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Original paper

Application of microfluidic perfusion culture for assessment of colistin nephrotoxicity

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Abstract

Recent microfluidic perfusion culture system provides a more physiological disease model and enables various evaluations. Colistin nephrotoxicity is of growing concern due to its high incidence and the increased use of colistin, but preventive and therapeutic options are lacking. Here, we compared the relative nephrotoxicity of different colistin dosing regimens, and assessed the effects of dipeptidyl peptidase IV inhibitors (DPP4i), as promising renoprotective agents, on colistin nephrotoxicity in a microfluidic culture device. We treated kidney tubular epithelial cells with colistin under physiological shear stress in bolus-simulating and continuous infusions over a 48 hour period. Evogliptin, a recently developed DPP4i, was administered into the lower channel to examine its renoprotective effects. We assessed cell viability, tight junction protein expression, transmembrane permeability, and kidney injury molecule-1 (KIM-1) levels for each group. The continuous infusion regimen resulted in significantly increased cell death, decreased zonula occludens-1 and occludin expression, higher transmembrane permeability, and higher KIM-1 levels compared with bolus-simulating regimen. The DPP4i decreased transmembrane permeability, but there was no difference in cell viability and KIM-1 levels. Overall, the bolus-simulating regimens showed lower cytotoxicity and preserved barrier integrity better than the continuous infusion regimen, and DPP4i might mitigate colistin-dependent transmembrane permeability.

Keywords Colistin, nephrotoxicity, microfluidics, organ-on-a-chip, acute kidney injury.

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Introduction

In the last decade, a microfluidic cell culture device, called "organ-on-a-chip", has attracted attention as a highly physiologically relevant *in vitro* model (BHATIA & al [1]). Specifically, kidney tubular epithelial cells exposed to physiological fluid shear stress showed physiologically relevant changes, including a significant increase in tight junctions, transporters, and enhanced cell polarity. Therefore, the organ-on-a-chip platform can have advantages for advancing models of renal physiology (ASHAMMAKHI & al [2]). Further, organ-on-a-chip technology enables various evaluations such as assessment of cell barrier integrity and transporter function (JANG & al [3]; KIM & al [4]). In this regard, the organ-on-a-chip has advantages for assessing the nephrotoxicity of diverse substances (KIM & al [4]; LI & al [5]; CHANG & al [6]). We previously evaluated gentamicin nephrotoxicity under microfluidic conditions, showing an association between the pharmacokinetic (PK) profile and nephrotoxicity (KIM & al [4]). Recently, there has been increasing concern on the potential nephrotoxicity of colistin due to its extensive use. Colistin therapy is associated with high rates of acute kidney injury (AKI) (ZAVASCKI & al [7]). However, PK and toxicity data of colistin are relatively limited. As a result, the optimal dosage regimen to minimize the nephrotoxicity remains uncertain. Only small observational studies have investigated colistin nephrotoxicity according to the dosing regimen (DALFINO & al [8]; DEWAN & al [9]; SORLI & al [10]) because of the difficulty of controlling multiple confounding factors in ICU settings and recruiting eligible patients. Moreover, (WALLACE & al [11])although several risk factors for colistin nephrotoxicity have been reported, current preventive and therapeutic strategies are insufficient (ORTWINE & al [12]). The effects of various antioxidants on colistin nephrotoxicity have been evaluated in several animal studies, but have not been demonstrated in clinical studies (GAI & al [13]). In recent years, dipeptidyl peptidase IV inhibitors (DPP4i) have received considerable attention as promising renoprotective agents (TANAKA & al [14]). DPP4i revealed renoprotective effects via anti-apoptotic and anti-oxidative effects (GLORIE & al [15]), which are also mechanisms involved in colistin nephrotoxicity (GAI & al [13]). However, little is known about the effects of DPP4i on colistin nephrotoxicity. Therefore, (TANAKA & al [14])we aimed to demonstrate the applicability of microfluidic culture systems in assessing the effects of DPP4i on colistin nephrotoxicity and dosing regimens to minimize colistin-induced kidney injury.

