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Pharmacokinetic profile that reduces nephrotoxicity of gentamicin in a perfused kidney-on-a-chip

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Abstract

Nephrotoxicity is often underestimated because renal clearance in animals is higher compared to in humans. This paper aims to illustrate the potential to fill in such pharmacokinetic gaps between animals and humans using a microfluidic kidney model. As an initial demonstration, we compare nephrotoxicity of a drug, administered at the same total dosage, but using different pharmacokinetic regimens. Kidney epithelial cell, cultured under physiological shear stress conditions, are exposed to gentamicin using regimens that mimic the pharmacokinetics of bolus injection or continuous infusion in humans. The perfusion culture utilized is important both for controlling drug exposure and for providing cells with physiological shear stress (1.0 dyn cm^{-2}). Compared to static cultures, perfusion culture improves epithelial barrier function. We tested two drug treatment regimens that give the same gentamycin dose over a 24 h period. In one regimen, we mimicked drug clearance profiles for human bolus injection by starting cell exposure at 19.2 mM of gentamicin and reducing the dosage level by half every 2 h over a 24 h period. In the other regimen, we continuously infused gentamicin (3 mM for 24 h). Although junctional protein immunoreactivity was decreased with both regimens, ZO-1 and occludin fluorescence decreased less with the bolus injection mimicking regimen. The bolus injection mimicking regimen also led to less cytotoxicity and allowed the epithelium to maintain low permeability, while continuous infusion led to an increase in cytotoxicity and permeability. These data show that gentamicin disrupts cell–cell junctions, increases membrane permeability, and decreases cell viability particularly with prolonged low-level exposure. Importantly a bolus injection mimicking regimen alleviates much of the nephrotoxicity compared to the continuous infused regimen. In addition to potential relevance to clinical gentamicin administration regimens, the results are important in demonstrating the general potential of using microfluidic cell culture models for pharmacokinetics and toxicity studies.

Introduction

Safety is a critical issue for developing new drugs. One-third of all drug failures during preclinical and clinical developmental stages are attributed to toxicity [1]. Nephrotoxicity that is not detected in conventional cell cultures or animal models, is one causes of unexpected drug attrition. Currently, selection of the drug administration protocol to be used in human clinical trials is based predominantly on animal tests of drug efficacy [2]. There are important differences, however, between animals and humans. For example, animal renal clearance is usually significantly higher than human renal clearance [3]. Thus, drugs that are safe and efficacious in animal studies may be toxic to humans if given using a similar drug administration dosage and regimen. Recent advances in organ-on-a-chip systems have led to development of *in vitro* kidney

models with improved renal cell morphology and function compared to conventional 2D cultures and show promise for nephrotoxicity assessment [4]. These systems, however, have not yet extensively explored the effects that different drug administration regimens of the same medication may have on nephrotoxicity. That is, although kidneys-on-a-chip have been exposed to drugs for toxicity evaluation, the studies did not use drug administration regimens that follow human pharmacokinetic profiles. Here, we present proof-of-principle experiments that demonstrate microfluidic kidney models may be able to fill some of the unmet need in the study of the role of pharmacokinetics and nephrotoxicity.

Gentamicin is an aminoglycoside antibiotic that is widely administered but has a major drawback in that nephrotoxicity can limit its use at efficacious dosage levels. Thus, finding drug administration regimens that minimizes nephrotoxicity is an important clinical need. While the conventional clinical regimen administers multiple daily doses, rat studies have shown that a once daily regimen minimizes kidney cortical accumulation of gentamicin [5, 6]. Meta-analyzes of existing human clinical trials, however, are inconclusive in that no obvious differences are found between once daily dosing regimens and conventional dosing methods in terms of nephrotoxicity and mortality [7–9]. Here, we test the hypothesis that a microfluidic kidney-on-a-chip would show less nephrotoxicity to the same amount of drug (as defined by area under the curve) when administered as a bolus versus continuously.

Microfluidic techniques can generate physiologic microenvironments for a variety of tissues and organs [10]. Such models, although still rather simple, include kidney-on-a-chip, proximal tubule and distal tubule-on-a-chip [4, 11]. We constructed a similar microfluidic device lined with kidney epithelial cells that could be exposed to various pharmacokinetic profiles in the system. Perfusion of fluid through this system generates shear stress to enhance tubular function. Another key use of microfluidics in our experiments is to use timed perfusion of different drug concentrations to mimic the pharmacokinetic profiles of drug concentrations in the blood stream in humans. For example, recently, cardiac and hepatic cells-on-a-chip were shown to respond differently to differently timed drug treatments [12]. In our experiments, by perfusing high to low concentrations of drug over time we mimic the clearance of drug from the blood stream by the kidney after bolus injection. By perfusing a constant concentration of drug we mimic continuous drug infusion. We evaluated the nephrotoxicity of these two different gentamicin regimens using immunohistochemistry, protein biomarkers measurements, and viability readouts. Our results show that this type of *in vitro* microfluidic pharmacokinetics studies may be useful in understanding the role of pharmacokinetic mechanisms of kidney toxicity in drug development.

Methods

Device fabrication

For the creation of the top channel, master molds were created through UV polymerization of photoresist (SU-8 100, Microchem, Newton, MA) spun on the surface of a silicon wafer. Polydimethylsiloxane (PDMS, Sylgard, DowCorning) was cast on the masters using a prepolymer to curing agent ratio of 1:10 w/w, and incubated at 60 °C for 12 h. The dimensions of the fluidic channel were 1 mm wide × 6 mm long × 100 μm high. The bottom channel was made with the same width and height. On the top channel layer, a cured 5 mm thick PDMS slab was attached for fixing connecting tubes. A porous polyester membrane (0.4 mm pores, 10 μm thick) cut out from Transwell^{TM} plates (Corning Inc., Corning, NY) was placed between the top and bottom structures, which were bound with a mixture of PDMS and toluene [4]. As far as we know, these membranes are the most transparent among commercial membranes that also promote attachment of the infused cells. Connecting slabs were plasma treated (Femto Science Inc., Suwon, Korea) to facilitate tight bonding [13].

Cell culture and flow experiments

As a non-microfluidic comparison, Madin-Darby canine kidney (MDCK) cells were seeded on Transwell membrane filters (Corning Inc., Tewksbury, MA) with DMEM medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Two different conditions were tested: (i) a short-term high concentration group was exposed with 18 mM of gentamicin for 4 h, followed by culture media without gentamicin for the following 20 h, and (ii) a long-term low concentration group was exposed to 3 mM gentamicin for 24 h (figure 2).

