tightness of Caco-2 monolayers was changed. This indicated that how tight the monolayer has formed is not related to permeability of transcellularly or paracellularly absorbed drugs that pass through the monolayer by simple. Next, to test if changes in integrity of the monolayer are related to changes in the expression of efflux proteins on the apical side, bidirectional (AP-to-BL and BL-to-AP) permeability coefficients and ER for P-gp marker substrates were determined in Caco-2 cell monolayers. We selected digoxin, quinidine and saquinavir as the P-gp marker substrates and determined the optimal donor concentration as 1 μM to prevent the saturation of P-gp mediated efflux [21, 22]. First, AP-to-BL permeability was determined, and we observed that all three P-gp substrates were absorbed less as the passage number was increased (Fig. 3A). For digoxin, the P_{app} value 4.0 x 10^{-6} cm/s in passage 30 was decreased to 3.0 x 10^{-6} cm/s, 3.0 x 10^{-6} cm/s and 3.0 x 10^{-6} in Caco-2 monolayers from passage number 60, 80 and 100, respectively. For quinidine, the P_{app} values were 21.6 x 10^{-6} cm/s in passage 30, and it was decreased to 15.8 x 10^{-6} cm/s, 15.7 x 10^{-6} cm/s and 14.0 x 10^{-6} cm/s in Caco-2 monolayers from passage number 60, 80 and 100, respectively.

For saquinavir, the P_{app} values were 0.7 x 10^{-6} cm/s in passage 30, and it was decreased to 0.2 x 10^{-6} cm/s, 0.2 x 10^{-6} cm/s and 0.2 x 10^{-6} cm/s in Caco-2 monolayers from passage number 60, 80 and 100, respectively. Next, we evaluated BL-to-AP permeability, and for all three P-gp substrates, the P_{app} values were increased as the passage number was increased (Fig. 3B). For digoxin, the P_{app} value 4.5 x 10^{-6} cm/s in passage 30 was increased to 6.4 x 10^{-6} cm/s, 6.9 x 10^{-6} cm/s and 7.4 x 10^{-6} in Caco-2 monolayers from passage number 60, 80 and 100, respectively. For quinidine, the P_{app} values were 29.1 x 10^{-6} cm/s in passage 30, and it was increased to 36.8 x 10^{-6} cm/s, 36.8 x 10^{-6} cm/s and 40.3 x 10^{-6} cm/s in Caco-2 monolayers from passage number 60, 80 and 100, respectively. For saquinavir, the P_{app} values were 3.0 x 10^{-6} cm/s in passage 30, and it was increased to 8.3 x 10^{-6} cm/s, 6.6 x 10^{-6} cm/s and 7.2 x 10^{-6} cm/s in Caco-2 monolayers from passage number 60, 80 and 100, respectively. Lastly, we determined the values of ER for P-gp substrates by calculating from the ratio of P_{app} in secretory (BL-to-AP) to that in absorptive (AP-to-BL) directions (Fig. 3C). The ER value for digoxin was 1.1 in passage 30 monolayers, and it was increased to 2.2, 2.3 and 2.4 in passage 60, 80 and 100 monolayers, respectively. For quinidine, the ER values were increased from 1.3 in passage 30 monolayers to 2.3, 2.3 and 2.9 in passage 60, 80 and 100 monolayers, respectively. For saquinavir, the ER value in passage 30 monolayers was 4.6, and it was increased to 36.6, 28.9 and 31.5 in passage 60, 80 and 100 monolayers, respectively. Interestingly, an increase in efflux ratio of P-gp substrates showed a positive correlation with an increase in TEER values of Caco-2 monolayers of different passages (Fig. 4), indicating that there is a relationship between changes in integrity of the monolayer and efflux of P-gp substrates. Altogether, through the bidirectional transport studies for P-gp substrates, we have concluded that P-gp substrates are pumped out to the apical side to the greater extent as the passage number is increased, and this is more likely because P-gp transporters are functionally more active in monolayers of over-passaged Caco-2 cells.

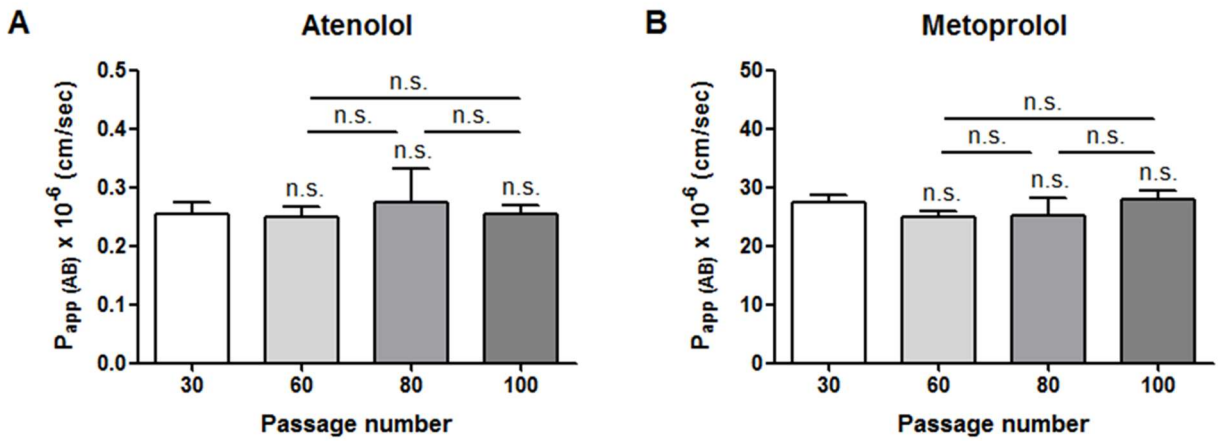


Fig. 2. Effect of the passage number of Caco-2 cells on the AP-to-BL permeability on simple diffusion drugs.