Methods and Materials

Organ-on-a-chip device design

In the present study, microfluidic technology was implemented in PDMS-based microchips. The device consisted of two channels of the same size (1-mm width, 6-mm length, 100-μm height), located above and below a porous polyester membrane (0.4-μm pores, 10-μm thickness) for cell culture (Figure 1A). The cell-seeded upper channel was designed to be longer to receive shear stress more stably over a 48 hour period. The membrane was cut out from TranswellTM plates (Corning Inc., Corning, NY, USA), which is transparent and collagen-treated to promote cell attachment. The molds for upper and lower channels were fabricated using an SU-8 photoresist (Microchem, Newton, MA, USA) to have the shape and thickness of the microchannels on a silicon wafer. Then, PDMS was spin-coated onto the molds and cured by heating. The PDMS castings with the microfluidic channels were placed above and below the membrane and fixed with toluene. A 5-mm-thick PDMS slab connected to two pairs of inlet and outlet tubes was plasma-bonded onto the upper PDMS casting. The inlet and outlet tubes with an inner diameter of 0.79 mm were connected to an infusion pump and a drain bottle, respectively. We initially selected the devices that do not have any void space by air between the interface of the top and bottom PDMS castings. The devices without any leakage under normal flow conditions were finally chosen for the cell experiment. A microscope was used to inspect the apparatus. The leakage pressure of the device was 48 kPa when we measured with a pressure sensor (PX309-015G5V, Omega Eng). This relatively high leakage pressure ensured the cell experiment without any leakage of solutions under our flow conditions (flow rate: 15 μL/min).

Cell culture and microfluidic conditions

To culture the cells in the microchip, the porous membrane was coated with 50 μL/mL of fibronectin for 4 h and 4×10^5 Madin-Darby canine kidney (MDCK) cells in 50 μL were repeatedly seeded into the upper channel to achieve cell confluence. DMEM supplemented with 10% fetal bovine serum was used as the cell culture medium. To apply the microfluidic conditions to the attached cells, the cells were exposed to a fluid shear stress of 1 dyne/cm^2 through the upper channel for 6 h prior to drug exposure. Fluid flow rate was calculated using the formula $\tau = 6 \mu Q / bh^2$, where τ is the fluid shear stress (dyne/cm^2), Q is the fluid flow rate (cm^3 s^{-1}), μ is the medium viscosity (0.7 cP), b and h are the channel width (0.1 cm) and height (0.01 cm), respectively. The negative control was cultured without drug exposure under microfluidic conditions with the same shear stress. In the experimental groups, colistin was administered in two different regimens (Figure 1B): a bolus-simulating regimen and a continuous infusion regimen. In the bolus-simulating regimen, colistin was perfused at a starting concentration of either 1138 μg/mL (B1) or 2133 μg/mL (B2), with the concentration halved every 9 h (the half-life of colistin in critically ill patients in previous studies (GARONZIK & al [16])) over a total of 45 h. In the continuous infusion regimen, colistin was constantly perfused at a concentration of 400 μg/mL (C) for 48 h. Although the total colistin exposure (defined by area under curve) in B1 and C was the same (19, 200 mg/L·h) for 48 hours, the total dose of colistin in B2 was 1.9 times greater (37,194 mg/L·h) than in B1. To evaluate the nephroprotective effects of the DPP4i, 50 nM of evogliptin (Suganon®, Dong-A Pharm., Korea),

recently developed DPP4i, was administered to the lower channel with the same colistin dosing regimen as C. The tested concentration of evogliptin was determined using the cell viability assay in static conditions. Evogliptin was diluted in culture medium and exchanged every 24 h for 48 h.