In microfluidic experiments, MDCK cells were seeded on porous membranes that were coated with fibronectin (a concentration of 10 μg ml^{-1}) over 3 h. For creating shear stress condition, cells were pre-exposed to a fluid flow of 1 dyn cm^{-2} for 6 h (actual flow rate of 15 μl min^{-1}) before drug administration. Fluid shear stress was calculated using the following equation: $\tau = 6\mu Q/bh^2$, where m is the medium viscosity (g cm^{-1} s^{-1}), Q is the volumetric flow rate (cm^3 s^{-1}), b is the channel width (cm), and h is the channel height (cm). All experiments were completed at least in triplicate.

Dosing schedules are shown in figure 1. The half-life of gentamicin in humans is ~2 h [14, 15]. The D1 regimen mimics exposure of kidney to a single daily dose of gentamicin. Drug perfusion concentrations were reduced by half every 2 h. After 8 h, for the following 16 h, drug-free culture medium was infused.

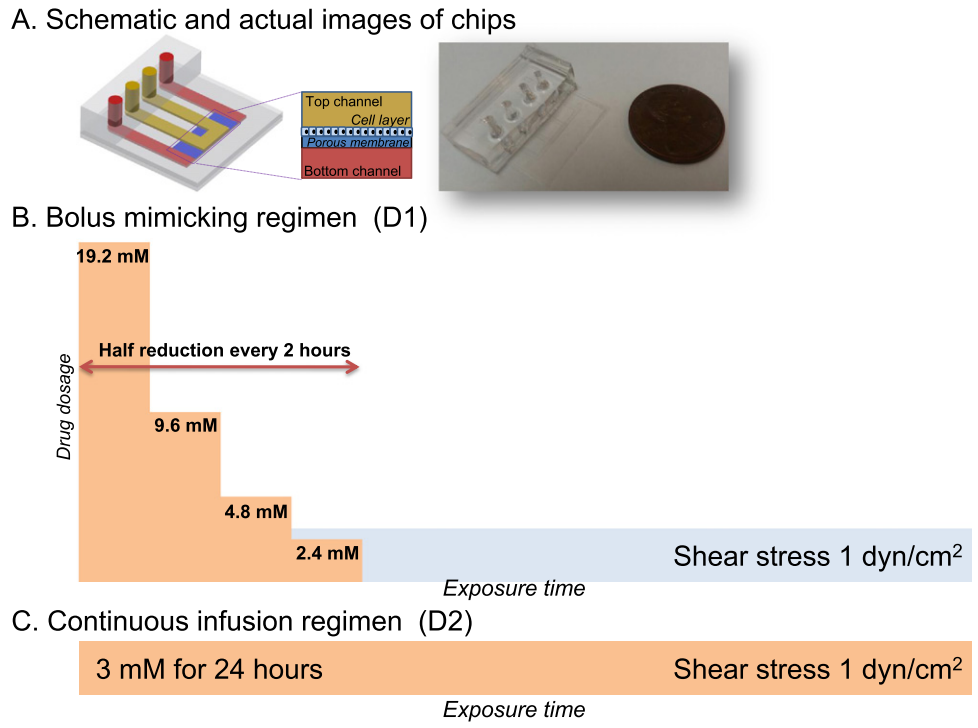

Figure 1. Schematic of study design. (A) Schematic design and actual image of a kidney-on-a-chip. (B) After 6 h shear stress, two different regimens were applied to the devices for 24 h. D1 mimicked drug levels in the human kidney after a single bolus regimen of gentamicin administration. Since gentamicin half-life in humans is 2 h, drug concentrations were reduced by half every 2 h for 8 h. For the following 16 h, drug free culture medium was infused with the same shear stress (1 dyn cm^{-2}). (C) D2 mimicked continuous infusion of gentamicin with the same shear stress (1 dyn cm^{-2}).

All of these infusions were performed at a shear stress of 1 dyn cm^{-2} over the full 24 h. These temporal changes in drug concentration are similar to the pharmacokinetic profiles observed in human studies of bolus injection [15]. In contrast, the D2 regimen mimics continuous infusion of gentamicin, with a fixed 3 mM level infused for 24 h with a constant shear stress of the same 1 dyn cm^{-2}. Importantly, both regimens have the same areas under the curve for drug exposure. We additionally tested two control regimens that do not include drug exposure: one with and one without cell exposure to fluid flow.

Immunofluorescence microscopy

After drug exposure, the polyester membranes with attached cells were quickly rinsed with PBS, and fixed in 3.7% paraformaldehyde for 15 min. The fixed cells were permeabilized in 0.5% Triton-X100 solution before incubation with antibodies directed against ZO-1 (monoclonal antibody conjugated to Alexa Fluor® 488, Invitrogen Corporation, Camarillo, CA), and occludin (Sigma-Aldrich; St. Louis, MO), overnight at 4 °C followed by incubation with fluorescently-labeled secondary antibodies for 1 h. The nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) or Hoechst. We used them for localization of apical proteins on the cells and detection of apoptotic cells since nuclei should be in the middle of the live or dead cells.

Fluorescein (Sigma Aldrich, St. Louis, MO) and rhodamine-DEX (molecular weight 10KDa) in media were perfused in the top channel of the device at a concentration of 0.025 mg ml^{-1} for measuring small and intermediate sized molecule transmembrane permeability, respectively. After 30 min, a portion of the fluorescent dyes permeates into the bottom channels; therefore, the top and bottom channels were separately imaged to quantify the fluorescein and rhodamine-DEX intensities (Nikon Eclipse TE300, Nikon Instruments, Inc., Melville, NY). Transmembrane permeability was estimated by the percentage (their fluorescence intensity at the only bottom channel exposed area, divided by their fluorescence intensity at the top and bottom channels-overlapped area). Z-sectioned fluorescent images were captured using Nikon A1Rsi Confocal Laser Scanning Microscope (Nikon Instruments, Inc., Melville, NY).