Apparent permeability coefficients for (A) paracellularly absorbed drug (atenolol) and (B) transcellularly absorbed drug (metoprolol). Data were represented as relative to that at passage number 30. The data were analyzed by a one-way ANOVA test followed by a Tukey post hoc test. Values are indicated as mean ± S.D. (n = 3); n.s., not significant. AB = AP-to-BL, BA = BL-to-AP.

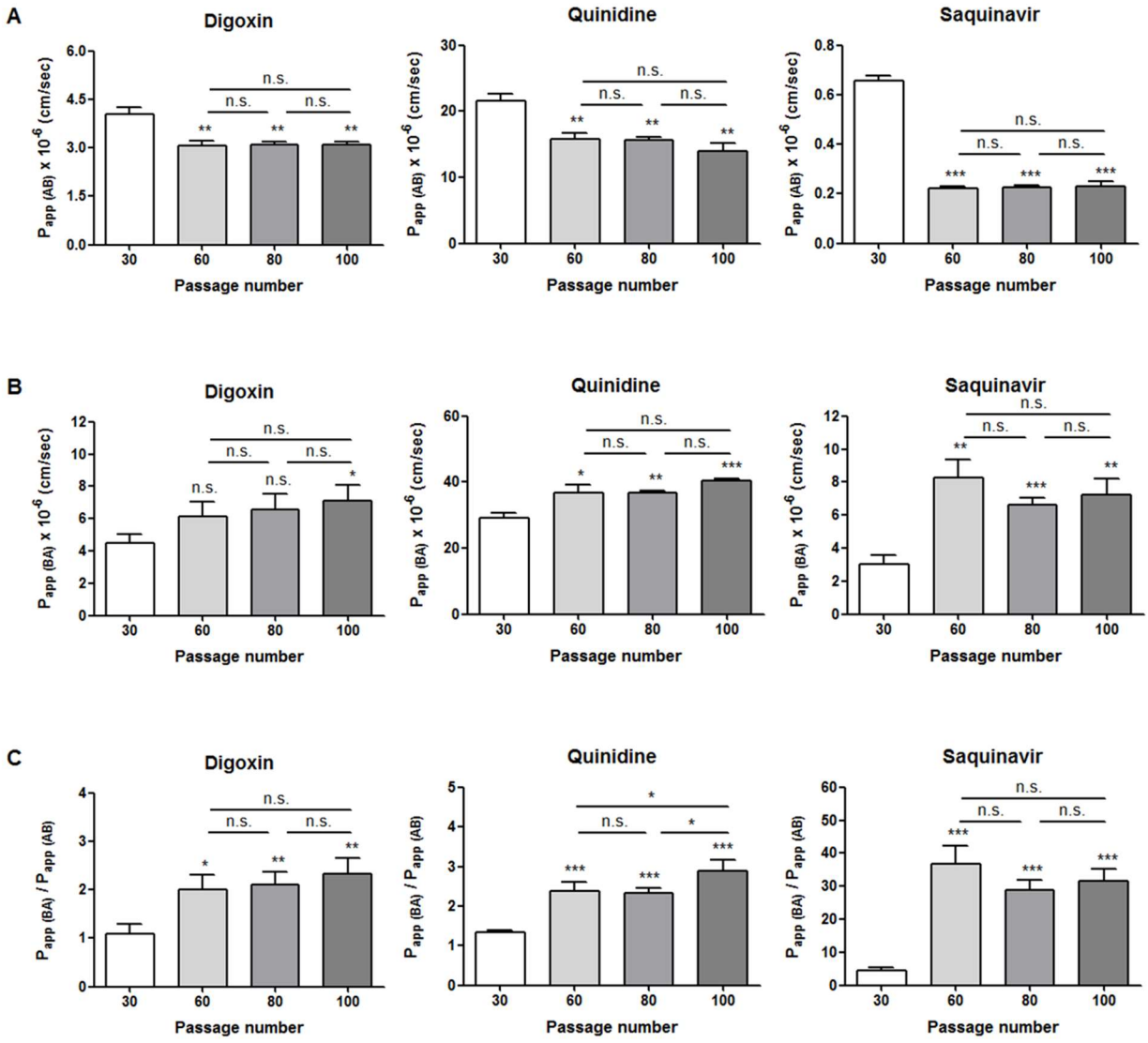


Fig. 3. Bidirectional permeability for P-gp substrates across Caco-2 cell monolayers from different passage number.

Efflux transport of (A) digoxin, (B) quinidine and (C) saquinavir. Data were represented as relative to that at passage number 30. The data were analyzed by a one-way ANOVA test followed by a Tukey post hoc test. Values are indicated as mean ± S.D. (n = 3); *P < 0.05, ** < 0.01, ***P < 0.001, n.s., not significant. AB = AP-to-BL, BA = BL-to-AP.