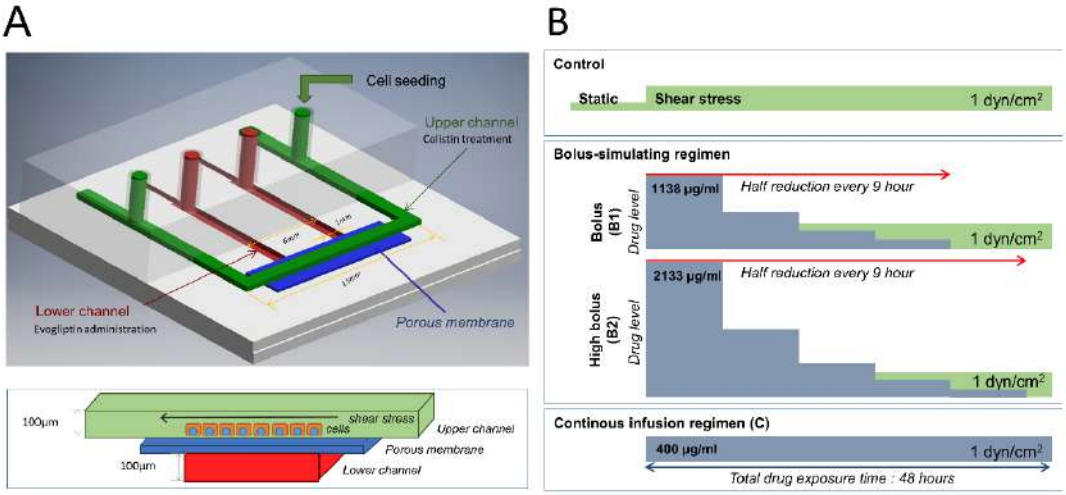

Figure 1. (**A**) Schematic illustration of the microfluidic culture device. (**B**) Different colistin dosing regimens; negative control, bolus-simulating regimens, and continuous regimes.

Cell toxicity assessment

Cell viability was evaluated with calcein AM, ethidium homodimer-1 and Hoechst stain using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Inc., Eugene, OR, USA) in each condition. The percentage of dead cells was calculated from the division of the number of ethidium-stained cells by the number of Hoechst-stained cells. In addition, tight junction protein expression and transmembrane permeability were assessed to verify morphological and functional changes in the epithelial barrier. The expression of the tight junction proteins zonula occludens-1 (ZO-1) and occludin was assessed using immunocytochemistry and confocal microscopy (Carl Zeiss LSM710, Carl Zeiss, Jena, Germany). For immunofluorescence staining, the cells attached to the porous membrane were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.5% Triton-X100 solution. The cells were then incubated overnight at 4°C with anti-occludin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-ZO-1 antibody (Abcam, Cambridge, MA, USA), followed by incubation with Alexa488-labeled goat anti-mouse IgG (Abcam) and Alexa568-labeled anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. To evaluate transmembrane permeability, albumin-fluorescein isothiocyanate conjugate (FITC-albumin, Sigma-Aldrich, St Louis, MO, USA) leakage from the upper channel to the lower channel was assessed using a fluorescence micro-plate reader (Gemini EM, Molecular Devices, San Jose, CA, USA) 30 min immediately after applying 50 μg/mL of FITC-albumin to the upper channel at 37°C. We also measured kidney injury molecule-1 (KIM-1), a sensitive renal tubular cell injury marker, in the medium collected from the upper channel after 24 h and 48 h using a KIM-1 ELISA kit (Mybiosource, San Diego, CA, USA) without dilution.

Image and statistical analysis

All image processing and analysis was performed using ImageJ (NIH, USA). Maximum image projections were used for the measurement of CTCF. Corrected total cell fluorescence was obtained using the formula CTCF = integrated density − (area of fluorescent cells × mean of background fluorescence). The Mann-Whitney U test was used to compare cell viability, CTCF, transmembrane permeability, and KIM-1 levels between experimental groups. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using R (version 3.4.3, the R foundation) and graphs were plotted using GraphPad Prism 7 (GraphPad Software Inc.).