Toxicity assessment

Each gentamicin regimen was applied to the upper channel under fluidic cultures for 24 h. After gathering apical medium from the device outlet, kidney injury molecule-1 (KIM-1) from the cells was measured using a commercially available ELISA kit (Enzo Life Science, Inc., NY, USA) a Synergy NEO multi-mode reader (Biotek Instruments, Inc., Winooski, VT). The viability data were obtained by fluorescence microscopy imaging using live/dead stain (LIVE/DEAD

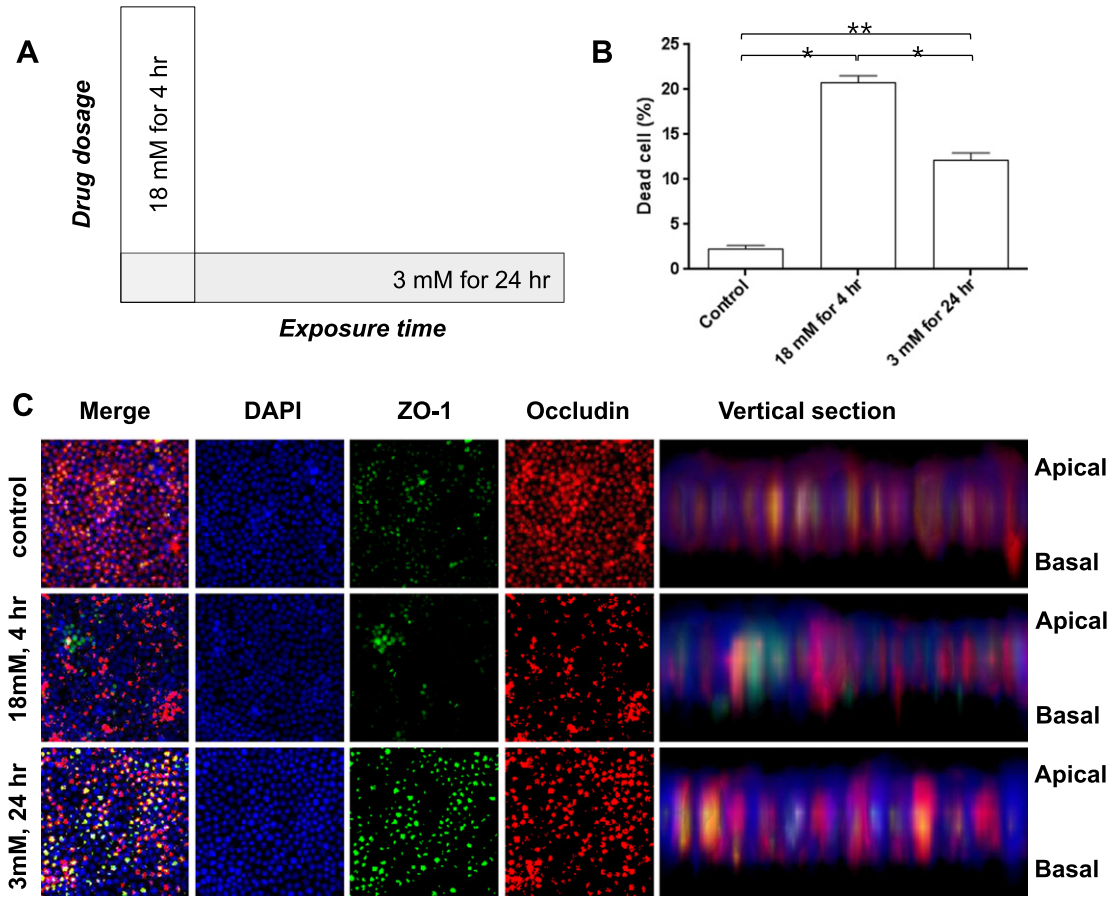

Figure 2. Static transwell culture data under two different gentamicin regimens. (A) Schematic design of the two different regimens. (B) Live/dead stains in each group. (C) Confocal stains of occludin and ZO-1 is distributed throughout the cells in each group suggesting lack of polarization. (×10 magnification). Short-term and high concentration regimen (18 mM for 4 h) of gentamicin is more cytotoxic and decreased occludin and ZO-1 expression, compared to the long-term low concentration regimen (3 mM for 24 h). $^{*}P < 0.05$, $^{**}P < 0.01$.

Viability/Cytotoxicity Kit, Molecular Probes, Inc., Eugene, OR). Data are presented relative to the total number of cells that remained adherent and were counted in the field of view. Intracellular fluorescence was measured using fluorescence microscopy.

Image and statistical analysis

All image analysis was performed using ImageJ and Fiji image software (National Institutes of Health, Bethesda, MD, USA) [16]. For the quantification of ZO-1 and occludin expression, we calculated the corrected total cell fluorescence using this formula:

Corrected total cell fluorescence = integrated density − (area of selected cell × mean fluorescence of background readings)

Z-sectioned fluorescent images of ZO-1 and occludin expression were acquired along the *x–y* axis of the cell monolayer, using a Nikon A1 Spectral Confocal System (Nikon Instruments Inc., Melville, NY). Images with the 20× objectives were taken and then the *x–z* optical sectioned images were reconstructed using FIJI. The maximum projection images for *x–z* optical sectioned images were used for quantification. For statistical analyzes, a one-way analysis of variance with Dunnett T3 multiple comparisons test was performed using SPSS and GraphPad InStat software (GraphPad Software Inc., San Diego, CA, USA). All data are presented as means ± standard error; differences between groups were considered statistically significant when $P < 0.05$.

Results

Ahead of kidney-on-a-chip experiments, we performed static cell culture experiments in Transwell™ plates to obtain preliminary results. Two different drug regimens that exposed kidney epithelial cells to the same total amount (same area under the curve) of gentamicin for 24 h, were applied (figure 2(A)). A short-term high concentration regimen (18 mM for 4 h) which mimics single bolus injection regimen was more cytotoxic than a long-term low concentration regimen (3 mM for 24 h) that mimics a continuous infusion regimen (figure 2(B)). In these experiments, we found that the long-term low concentration exposure led to less toxicity compared to a short high concentration infusion. Unfortunately, we also found that the cells were not well polarized in transwell