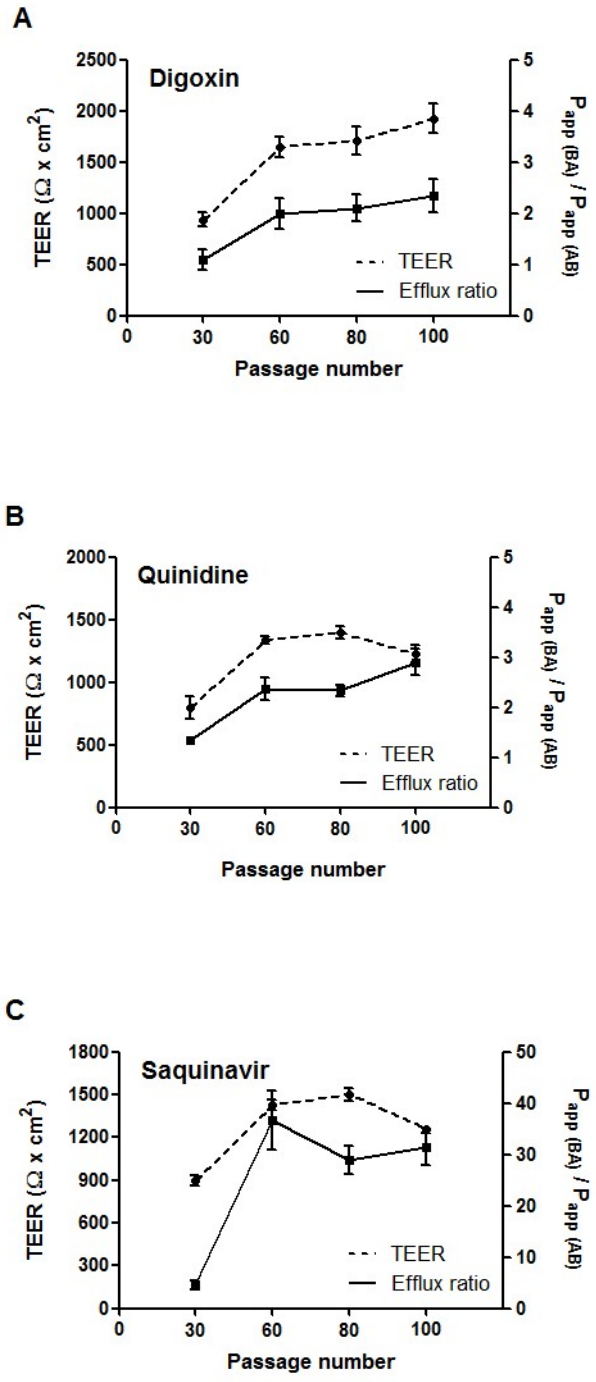


Fig. 4. Correlation between the TEER values and the efflux ratio of P-gp substrates.

Values for (A) digoxin, (B) quinidine and (C) saquinavir across Caco-2 cells from different passage number. Data represent the mean of three independent monolayers. AB = AP-to-BL, BA = BL-to-AP.

3. Protein expression of P-gp

Next, we have compared the expression level of P-gp between Caco-2 monolayers from early- and late-passage number to figure out whether the increased efflux ratio of P-gp substrates by increased passage number was due to the increased expression of P-gp in the apical side of the monolayer. Based on the results from the bidirectional transport experiments, we assigned the early-passage number to passage 30 and late-passage number to passage 80. We used the membrane protein extraction kit from abcam and followed the manufacturer's protocol to extract total membrane protein from the Caco-2 cell monolayer from passage 30 and 80 Caco-2 cells. As the result, the expression level of P-gp was higher in the monolayer from the late-passage Caco-2 cells (Fig. 5). This indicated that the expression of P-gp was upregulated in the monolayers from late-passage Caco-2 cells and increased efflux of P-gp substrates in the monolayers from passages over 60 Caco-2 cells was due to the increased P-gp expression on the apical side of the monolayer.

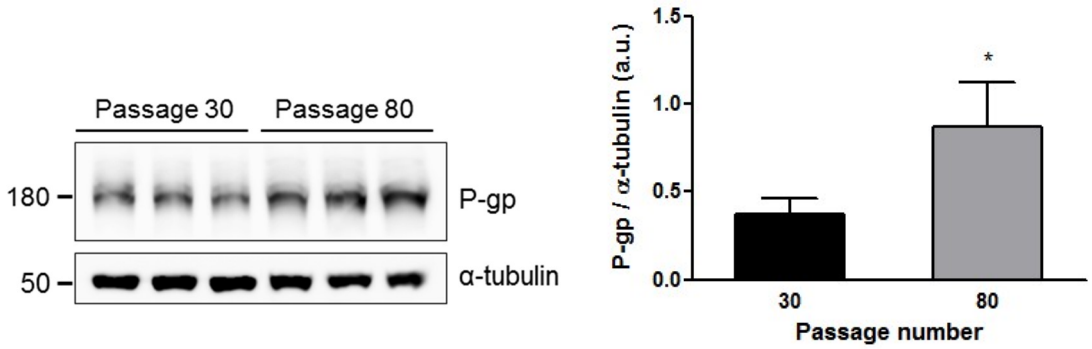


Fig. 5. Western blot analysis for P-gp expression in Caco-2 monolayers.

MDR1 P-glycoprotein (170 kDa) and α-tubulin (50 kDa) for Caco-2 monolayers from early passage number (30) and late passage number (80) were determined. The amount of P-gp expression was normalized to α-tubulin. Mean comparisons were analyzed by Student's *t* test. Values are indicated as mean ± S.D. (n = 3); *P < 0.05.