Results and Discussions

Colistin nephrotoxicity according to different dosing regimens

First, we examined cell viability and the barrier integrity of the epithelial monolayer in the organ-on-a-chip according to the different dosing regimens. After 48 h of colistin treatment, the percentage of dead cells was approximately 0.34% in the negative control subjected to physiological shear stress, which was lower than that in static conditions (1.08%, Figure 2). In both bolus-simulating regimens starting at 2133 μg/mL (B2) and 1138 μg/mL (B1), the extent of cell death was similar (1.64%), despite the different total drug exposure. Both regimens were more cytotoxic than the negative control, but they were significantly less cytotoxic than regimen C (3.44%, P = 0.026 vs. regimen B1 and P = 0.010 vs. regimen B2). The continuous infusion regimen induced the greatest cell death, about twice that of bolus regimens. In immunofluorescence staining of the tight junction components ZO-1 and occludin, higher intensity levels were observed under shear stress conditions compared to static conditions (Figure 3a), as shown by previous studies (MAGGIORANI & al

[17]). The tight junction expression was diminished after colistin treatment. In particular, the decrease in fluorescence intensity was more pronounced in regimen C. The corrected total cell fluorescence (CTCF) supported these observations (Figure 3b and 3c). CTCF analysis also showed a significant decrease in tight junction components in regimen C, but not in regimens B1 or B2. The transmembrane permeability of FITC-albumin followed a similar pattern (Figure 3d), suggesting more injury in regimen C. The continuous infusion regimen exhibited significantly increased permeability compared to two bolus regimens or negative control. On the other hand, KIM-1 levels were similar in regimens B1 and C during the first 24 h and significantly greater in the regimen B2 (Figure 3e). After 48 h, KIM-1 levels in collected media decreased in both B1 and B2, but not in regimen C. At 48 h, KIM-1 levels were significantly higher in regimen C than in regimen B1.

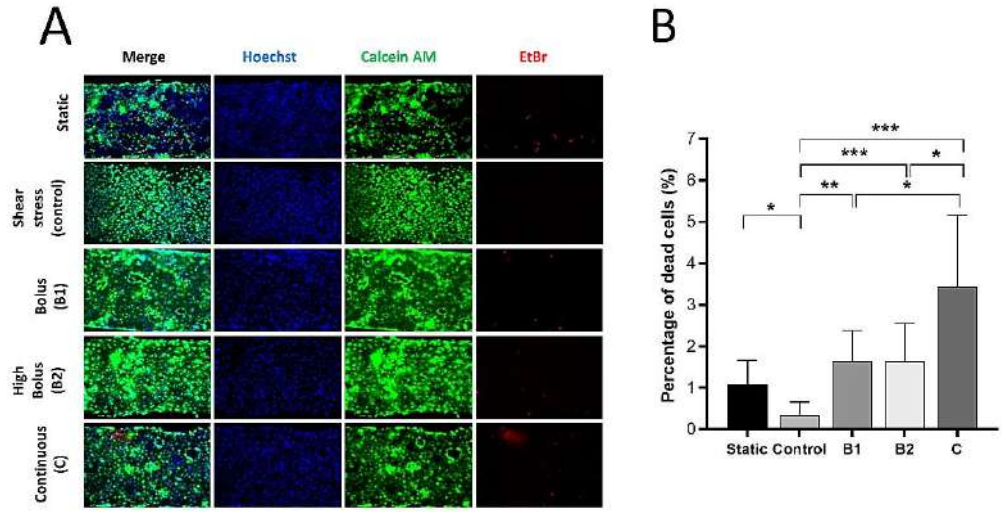

Figure 2. (**A**) Fluorescence images of live/dead staining of tubular epithelial cells. (**B**) The percentage of dead cells in different dosing regimens. (*p < 0.05; **p < 0.01; ***p < 0.001).