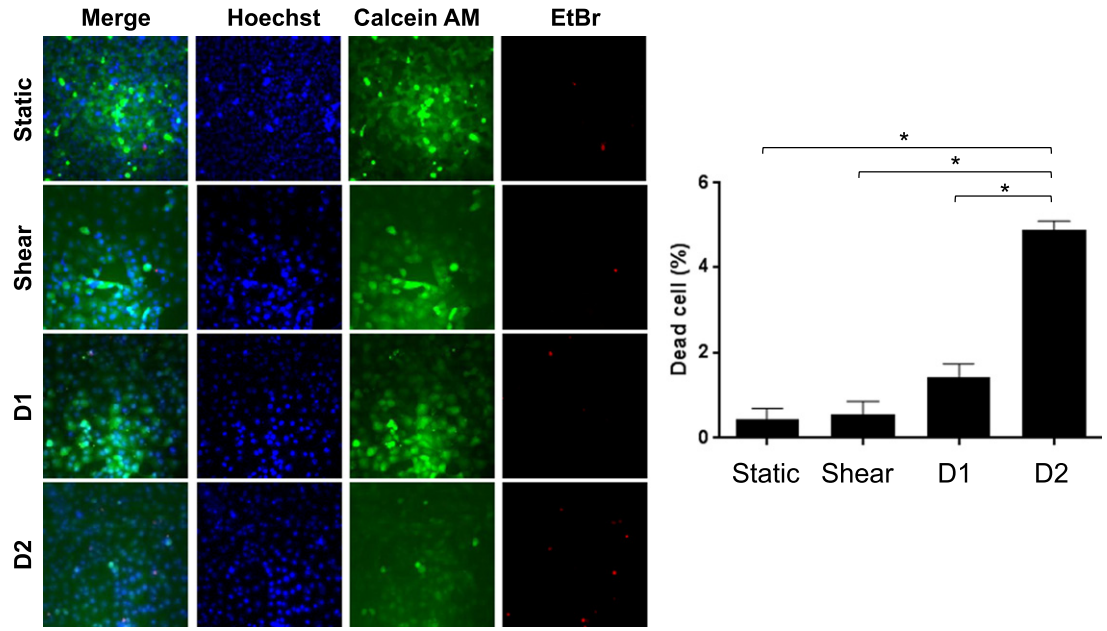

Figure 3. Live/dead stain of each group. The static and shear groups are measured before exposure to gentamicin, and D1 and D2 groups are measured 24 h after exposure to gentamicin. Three groups, except D2, show similar dead cell percentages, which are below 2%. In contrast, dead cells in D2 group are over 4%, which is significantly different, compared to the other groups. (×10 magnification). $^{*}P < 0.05$.

cultures as visualized by lack of apically-polarized staining of occludin and ZO-1 expression regardless of whether the cells were treated with drug or not (figure 2(C)). These promising results, with regards to study of pharmacokinetics *in vitro*, of observing differential response of cells to different drug exposure regimen, together with observations on sub-optimal cell polarization led us to pursue a kidney-on-a-chip experiment. We hypothesized that well-polarized cells, as may be obtained by microfluidic perfusion culture, may uptake and secrete molecules on their apical or basolateral membranes in a more physiologic fashion [17, 18] enhancing the observed pharmacokinetic differences.

Our kidney-on-a-chip construction follows methods from Jang *et al* where it is described that distal renal tubule cells exposed to ~1 dyn cm^{-2} enhance tight junctional protein expression and cell polarization [11]. We also confirmed increased immunofluorescence of tight junctional proteins such as occludin and ZO-1after a 6 h exposure to flows of 1 dyn cm^{-2}. Cross-sectional confocal images also showed increased prominence of apical expression of these markers in the cells. In addition, transmembrane permeability of both small molecules (fluorescein, 376 Da) and intermediate-sized molecules (rhodamine-DEX, 10K Da) was reduced by ~20% after the 6 h shear stress protocol. The kidney-on-a-chip allows convenient direct observation of kidney cell health and injury. Among four regimens tested, including two control cultures with and without fluid shear stress exposure, regimen D2 showed the highest cell death rate of 4% while the other conditions had less than 2% cell death at 24 h (figure 3).

KIM-1 is one of several sensitive kidney injury markers reported recently [19]. We chose KIM-1 as an additional readout of nephrotoxicity in our kidney-on-a-chip experiments; taking readings at 6 and 24 h after start of drug administration. The 6 h time point was chosen because until 8 h after drug administration, the D1 regimen had higher levels of gentamicin than the D2 regimen. At 6 h, KIM-1 levels from the D1 drug regimen were much higher than those from the D2. This is consistent with the notion that the higher levels of gentamicin at the early time points of the D1 regimen are more toxic and contributed to the release of KIM-1 from the cells. In contrast, at 24 h, KIM-1 levels in D1 were lower than those in D2 (figure 4). This is consistent with the 24 h live/dead stain data which also shows the D1 regimen to be overall less toxic (figure 3).

Occludin and zonula occludens (ZO) are major components of the tight junction and their disruption affect cell survival and function [20, 21]. In our experiments, occludin and ZO-1 were strongly expressed on the apical membrane after the physiologic levels of shear stress (figure 5). These increases in occludin and ZO-1 likely decrease transmembrane permeability of low and intermediate molecular weight compounds at 24 h (figure 6). Additionally, the D2 regimen led to a significant decrease in junctional protein expression compared to the D1 regimen at 24 h (figure 5).

Discussion

Chemical compounds are excreted through the kidney and/or biliary tract. Such clearance mechanisms together with method of drug administration have a

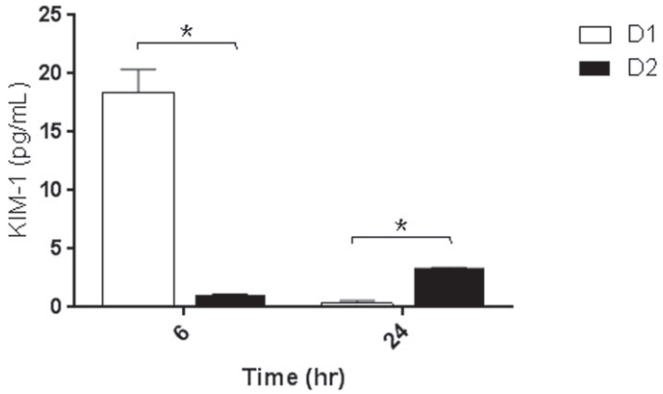

Figure 4. Kidney injury molecule (KIM)-1 ELISA. At 6 h, KIM-1 levels in the D1 regimen are higher than those in D2. After 24 h, KIM-1 levels in D1 are dramatically decreased and lower than those in D2. $^{*}P < 0.05$.