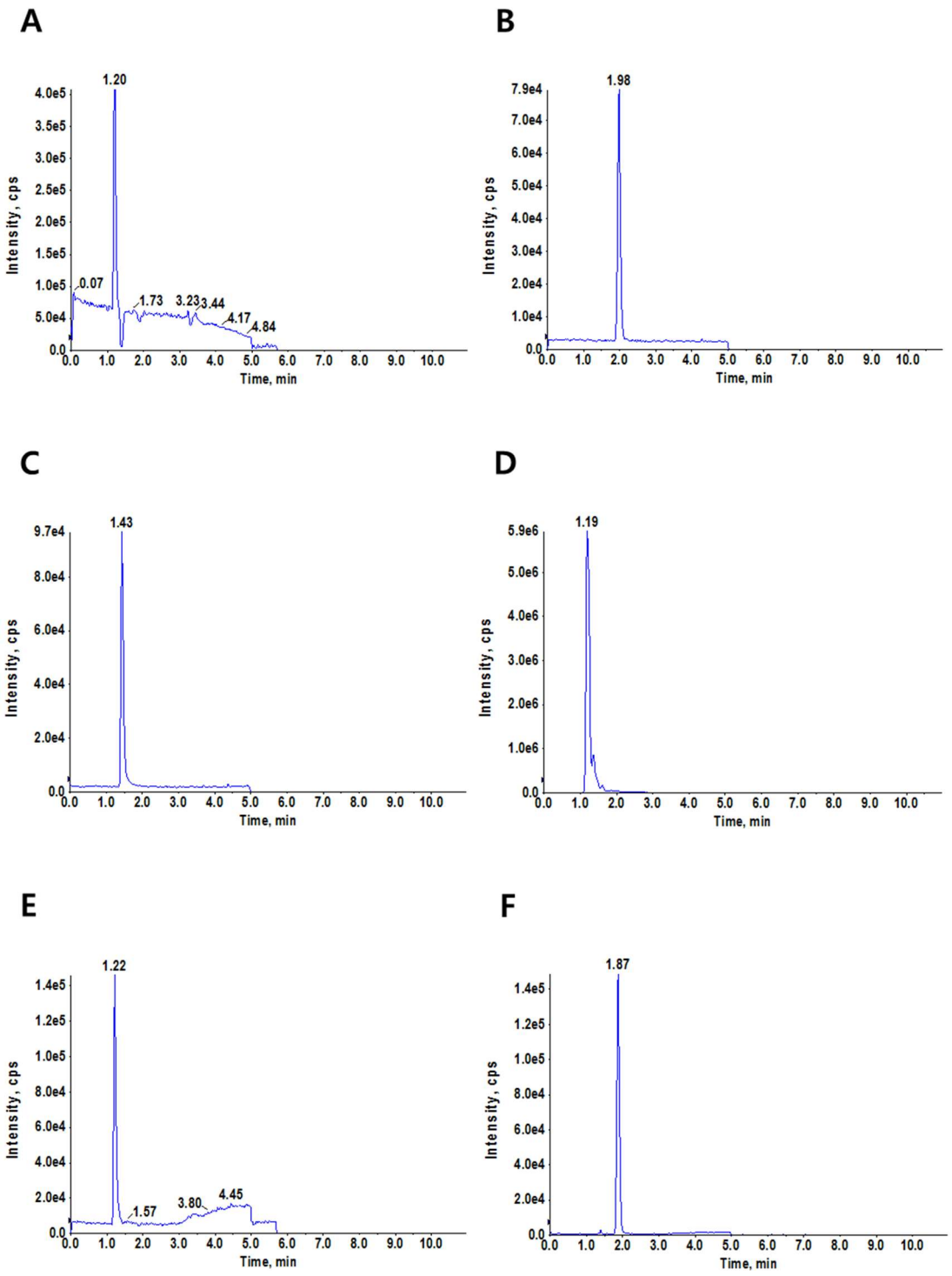
4. LC-MS/MS Analysis of TCA metabolites

4.1. Method Development

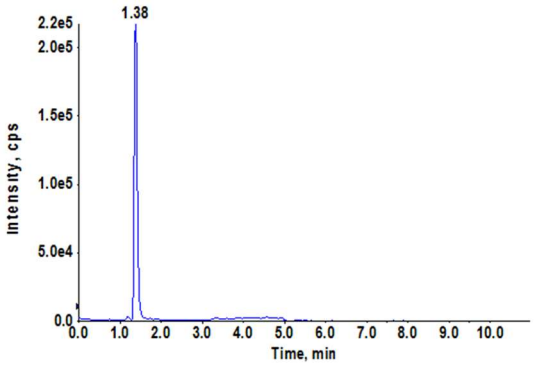
Before quantitation of TCA metabolites, the method for each metabolite was developed on LC-MS/MS. 1 uM stock solutions of each metabolite were prepared in HPLC grade water and they were infused to 5500 QTRAP Mass spectrometry to obtain the optimal MRM method for each metabolite. Method for Acetyl CoA, Alanine, Glutamine and Serine were developed under the positive polarity and a-ketoglutarate, citrate, isocitrate, fumarate, glucose, lactate, malate, oxaloacetate, pyruvate and succinate were developed under the negative polarity. Aspartate and glutamate were developed under both positive and negative polarities. Multiple MRM methods for each metabolite were obtained based on various Q1-Q3 pairs found through product ion scan. Then, chromatographic peaks of each metabolite were evaluated through LC-MS/MS system. Agilent 1290 series HPLC system equipped with on-line degasser, binary pump, thermostatted well-pleate autosampler and column compartment was used for our LC-MS/MS system. 2 uL volume of each metabolite was injected and flowed through Thermo Hypersil 2.1 x 150 mm column by the flow rate of 0.25 mL/min under the mobile phase of 1 mM Ammonium Acetate in water or methanol, and the chromatograms were examined. The method that resulted in the highest and sharpest analyte peak was chosen for the optimal method for each metabolite (Fig. 6), and the information of methods is described in table 1.