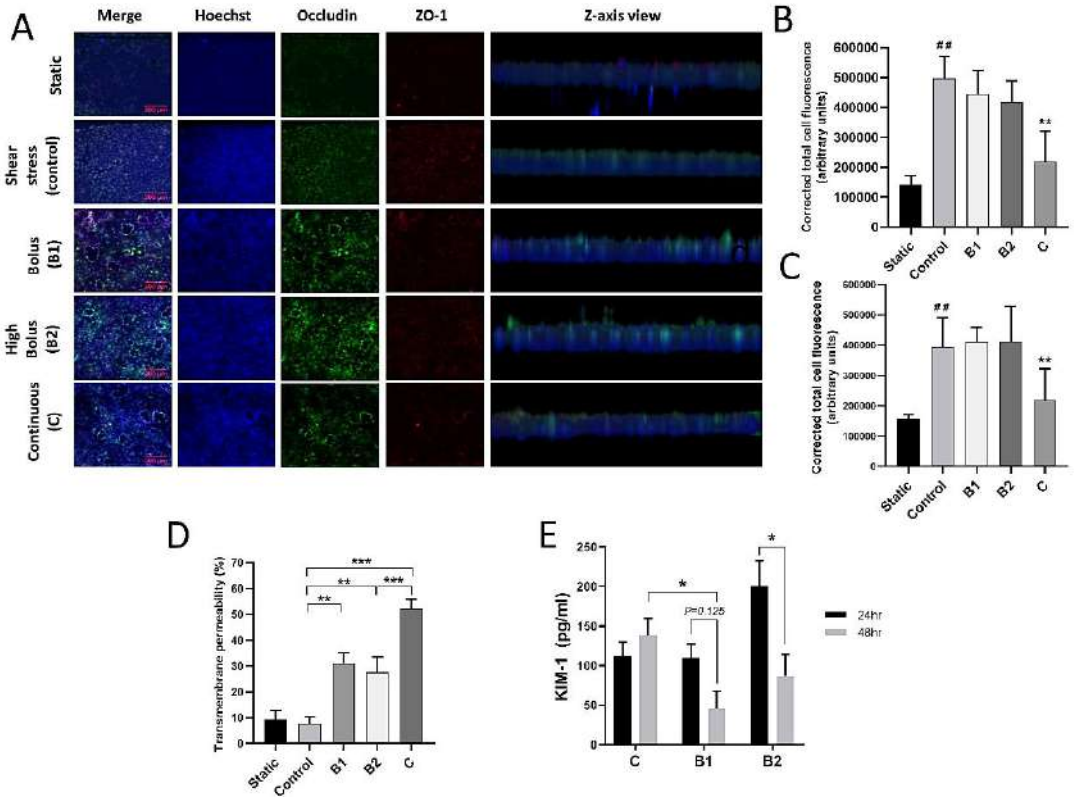

Figure 3. (**A**) Immunofluorescence stain for occludin (green), ZO-1 (red) (scale bar = 100 μm). The corrected total cell fluorescence (CTCF) of (**B**) occludin and (**C**) ZO-1, (**D**) The transmembrane permeability of albumin-FITC, (**E**) KIM-1 concentrations. The data are presented as the mean value ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001).

Overall, these results showed that the continuous infusion regimen resulted in higher cytotoxicity and deteriorated epithelial barrier function compared to bolus-simulating regimens under microfluidic conditions. Since the continuous infusion regimen maintained a higher drug concentration than the trough concentration of any bolus regimen, our results agreed with previous studies in which the colistin trough concentration was found to be an independent risk factor for AKI (SORLI & al [10]). Therefore, it is reasonable to hypothesize that the drug-free period or minimum drug concentration is an important determinant of colistin-associated nephrotoxicity. Meanwhile, two bolus-simulating regimens with different total colistin doses yielded similar nephrotoxicity after 48 h. However, we cannot conclude that total colistin dose is not associated with the extent of tubular cell injury. Our results may be due to the limited duration of the experiment, which was not long enough to identify nephrotoxicity from the cumulative colistin dose.