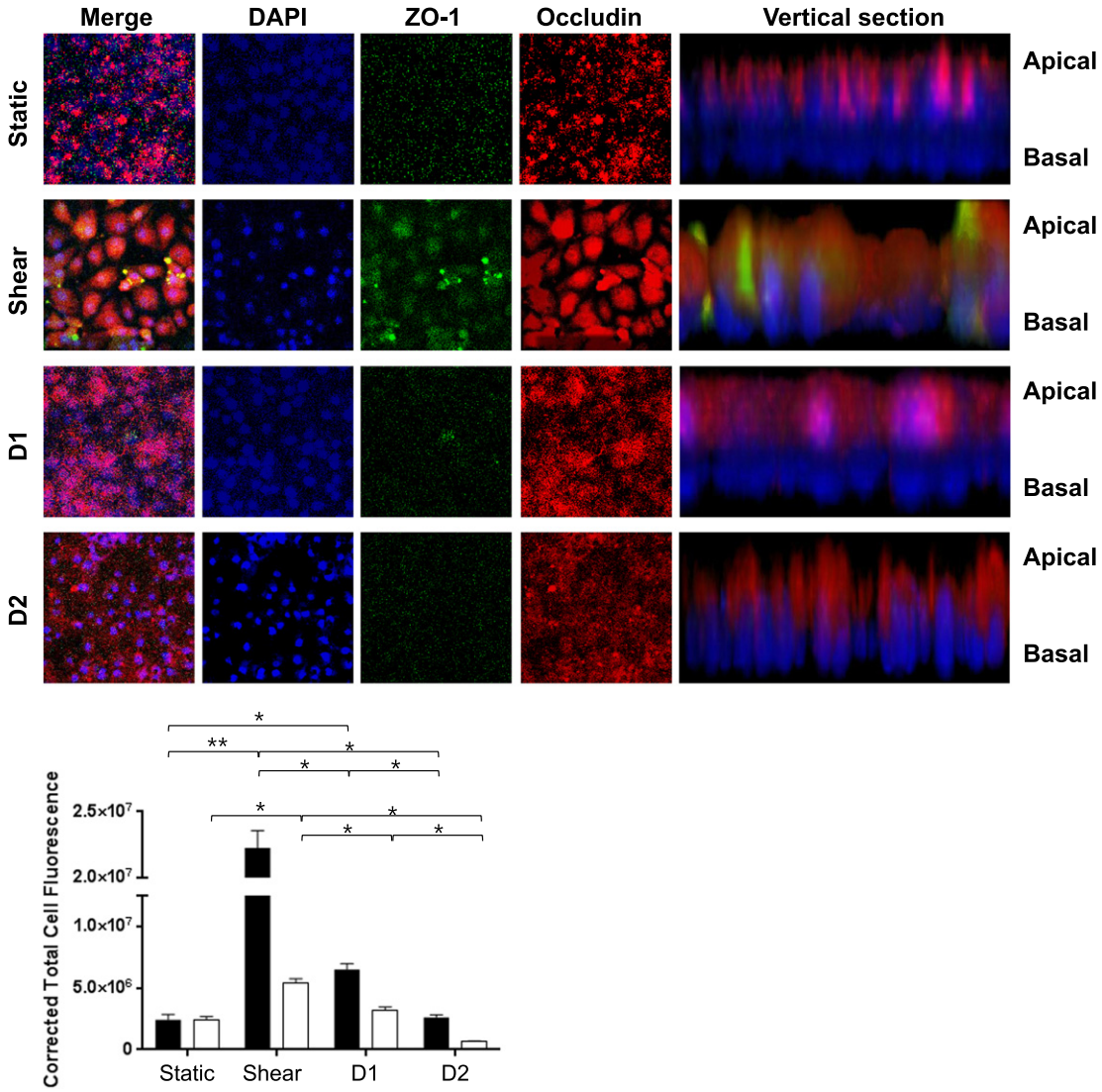

Figure 5. Junctional protein expression of each group. The static and shear groups are measured before exposure to gentamicin, and D1 and D2 groups are measured 24 h after exposure to gentamicin. All groups show improved polarization compared to Transwell™ cultures. Shear stress increased both occludin (black box) and ZO-1 (white box) expression. The two gentamicin regimens reduce expression of the two junctional proteins. However, the D2 regimen led to much more decrease in junctional proteins, compared to the D1 regimen. (×20 magnification). $P^{*} < 0.05$, $P^{**} < 0.01$.

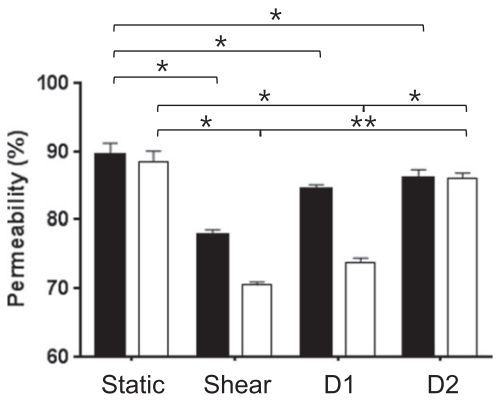

Figure 6. Permeability test using fluorescein (376 Da, black box) and rhodamine-dex (10K Da, white box). The static and shear groups are measured before exposure to gentamicin, and D1 and D2 groups are measured 24 h after exposure to gentamicin. Shear stress in absence of drug leads to decrease in the permeability of both small and middle-sized molecular weight molecules. In the D1 drug exposure regimen, both the small molecule permeability and middle-size molecule permeability is statistically the same as with the shear group that did not have drug exposure. In contrast, the D2 regimen causes a statistically-significant increase in the middle-sized molecule permeability, compared to the no-drug shear group, making the permeability of middle-sized molecules similar to levels in the static cultures. $^{*}P < 0.05$, $^{**}P < 0.01$.

profound effect on how drug concentrations within the body change over time. Importantly, drug toxicity is affected by such pharmacokinetic profiles. While a variety of organs-on-a-chip systems have been developed to recreate physiological tissue organization [22] or inter-organ interactions [23], there are few if any studies that have demonstrated that different pharmacokinetic profiles can alter toxicity. For example, cisplatin toxicity was tested a kidney-on-a-chips that exposes cells to physiologic shear stress to induce tissue-like cellular structures and function [4, 11]. This study, however, only tested exposure to constant level of cisplatin for 24 h [4]. It did not compare or consider pharmacokinetic profiles associated with the commonly used shorter infusions with hydration-assisted rapid renal clearance of cisplatin. Here we use external fluid pumping mechanisms to recreate two types of pharmacokinetic profiles corresponding to bolus injection or continuous infusion and test nephrotoxicity of gentamicin. This is an antibiotic for which there are disputes on whether bolus injection or continuous infusion is better with regards to reducing nephrotoxicity. Furthermore, because of differences in clearance rates between animal models and humans, animal studies cannot provide definitive conclusions as to which mode of administration is better. Here, we exposed a kidney-on-a-chip to physiological pharmacokinetic profiles for gentamicin based on published human half-life of gentamicin and compared nephrotoxicities (figure 1). Thus, we investigated whether physiological fluid shear stress-mediated cell polarization would be critical for studies of gentamicin where apical endocytosis will play a key role in nephrotoxicity [24].