Table 1. LC-MS/MS parameters of each TCA metabolite

Analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Declustering energy	Collison energy	Polarity
α-Ketoglutarate	1.20	145	101	-35	-17	Negative
Alanine	1.98	90	44	31	15	Positive
Aspartate	1.43	134	74	26	20	Positive
Citrate	1.19	191	111	-54	-20	Negative
Fumarate	1.22	115	71	-80	-11	Negative
Glucose	1.87	179	89	-40	-14	Negative
Glutamate	1.38	146	102	-55	-20	Negative
Glutamine	2.02	147	84	31	23	Positive
Isocitrate	1.18	191	73	-54	-30	Negative
Lactate	1.41	89	43	-50	-17	Negative
Malate	1.21	133	115	-40	-16	Negative
Oxaloacetate	1.21	131	87	-35	-14	Negative
Pyruvate	1.22	87	32	-40	-12	Negative
Serine	2.02	106	60	35	13	Positive
Succinate	1.22	117	73	-40	-17	Negative


A
1.20
0.07
1.73
3.23
3.44
4.17
4.84
Intensity, cps
Time, min
B
1.98
Intensity, cps
Time, min
C
1.43
Intensity, cps
Time, min
D
1.19
Intensity, cps
Time, min
E
1.22
1.57
3.80
4.45
Intensity, cps
Time, min
F
1.87
Intensity, cps
Time, min

G


1.38
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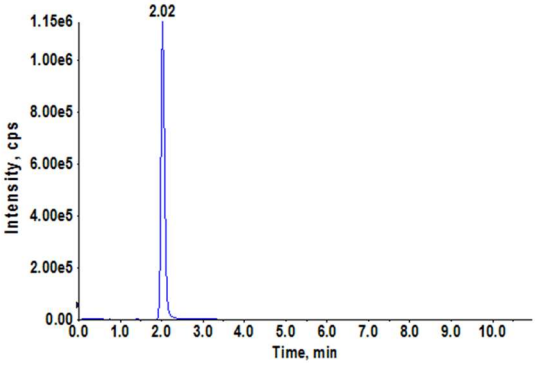
H


2.02
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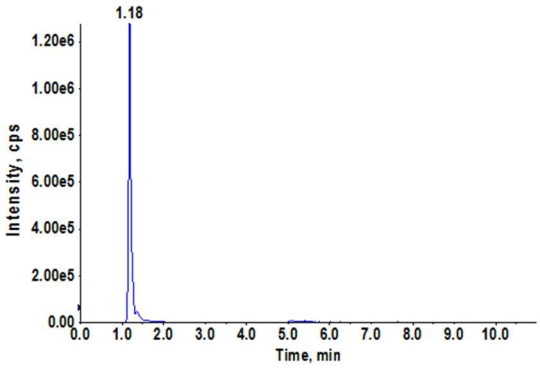
I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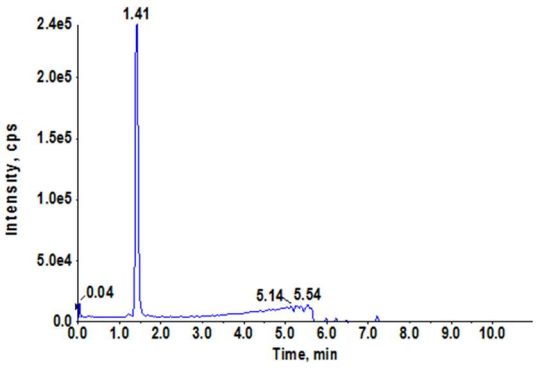
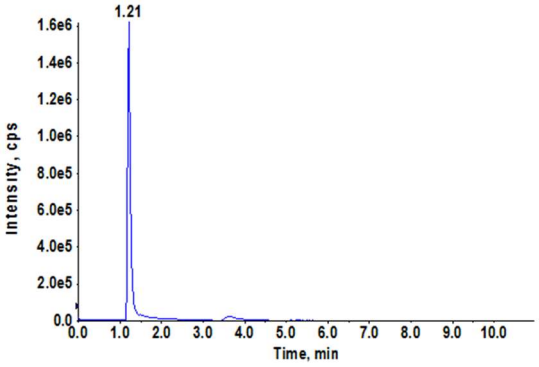
J


1.41
0.04
5.14, 5.54
Intensity, cps
Time, min

K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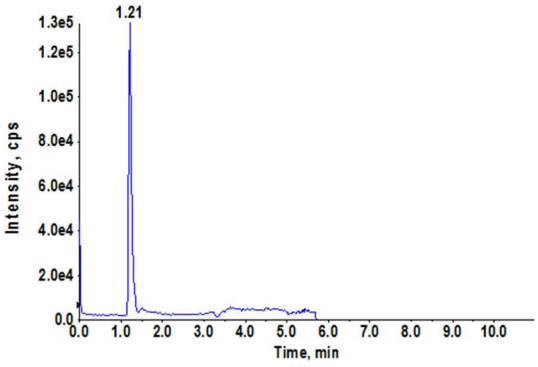
Time, min