Despite the uncertainty of the mechanism, colistin nephrotoxicity is reported to be associated with oxidative damage and caspase-dependent apoptosis (OZKAN & al [18]; ORDOOEI JAVAN & al [19]). Tight junction proteins are known to be sensitive to oxidative stress (REYES & al [20]). Others previously observed that ischemic and toxic AKI resulted in decreased expression and disassembly of tight junction proteins, which is similar to our findings (TRUJILLO & al [21]; DENKER & al [22]). Colistin also increases tubular epithelial cell permeability, leading to cell swelling and lysis (ORDOOEI JAVAN & al [19]) and can be involved in cell death. The lower cytotoxicity of the bolus-simulating regimen can be explained in two ways based on the published literature. First, it may reflect recovery during the non-drug exposure period in bolus-simulating regimens with physiological shear stress. Jang et al reported that tubular cell damage induced by cisplatin exhibited a more remarkable recovery under shear stress conditions than static conditions during the drug-free period (JANG & al [3]). Second, lower nephrotoxicity in the bolus-simulating regimen may be associated with membrane transporter saturation, which is a generally accepted finding for aminoglycoside dosing (DRUSANO & al [23]). Colistin undergoes extensive tubular reabsorption involving specific transporters like human oligopeptide transporter 2 (PEPT2) and, carnitine/organic cation transporter 2 (OCTN2, SLC22A5) after glomerular filtration (LU & al [24]; VISENTIN & al [25]). Supporting this assumption, PEPT2 showed saturable reabsorption characteristics (LU & al [24]).

Protective effects of DPP4 inhibitor on colistin nephrotoxicity

We next evaluated the effects of DPP4i on colistin toxicity under organ-on-a-chip culture conditions with fluid shear stress. The DPP4i evogliptin (50nM) was administered into the lower channel during the colistin treatment (400 μg/mL for 48 h). The drug administration into the lower channel was intended to mimic drug diffusion through interstitial space. Using our described method, we evaluated tight junction protein expression, cell death, and epithelial barrier integrity. As seen in Figure 4a, higher immunofluorescence intensity of occludin and ZO-1 were observed in the evogliptin treatment group compared to the colistin-only-treated group. The corrected total cell fluorescence (CTCF) of occludin and ZO-1 was also higher in the evogliptin-treated groups. Further, with regards to transmembrane permeability, evogliptin showed beneficial effects, decreasing transmembrane permeability from 52.13% to 34.9%. However, the dead cell percentage did not significantly differ with evogliptin treatment (Figure 4), and KIM-1 levels in collected media 24 h or 48 h after drug treatment showed no difference with or without evogliptin treatment.