Gentamicin was discovered in 1963 [25], but still remain an important antibiotic in selected indications. Among pharmacokinetic parameters, including area under the curve, maximum concentration, and exposure time, peak concentration is known to be a major determinant of efficacy of gentamicin [26]. In contrast, clinical response to gentamicin in some infections can be better with multiple dosing regimens than with once daily regimens [27]. Animal data using gentamicin favor single dose regimen due to less nephrotoxicity [28], However, it is possible that toxicity may be underestimated, since animal renal clearance is usually higher than human renal clearance [3]. Meta-analysis data shows multiple dosing regimens and once daily regimens of gentamicin are equivalent for efficacy and toxicity; however, those trials had small sample sizes and other methodological limitations [7, 8]. Because human clinical trials are expensive and time consuming, what the best drug regimen is for gentamicin administration is still controversial. Here, we use a simple, inexpensive, and readily imaged and evaluated kidney-on-a-chip to compare nephrotoxicity of two different gentamicin regimens that have the same overall drug exposure.

As shown in figure 3, regimen D2 showed higher cell death rate than other conditions by approximately 2%. Although the difference (2%) in this 24 h experiment is small, such differences may become more significant over the course of the normal 7–14 day administration protocol. Additionally, we believe this level of relatively high cell viability is more realistic and relevant to state of cells in actual patients, where sub-lethal cell injury may predominate over lethal cell injury. However, this necessitates use of sub-lethal kidney cell injury markers to compare drug exposure regimens. Interestingly, bolus injection mimicking regimen in kidney-on-a-chip showed better survival rates, while the same regimen in transwell plate showed worse survival rates. In addition, death rates in the Transwell^{TM} culture data were much higher than those in the organ-on-a-chip, regardless of drug regimens. This suggests that the kidney-on-a-chip may be supplying cells with an advantageous microenvironment for survival. In particular, static and macroscopic Transwell^{TM} cultures gave a more diffuse and less polarized occludin and ZO-1 pattern suggesting sub-optimal and non-physiologic expressions of junctional proteins that may compromise barrier function and also be associated with lower cell viability. Even cells in the static condition in the kidney-on-a-chip look more polarized than in transwell conditions. Recent results show that channel culture enhances barrier function of endothelial cells even without extensive shearing [29]. This also appears to be the case with our kidney-on-a-chip. This could be due the narrow channel widths altering shapes of cells, enhanced autocrine retention effect, or some minimal shear

effects of inevitable flow during media exchange. The ability to induce polarization of kidney cells under *in vitro* culture systems is an important benefit of kidneys-on-a-chip.

To further obtain insights into sub-lethal nephrotoxicity, we explored use of other biomarkers of kidney injury. In most clinical trials, the elevation of serum creatinine is used as the gold standard of nephrotoxicity. Serum creatinine is produced endogenously and is excreted through the kidney. If kidney function is decreased, serum creatinine levels are indirectly elevated. However, kidney cell injury does not always match the elevation of serum creatinine, which is a rather late injury marker [30]. This is the reason researchers still search for more sensitive biomarkers of kidney injury [31]. This biomarker limitation may also be contributing to the controversy regarding the best gentamicin regimen in terms of reducing nephrotoxicity. A more recent biomarker, KIM-1 is expressed at very low levels in normal kidney, but increases after kidney ischemia or nephrotoxin exposure [19, 32]. Animal models of gentamicin nephrotoxicity demonstrated that urinary KIM-1 is a more sensitive kidney injury marker than serum creatinine [19]. High levels in urinary KIM-1 are closely correlated with gentamicin-induced damage to renal tubules [33]. Furthermore, renal recovery after gentamicin exposure results in KIM-1 biomarker values returning to baseline levels [34].

Our KIM-1 data demonstrated that the cells in the D1 regimen, although exposed to more toxic conditions for the initial 6 h, recover in the following 16 h. In the D2 regimen, cells are exposed to less toxic condition for the initial 6 h, but KIM-1 levels kept increasing to ultimately become higher than in the D1 regimen at 24 h. It suggests that shear stress may be helpful for recovery of damaged cells, which is consistent with the previous report [4], but it may facilitate the absorption of gentamicin through apical endocytosis, which paradoxically leads to the accumulation of cell damages. The ability to use clinically relevant biomarkers for evaluation of the kidney-on-a-chip is important for translation of the on-chip experiments to clinical situations.

Advantage of toxicity testing using a kidney-on-a-chip is the ability to visualize or measure parameters that are difficult to quantify using *in vivo* models. In the kidney, expression of occludin and ZO-1 is critical for maintaining appropriate physiological concentration of ions, solutes and water [35]. Occludin expression is prominent in the border of the MDCK cells and transepithelial electrical resistance correlates with occludin abundance [36]. Shear stress on the cells increases occludin phosphorylation [37, 38], and increases ZO-1 expression [39]. However, the effect of shear stress on cell permeability is cell type-dependent [40]. Although occludin phosphorylation in vascular endothelial cells increase vascular permeability, occludin phosphorylation in MDCK cells is necessary for maintenance of barrier function [41]. ZO-1 controls solute permeability in tight junctions and stabilizes barrier function in MDCK cells [42].

Our experiments found that the physiologic levels of shear stress increased apical expressions of tight junctional proteins and decreased transmembrane permeability. Accumulated gentamicin is released into the cytosol where it acts on mitochondria and activates the mitochondrial pathway of apoptosis, induces oxidative stress, and reduces the ATP reserve [43, 44]. ATP depletion in MDCK cells are linked to decrease in occludin and ZO-1, leading to barrier dysfunction [41, 45]. Although both regimens of gentamicin attenuated the shear–stress effects on the cells, the single dose, D1 regimen of gentamicin administration appear to reduce drug accumulation and associated toxicities in kidney cells [15] explaining the improved barrier function of cells exposed to the D1 regimen over the D2 regimen cells.

How may these results relate to clinical practice? Gentamicin is known to show post-antibiotic effects, which is the persistent inhibition of bacterial growth after brief exposure to drugs. The anti-bacterial efficacy of gentamicin is, thus, highly dependent on peak drug concentration [27, 46]. Therefore, it is generally accepted and observed in publications that a once daily drug administration regimen is at least as effective as multiple daily dose regimens [8]. If the two dosing regimens are similar in terms of antibiotic efficacy, then a less toxic regimen should be favored. Our data using a kidney-on-a-chip suggest that a once daily dosing may be less toxic to cells compared to a continuous injection regimen.