L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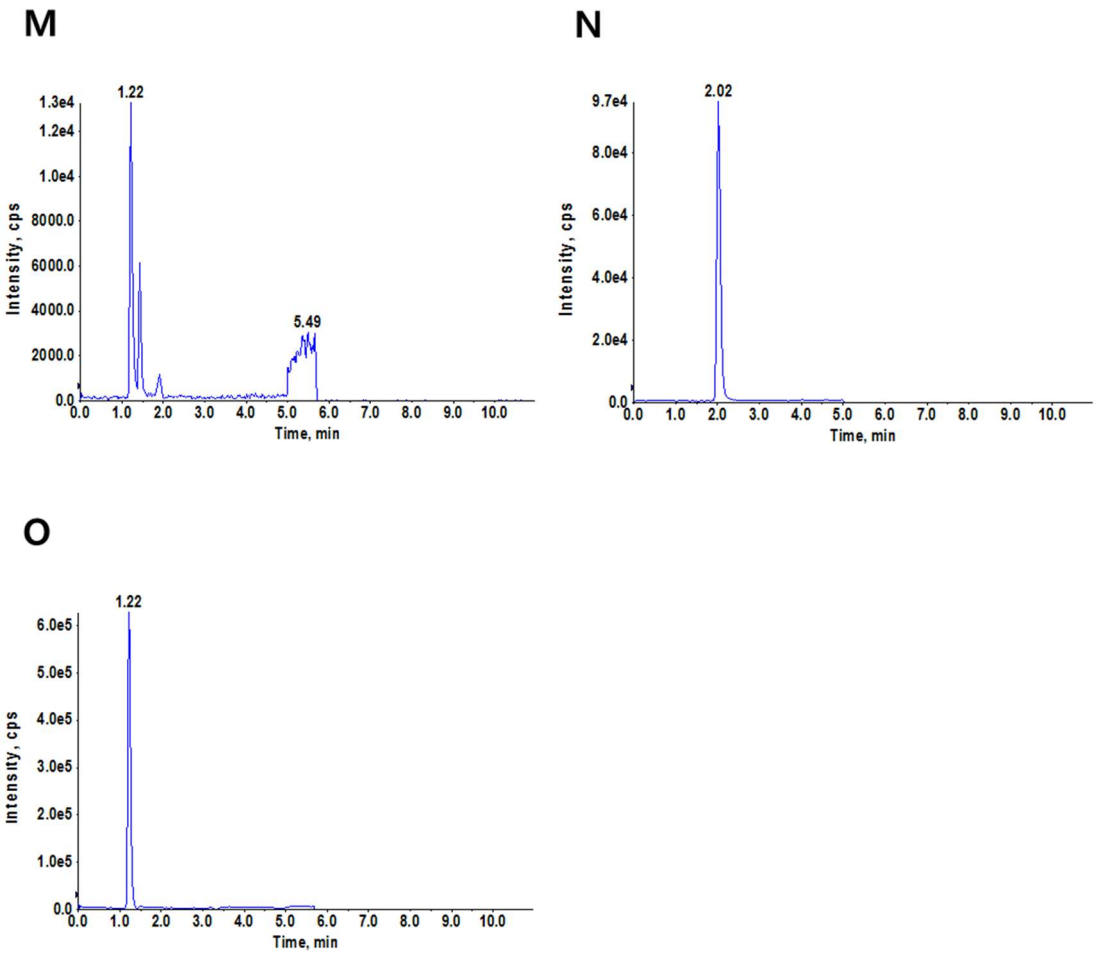

Fig. 6. Representative chromatograms of TCA metabolites. (A) a-Ketoglutarate. (B) Alanine. (C) Aspartate. (D) Citrate. (E) Fumarate. (F) Glucose. (G) Glutamate. (H) Glutamine. (I) Isocitrate. (J) Lactate. (K) Malate. (L) Oxaloacetate. (M) Pyruvate. (N) Serine. (O) Succinate.

4.2. Metabolite pool size of TCA metabolites

The validated method was used for quantification of TCA intermediates in Caco-2 cell monolayer from each early- and late-passage cells. The concentration of each metabolite was quantified using the 2-point standard curve (1 μM and 5 μM) for each metabolite. Standard curve was fitted in linear or quadratic regression and the concentration of samples was extrapolated from the fitted standard curve. Since TCA metabolites are endogenous metabolites, the amount of metabolite found in the sample was subtracted from the blank, 1 μM and 5 μM samples to make the standard curve pass through the zero. Out of total 15 TCA metabolites of which LC-MS/MS methods were developed, oxaloacetate and α-ketoglutarate were excluded from the quantification because they were not detected in the sample. The concentration of TCA metabolite extrapolated from the standard curve and measured in the unit of μM was further calculated into the metabolite pool size (nmol/mg) by normalizing with the total volume of the sample and protein content. Aspartate, serine, pyruvate, citrate, isocitrate, fumarate, malate, succinate were present in a relatively low level of metabolite pool size, all under 50 nmol/mg. Metabolite pool sizes of alanine, glutamate and glutamine were detected in a moderate level, whereas glucose and lactate were present in a pool size higher than 1000 nmol/mg (Fig. 7). The levels of glutamate, glutamine, lactate, serine and succinate were significantly reduced in passage 80 Caco-2 monolayer, and the levels of all other intermediates were remained the same, as compared to passage 30. The level of glutamate was decreased from 50.33 nmol/mg to 44.84 nmol/mg; the level of glutamine was decreased from 101.75 nmol/mg to 76.49 nmol/mg; the level of lactate was decreased from 1804.61 nmol/mg to 1515.34 nmol/mg; the level of serine was decreased from 19.95 nmol/mg to 14.80 nmol/mg; the level of succinate was decreased from 2.49 nmol/mg to 2.08 nmol/mg (Fig. 7). These changes in metabolite pool size indicated that TCA energy metabolism was altered in Caco-2 monolayer as the passage number was increased from 30 to 80