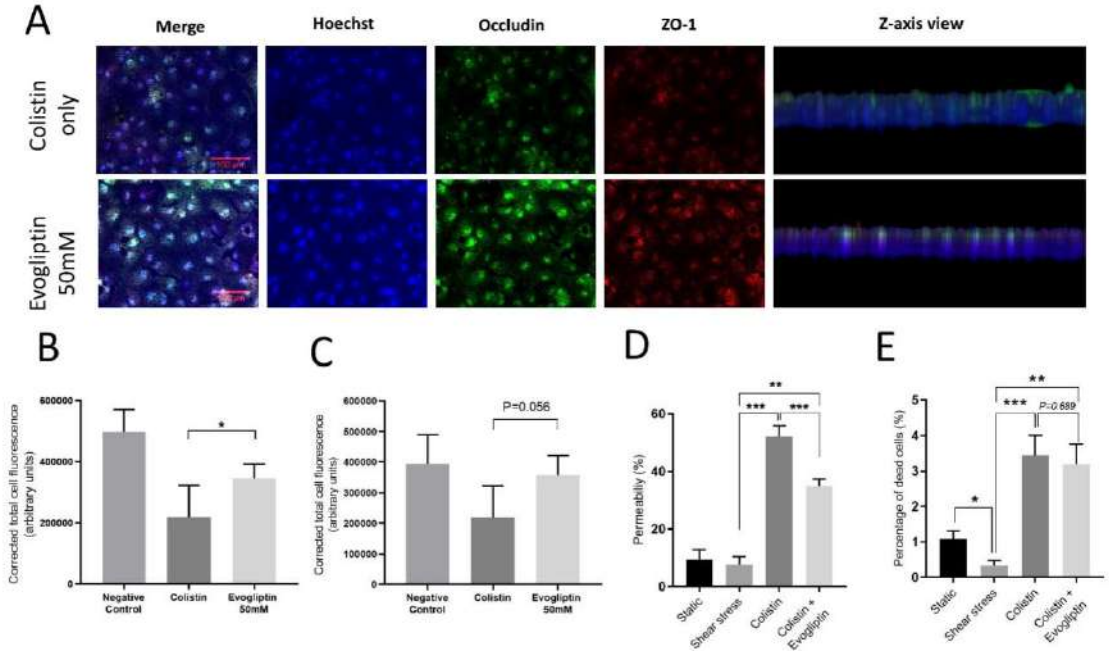

Figure 4. (**A**) Immunofluorescence stain for occludin (green), ZO-1 (red) (scale bar = 100 μm). The corrected total cell fluorescence (CTCF) of (**B**) occludin and (**C**) ZO-1 (b) The percentage of dead cells (**D**) The transmembrane permeability of albumin-FITC, (**E**) KIM-1 concentrations. The data are presented as the mean value ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001).

DPP4i was reported to have a renoprotective effect in addition to anti-diabetic effects (TANAKA & al [14]). In particular, DPP4i promoted renal recovery and inhibited cell death and inflammation in animal models of drug-induced AKI. (KANASAKI [26]) (CHOI & al [27]; IWAKURA & al [28]). We did not notice any significant differences in kidney injury markers or cell viability in this study, but we did find beneficial effects of DPP4i on transmembrane permeability and tight junction protein levels. Although there are few studies of DPP4i in relation to tight junctions in the kidney, several studies on the retina revealed that DPP4i reduce vascular permeability and inhibit the degradation of tight junctions in the retina (GONCALVES & al [29]; GONCALVES & al [30]). Further, ZO-1 and occludin expression in the retina increased in response to DPP4i (GONCALVES & al [29]). Therefore, it is possible that a similar effect occurs in renal tubules, but further investigations are needed to confirm this hypothesis. Our study has several limitations. Although MDCK cells are well-established cell lines suitable for microfluidic experiments (MAGGIORANI & al [17]; CATTANEO & al [31]), these cells are not human cells. Moreover, our experimental design is still simple compared to the human renal tubular structure. We also only evaluated cell injury and barrier functions, and hence detailed mechanisms need to be uncovered in future studies. Nevertheless, the organ-on-a-chip technology used in this study can provide a more physiological microenvironment, and these approaches could complement existing studies and provide more reliable evidence. Importantly, we varied drug concentrations over time to simulate the *in vivo* PK profile of colistin under physiological conditions. This may be one of strengths of this study.

Conclusions

In this study, we showed an application of the microfluidic organ-on-a-chip device for evaluating different dosing regimens and effects of DPP4i on colistin-associated nephrotoxicity, focusing on the epithelial barrier integrity and cytotoxicity. The bolus-simulating regimens showed lower cytotoxicity and preserved barrier integrity better than the continuous infusion regimen. DPP4i also might mitigate transmembrane permeability induced by colistin.

Acknowledgments

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