There are several major limitations to the conclusions we draw from our experiments. While we mimicked human pharmacokinetics, we used MDCK cells rather than primary human kidney cells in the kidney-on-a-chip. We do note that MDCK cells are commonly used for initial evaluation of kidney injury and that the microfluidic culture under fluid perfusion and shear stress conditions induced a more physiological cell polarization and maintenance of barrier function that is superior to conventional *in vitro* cell cultures. Another limitation is that our chip only mimics the kidney tubules, which is the main known target of gentamicin nephrotoxicity, but not the glomerulus or vasculature.

On a more positive and forward-looking note, the reader is reminded that it is difficult to recreate human renal clearance rates in animal models, and that this critical effect on pharmacokinetics may lead to discrepancies between animal models and ultimate human results [2]. Thus, while our cells are still non-human, a major point of our work is recreation of human-like drug clearance profiles. Furthermore, while we focus on gentamicin, it is estimated that ~25% of the 100 most used drugs in intensive care units are potentially nephrotoxic [47], and that nephrotoxicity is responsible for 10%–20% of acute

renal failure cases [48]. These facts point to the need for new tools, such as described in this paper, that provide better understanding of the mechanisms of drug-induced nephrotoxicity.

Conclusions

The same drug, administered differently, can lead to significantly different outcomes. A major reason is associated with the different pharmacokinetic profiles that result from different administration regimen. Unfortunately, the effects of different drug administration regimen are difficult to test using animal models, at least in part, due to differences in drug clearance rates between humans and animals. Even if the pharmacokinetic profiles in humans are known, it can be difficult to reproduce such profiles in animal models. This work illustrates that different human-like pharmacokinetic profiles are readily recreated *in vitro* in a kidney-on-a-chip system. Importantly, we demonstrate, using physiologically and clinically-relevant sub-lethal cell injury markers, that the drug administration regimen can play a significant role in nephrotoxicity. More specifically, administration of gentamicin led to cell injury as measured by reductions in tight junctional protein, increase in permeability and expression of a clinically relevant kidney injury marker, KIM-1. The degree of cell injury, however, was dependent on the drug administration regimen, even when the overall dosage (area under the curve) was the same. A once-daily dosing regimen of gentamicin was favored over a continuous infusion regimen.

While this work focuses on gentamicin, the methods described should be readily applicable to a wide range of drug-induced nephrotoxicity studies. Even more broadly, the concept of efficiently comparing human-like pharmacokinetic profiles using organs-on-a-chip system opens the way for a wide range of drug efficacy and toxicity studies in many different *in vitro* organ systems.

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References

[1] Kola I and Landis J 2004 Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug. Discov.* **3** 711–5

[2] Andes D and Craig W A 2002 Animal model pharmacokinetics and pharmacodynamics: a critical review *Int. J. Antimicrob. Agents* **19** 261–8

[3] Paine S W, Menochet K, Denton R, McGinnity D F and Riley R J 2011 Prediction of human renal clearance from preclinical species for a diverse set of drugs that exhibit both active secretion and net reabsorption *Drug Metab. Dispos.* **39** 1008–13

[4] Jang K J, Mehr A P, Hamilton G A, McPartlin L A, Chung S, Suh K Y and Ingber D E 2013 Human kidney proximal tubule-on-a-chip for drug transport and nephrotoxicity assessment *Integr. Biol.* **5** 1119–29

[5] Giuliano R A, Verpooten G A and De Broe M E 1986 The effect of dosing strategy on kidney cortical accumulation of aminoglycosides in rats *Am. J. Kidney Dis.* **8** 297–303

[6] Aronoff G R, Pottratz S T, Brier M E, Walker N E, Fineberg N S, Glant M D and Luft F C 1983 Aminoglycoside accumulation kinetics in rat renal parenchyma *Antimicrob. Agents Chemother.* **23** 74–8

[7] Hatala R, Dinh T and Cook D J 1996 Once-daily aminoglycoside dosing in immunocompetent adults: a meta-analysis *Ann. Intern. Med.* **124** 717–25

[8] Mavros M N, Polyzos K A, Rafailidis P I and Falagas M E 2011 Once versus multiple daily dosing of aminoglycosides for patients with febrile neutropenia: a systematic review and meta-analysis *J. Antimicrob. Chemother.* **66** 251–9

[9] Bailey T C, Little J R, Littenberg B, Reichley R M and Dunagan W C 1997 A meta-analysis of extended-interval dosing versus multiple daily dosing of aminoglycosides *Clin. Infect. Dis.* **24** 786–95

[10] Bhatia S N and Ingber D E 2014 Microfluidic organs-on-chips *Nat. Biotechnol.* **32** 760–72

[11] Jang K J and Suh K Y 2010 A multi-layer microfluidic device for efficient culture and analysis of renal tubular cells *Lab Chip* **10** 36–42

[12] Giobbe G G, Michielin F, Luni C, Giulitti S, Martewicz S, Dupont S, Floreani A and Elvassore N 2015 Functional differentiation of human pluripotent stem cells on a chip *Nat. Methods* **12** 637–40

[13] Chueh B H, Huh D, Kyrtsos C R, Houssin T, Futai N and Takayama S 2007 Leakage-free bonding of porous membranes into layered microfluidic array systems *Anal. Chem.* **79** 3504–8

[14] Siber G R, Echeverria P, Smith A L, Paisley J W and Smith D H 1975 Pharmacokinetics of gentamicin in children and adults *J. Infect. Dis.* **132** 637–51

[15] Verpooten G A, Giuliano R A, Verbist L, Eestermans G and De Broe M E 1989 Once-daily dosing decreases renal accumulation of gentamicin and netilmicin *Clin. Pharmacol. Ther.* **45** 22–7

[16] Schneider C A, Rasband W S and Eliceiri K W 2012 NIH image to ImageJ: 25 years of image analysis *Nat. Methods* **9** 671–5

[17] Saito Y, Murata-Kamiya N, Hirayama T, Ohba Y and Hatakeyama M 2010 Conversion of *Helicobacter pylori* CagA from senescence inducer to oncogenic driver through polarity-dependent regulation of p21 *J. Exp. Med.* **207** 2157–74

[18] Finlay B B, Gumbiner B and Falkow S 1988 Penetration of *Salmonella* through a polarized Madin-Darby canine kidney epithelial cell monolayer *J. Cell. Biol.* **107** 221–30