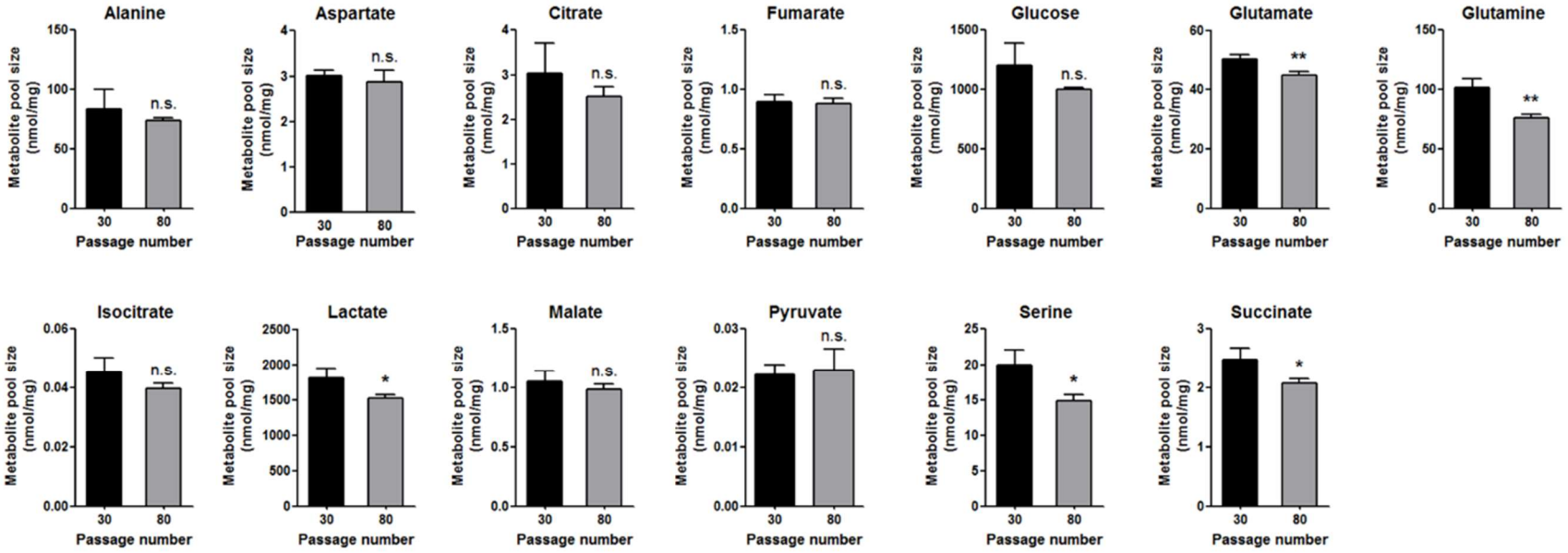

Fig. 7. Metabolite level of TCA intermediates in Caco-2 monolayers of passage 30 and 80 Caco-2 cells.

The concentration of metabolites was normalized to total protein content for comparisons of metabolic pool size. Mean comparisons were analyzed by Student's *t* test. Values are indicated as mean ± S.D. (n = 3); *P < 0.05, ** < 0.01. n.s., not significant.

Discussion

Caco-2 permeability test has been the standard in vitro model of intestinal absorption but heterogeneity in results across labs and institutions is yet an issue to overcome due to lack of standardization of the assay. Out of many factors that would influence the outcomes of the assay, over-passaging has been one of the experimental procedures misconducted by many scientists in attempts to continuously subculture on culture dish for maintenance of the cell line. Over-passaging results in increased passage number of the cells and it has been reported through many studies that passage number affects many characteristics of Caco-2 cells [15, 23-25]. Through this study, we have found that over-passaging of Caco-2 cells causes changes in integrity of the monolayer differentiated Caco-2 cells by increasing the expression of P-gp, a type of efflux proteins and altering TCA energy metabolism.

TEER values indicate how tight the monolayer has formed and we have found that Caco-2 monolayer of late passage Caco-2 cells has higher TEER values than the monolayer of early-passage Caco-2 cells. A higher proliferative rate in late-passage Caco-2 cells has been reported [26], and higher TEER values measured in late-passage Caco-2 monolayers in our study could also be explained by higher density of cells in the late passage due to a higher rate of proliferation. However, since permeability of simply diffusing drugs like atenolol and metoprolol was not affected by increased passage number, we have concluded that a higher density of Caco-2 cells is not the major factor that influences the outcomes of this assay. We additionally have found that TEER values are also affected by expression of efflux proteins by showing that the protein expression of P-gp is increased as the passage number is increased, and we have concluded that changes in P-gp expression is the major changes that affects the integrity of Caco-2 monolayers since efflux ratio of efflux substrates was increased in the similar manner.

An increase in P-gp is a phenomenon that can be observed in cancer cells [27]. As normal cells turn into cancer cells, they acquire characteristics of a higher drug-resistance by expressing more efflux proteins in the plasma membrane. Drug-resistance in cancer cells has been the biggest problem to overcome in chemotherapy since many anti-cancer drugs are substrates for efflux proteins. Cancer cells not only acquire defense mechanism but also go through energy metabolic reprogramming to increase the efficiency of energy consumption. Cancer cells take up glucose at higher rates than normal tissue yet use less glucose for oxidative phosphorylation and favor the incomplete oxidation of glucose through the glycolytic pathway even in the presence of oxygen (Warburg effect) [28, 29]. Glutaminolysis is also enhanced to provide biosynthetic precursors for cancer cells. Cancer cells display enhanced and unusual metabolic activities compared with normal differentiated cells, as they reprogram their metabolic machinery to satisfy their bioenergetics and biosynthetic requirements [30, 31]. Pyruvate generated from the glycolytic pathway is converted to lactate, rather than being used in the TCA cycle. Although the requirement for mitochondrial ATP production is decreased in cancer cells, the demand for biosynthetic precursors and NADPH is increased. To compensate for these changes and to maintain a functional TCA cycle, cancer cells often rely on elevated glutaminolysis.

Based on our LC-MS/MS analysis of the metabolite level, metabolic pool size of glutamine was decreased in monolayers of late-passage Caco-2 cells compared to that in monolayers of early-passage Caco-2 cells. Since the metabolite analysis was done on the total cell lysates, the metabolic pool size represents the amount of metabolites measured in the cytosol of the cell at one point, and a decrease in metabolic pool size can mean the increased utilization of that metabolite. Therefore, decreases in metabolite pool size of glutamine and its subsequent metabolites, glutamine and succinate indicate that differentiated late-passage Caco-2 cells probably utilize more glutamine and glutamate than differentiated early-passage Caco-2 cells. This can be evidence for glutaminolysis, a phenomenon observed in cancer cells. In

addition to changes in the level of glutamine and its subsequent metabolites, the level of lactate was decreased in passage 80 Caco-2 monolayer. This decrease in lactate level in late-passage Caco-2 monolayer may be explained by an increase in lactate release observed in cancer cells which turn most of their pyruvate into lactate then release it due to over-consumption of glucose [32]. Lastly, the metabolic pool size of serine was decreased in the late-passage Caco-2 monolayer, and such decreases in the level of serine resemble the accelerated breakdown of serine in cancer cells. In cancer cells, breakdown of serine is elevated to generate a major pool of one-carbon units for synthesis of nucleotides that are fundamental for DNA and RNA synthesis for rapid cell division [33]. Altogether, these changes in TCA energy metabolism can indicate that Caco-2 cells, which differentiate into normal enterocytes, differentiate further into cancerous enterocytes when their passage is high. To obtain stronger evidence that Caco-2 cells become to resemble cancer cells as they passage over a long period of time, a further metabolite study using isotope tracing should be performed to observe not only the metabolite level but also the metabolic flux.

A transport assay using Caco-2 cells is widely used in vitro model of intestinal absorption for many types of drugs, including simply diffusing drugs and efflux substrates. Through this study, we suggest that for transport assays for transcellularly or paracellularly absorbed drugs, differentiated Caco-2 cells of any passage can be used. On the other hand, to predict drug absorption in cancer patients or identify P-gp substrates, late-passage Caco-2 cells (passage number 80 or higher) are recommended for a better prediction of their permeability in vivo.

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국문요약

장 흡수는 경구 약물의 생체 이용률을 결정하는 중요한 속성이며, 분화 시 사람의 소장 상피 세포와 흡사해지는 Caco-2 로 이루어진 단층 시스템은 경구로 투여되는 화합물의 장 흡수를 스크리닝 하기 위한 가장 이상적인 in vitro 시험 방법이다. 이런 Caco-2 세포는 계대 수가 증가할수록 약물의 저항성이 높아진다는 것이 알려져 있음에도 불구하고, Caco-2 세포를 이용한 시험에 어떤 계대 수의 세포를 사용해야 하는지는 아직 표준화되지 않은 상태이다. Caco-2 세포는 암세포 유래이지만 분화 시 정상 소장 상피 세포와 흡사해지는데, 과연 세포의 장기 계대 배양이 Caco-2 세포를 암화 된 소장 상피 세포로 분화시키는지 알아보기 위해 계대 수가 높은 Caco-2 세포로 이루어진 소장 상피 세포 단층의 약물 efflux 정도와 에너지 대사를 평가했다. 계대 수가 높은 Caco-2 단층에서 내피 전기 저항 (TEER) 이 계대 수가 낮은 세포의 단층보다 높게 나왔고, 이를 통해 계대 수가 높아지면 세포의 단층이 더 단단해진다는 것을 알 수 있었다. 또한, 계대 수가 높은 세포 단층에서 efflux protein 인 P-glycoprotein (P-gp)의 단백질 발현이 높아져 있었고, 이에 따라 P-gp 물질들의 efflux 정도가 계대 수가 높은 Caco-2 단층에서 높아져 있었다. 세포 단층의 에너지 대사 분석을 위해 LC-MS/MS 를 이용하였고, 분석 결과, glutamate, glutamine, lactate, serine 그리고 succinate 의 대사체 pool size 가 계대 수가 높은 Caco-2 단층에서 낮아져 있는 것을 확인하였다. 이번 연구에서 낮은 계대 수와 높은 계대 수의 Caco-2 로 이루어져 있는 소장 상피 세포 단층의 비교 연구를 대사체 분석 측면에서도 진행하였고, 이를 통해 암화된 상태에서의 약물 흡수를 보다 더 정확하게 예측하기 위해서는 계대 수가 높은 Caco-2 를 이용해야 한다는 것을 알 수 있었다.

감사의 글

학문의 깊이를 깨닫게 도와주시고 본 연구를 완료할 수 있도록 이끌어 주셨던 저의 지도교수님이신 오수진 교수님께 먼저 머리 숙여 감사의 마음을 전합니다. 연구자로서 부족한 점이 많았던 저에게 믿음과 응원을 아낌없이 주셨던 덕분에 무사히 논문을 작성하고 석사과정을 마칠 수 있었습니다. 논문에 관심을 가지고 지도해 주시며, 실험 계획 및 결과 해석에 도움을 주셨던 이지윤 박사님께도 감사의 인사를 전합니다. 또한, 바쁘신 와중에도 따뜻한 격려와 조언과 함께 본 연구를 심사해 주신 정성윤 교수님과 황정진 교수님께 진심으로 감사드립니다.

처음 연구실에 왔을 때 잘 적응할 수 있도록 도와준 김지선 행정 선생님과 힘들 때 도움의 손을 내밀어 준 우리 연구실 동료들, 유동구 연구원과 신은진 연구원에게 감사합니다.

마지막으로, 늦은 나이에 다시 학업에 충실하고 지식을 습득할 수 있도록 딸 그리고 며느리의 선택을 믿어 주시고 지원해 양가 부모님과 가족분들 모두에게 감사 인사드립니다. 누구보다 아내인 저를 응원해 주고 사랑과 믿음으로 무한한 용기를 북돋아 주며 저의 행복을 빌어주는 남편 이웅휘에게 사랑과 고마움을 전합니다.

많은 분들의 사랑과 도움으로 맺어진 결실인 만큼, 그 은혜를 잊지 않고 앞으로 겸손한 자세로 사회에 큰 도움이 될 수 있는 사람이 되겠습니다.

감사합니다.