[19] Zhou Y, Vaidya V S, Brown R P, Zhang J, Rosenzweig B A, Thompson K L, Miller T J, Bonventre J V and Goering P L 2008 Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney following acute exposure to gentamicin, mercury, and chromium *Toxicol. Sci.* **101** 159–70

[20] Feldman G J, Mullin J M and Ryan M P 2005 Occludin: structure, function and regulation *Adv. Drug. Deliv. Rev.* **57** 883–917

[21] Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S and Tsukita S 1994 Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions *J. Cell. Biol.* **127** 1617–26

[22] Huh D, Matthews B D, Mammoto A, Montoya-Zavala M, Hsin H Y and Ingber D E 2010 Reconstituting organ-level lung functions on a chip *Science* **328** 1662–8
[23] Sung J H and Shuler M L 2009 A micro cell culture analog (microCCA) with 3-D hydrogel culture of multiple cell lines to assess metabolism-dependent cytotoxicity of anti-cancer drugs *Lab Chip* **9** 1385–94
[24] Raghavan V, Rbaibi Y, Pastor-Soler N M, Carattino M D and Weisz O A 2014 Shear stress-dependent regulation of apical endocytosis in renal proximal tubule cells mediated by primary cilia *Proc. Natl Acad. Sci. USA* **111** 8506–11
[25] Weinstein M J, Luedemann G M, Oden E M, Wagman G H, Rosselet J P, Marquez J A, Coniglio C T, Charney W, Herzog H L and Black J 1963 Gentamicin, a new antibiotic complex from micromonospora *J. Med. Chem.* **6** 463–4
[26] Moore R D, Lietman P S and Smith C R 1987 Clinical response to aminoglycoside therapy: importance of the ratio of peak concentration to minimal inhibitory concentration *J. Infect. Dis.* **155** 93–9
[27] Craig W A 1998 Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men *Clin. Infect. Dis.* **26** 1–10
[28] Freeman C D, Nicolau D P, Belliveau P P and Nightingale C H 1997 Once-daily dosing of aminoglycosides: review and recommendations for clinical practice *J. Antimicrob. Chemother.* **39** 677–86
[29] Wang J D, Khafagy E S, Khanafer K, Takayama S and ElSayed M E 2016 Organization of endothelial cells, pericytes, and astrocytes into a 3D microfluidic *in vitro* model of the blood–brain Barrier *Mol. Pharm.* **13** 895–906
[30] Waikar S S, Betensky R A, Emerson S C and Bonventre J V 2012 Imperfect gold standards for kidney injury biomarker evaluation *J. Am. Soc. Nephrol.* **23** 13–21
[31] Murray P T *et al* 2014 Potential use of biomarkers in acute kidney injury: report and summary of recommendations from the 10th *Acute Dialysis Quality Initiative Consensus Conference Kidney Int.* **85** 513–21
[32] Ichimura T, Bonventre J V, Bailly V, Wei H, Hession C A, Cate R L and Sanicola M 1998 Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury *J. Biol. Chem.* **273** 4135–42
[33] Vaidya V S *et al* 2010 Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies *Nat. Biotechnol.* **28** 478–85
[34] Ozer J S *et al* 2010 A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function *Nat. Biotechnol.* **28** 486–94
[35] Hwang I, An B S, Yang H, Kang H S, Jung E M and Jeung E B 2013 Tissue-specific expression of occludin, zona occludens-1, and junction adhesion molecule A in the duodenum, ileum, colon, kidney, liver, lung, brain, and skeletal muscle of C57BL mice *J. Physiol. Pharmacol.* **64** 11–8
[36] Gonzalez-Mariscal L, Namorado M C, Martin D, Luna J, Alarcon L, Islas S, Valencia L, Muriel P, Ponce L and Reyes J L 2000 Tight junction proteins ZO-1, ZO-2, and occludin along isolated renal tubules *Kidney Int.* **57** 2386–402
[37] DeMaio L, Chang Y S, Gardner T W, Tarbell J M and Antonetti D A 2001 Shear stress regulates occludin content and phosphorylation *Am. J. Physiol. Heart Circ. Physiol.* **281** H105–13
[38] Walsh T G, Murphy R P, Fitzpatrick P, Rochfort K D, Guinan A F, Murphy A and Cummins P M 2011 Stabilization of brain microvascular endothelial barrier function by shear stress involves VE-cadherin signaling leading to modulation of pTyr-occludin levels *J. Cell. Physiol.* **226** 3053–63
[39] Nikmanesh M, Shi Z D and Tarbell J M 2012 Heparan sulfate proteoglycan mediates shear stress-induced endothelial gene expression in mouse embryonic stem cell-derived endothelial cells *Biotechnol. Bioeng.* **109** 583–94
[40] Cummins P M 2012 Occludin: one protein, many forms *Mol. Cell. Biol.* **32** 242–50
[41] Tsukamoto T and Nigam S K 1999 Role of tyrosine phosphorylation in the reassembly of occludin and other tight junction proteins *Am. J. Physiol.* **276** F737–50
[42] Van Itallie C M, Fanning A S, Bridges A and Anderson J M 2009 ZO-1 stabilizes the tight junction solute barrier through coupling to the perijunctional cytoskeleton *Mol. Biol. Cell.* **20** 3930–40
[43] Morales A I, Detaille D, Prieto M, Puente A, Briones E, Arevalo M, Leverve X, Lopez-Novoa J M and El-Mir M Y 2010 Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway *Kidney Int.* **77** 861–9
[44] Girton R A, Sundin D P and Rosenberg M E 2002 Clusterin protects renal tubular epithelial cells from gentamicin-mediated cytotoxicity *Am. J. Physiol. Renal. Physiol.* **282** F703–9
[45] Tsukamoto T and Nigam S K 1997 Tight junction proteins form large complexes and associate with the cytoskeleton in an ATP depletion model for reversible junction assembly *J. Biol. Chem.* **272** 16133–9
[46] Vogelman B and Craig W A 1986 Kinetics of antimicrobial activity *J. Pediatr.* **108** 835–40
[47] Taber S S and Mueller B A 2006 Drug-associated renal dysfunction *Crit. Care Clin.* **22** 357–74
[48] Brivet F G, Kleinknecht D J, Loirat P and Landais P J 1996 Acute renal failure in intensive care units—causes, outcome, and prognostic factors of hospital mortality; a prospective, multicenter study. French study group on acute renal failure *Crit. Care Med.* **24** 192–8