

MONITORING NEPHROTOXICITY ON A PROXIMAL TUBULE MICROPHYSIOLOGICAL SYSTEM BY TRANS-EPITHELIAL ELECTRICAL RESISTANCE MEASUREMENTS

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ABSTRACT

Drug-induced nephrotoxicity assessment relies on general cytotoxicity assays to make measurements at arbitrary time points. Therefore, a method to evaluate the dynamics of nephrotoxicity is required. We developed a proximal tubule microphysiological system (MPS) integrated with ITO electrodes to measure trans-epithelial electrical resistance (TEER). Time course of TEER was monitored when cisplatin was introduced into the top or bottom channels. We detected differences of cisplatin-induced nephrotoxicity depending on the drug concentration and cell polarity. These results indicate that TEER measurements in the proximal tubule MPS can evaluate drug-induced nephrotoxicity in real time.

KEYWORDS

TEER, renal proximal tubule epithelial cell (RPTEC), nephrotoxicity, MPS, cisplatin, cell polarity

INTRODUCTION

Drug-induced nephrotoxicity is one of the most common adverse events reported during the drug development stages that can be a dose limiting factor for chemotherapeutic drugs, e.g. cisplatin [1]. While it accounts for 2% of drug development failures in pre-clinical tests, it reaches to 19% of failures in clinical test [2][3]. The failure in drug development is mainly occurred by the reliance of non-clinical tests on animal models, since human physiology is profoundly different from the animal models [4].

To overcome this issue, human-derived cell-based tests have been widely recommended for the nephrotoxicity assessment. MPS containing cell cultures have been widely utilized to mimic the in-vivo environment physically and chemically by using microfluidic device technology [5]. The proximal tubule, which is a segment of the kidney, is of particular interest for studies on nephrotoxicity because active clearance, reabsorption and local interstitial accumulation of drugs occur primarily at this segment [6]. Therefore, development of a proximal tubule MPS model is required for drug development.

Nephrotoxicity assessment in non-clinical tests usually relies on general cytotoxicity assays such as cell death, the degree of cytoskeletal defects or mitochondrial function. These cytotoxicity assays can assess only advanced stages of kidney injury elicited by high doses of the drug and therefore might fail to detect subtle nephrotoxicity that could still lead to acute or chronic kidney injury in vivo [7]. In addition, the assays are end-point measurements which perform a single measurement at an arbitrary time point, resulting in limited kinetic and prognostic information.

Electrical measurements for cells are label-free and non-invasive methods, therefore they can assess complex physiological states in real time [8]. In MPS research, TEER measurement is a widely accepted technique to confirm the barrier integrity and the permeability of the tight junctions. The electrical resistance is usually measured across the cellular monolayer [9]. Abnormalities in early stages of nephrotoxicity alter ion channel activity, cytoplasm conductivity and resistance.

This study suggests that TEER measurements can be applied for the real-time assessment of the drug-induced nephrotoxicity. Cisplatin with well-known drug properties was used as the drug [10]. Cisplatin concentrations with the proximal tubule exceed those in blood suggesting an active accumulation of drug. The accumulation of cisplatin occurs more from the basolateral side than from the apical. Copper transporter 1 (Ctr 1) and organic cation transporter 2 (OCT2) are highly expressed in the basolateral membrane of the proximal tubule epithelial cell. The transporters dominantly transports cisplatin. Thus, the stronger toxic effect is observed for cisplatin when it was applied at the high concentration [11] or on the basolateral side [12]. We established the proximal tubule MPS which has the top and bottom channels and ITO electrodes to measure TEER. TEER values were measured when cisplatin was introduced into the top or bottom channels.

EXPERIMENTAL METHODS

Device fabrication

Our MPS is a five-layer structure consisting of two glass substrates with ITO electrodes, two polydimethylsiloxane (PDMS) sheets, and a porous PET membrane (Figure 1a, b). In addition, reservoirs for storing the culture medium are attached on the top glass substrate. Two independent ITO electrodes were patterned on the top and bottom glass substrates. The top glass substrate and the top PDMS sheet were provided with holes for Inlet and Outlet. Two PDMS sheets of 1-mm thickness have top and bottom microfluidic channels. Channels with height of 1 mm, width of 1 mm, and length of 15 mm were fabricated by laser machining. The porous membrane whose pore size was 3 μ m laser cut to 18 mm in length and 10 mm in width to cover the straight part of the channel (PET3047100, STERLITECH). Each layer was bonded with PDMS prepolymer.

Cell culture

The human renal proximal tubule epithelial cells were immortalized with pLXSN-hTERT retroviral transfection (RPTEC/TERT1, ATCC). In the case of the cell culture in a T-25 flask (TPP), they were passaged in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco) supplemented with hTERT Immortalized RPTEC Growth Kit (ACS-4007, ATCC) and 2 μ L/mL G-418 (04727878001, Roche). To culture cells

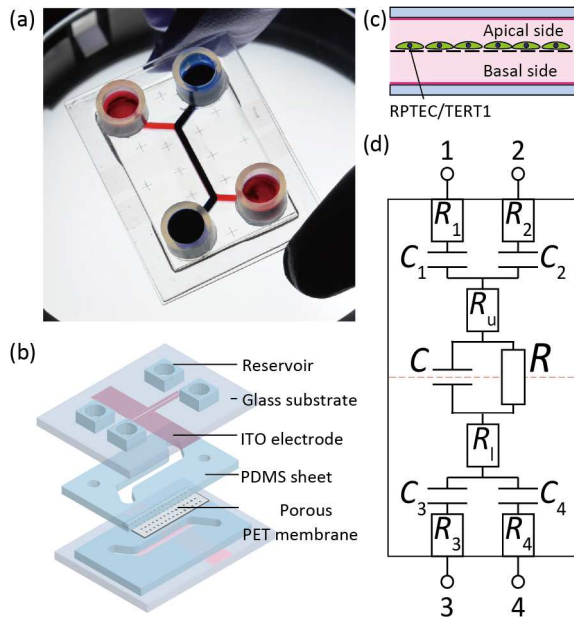


Figure 1: Design of our microfluidic device. (a) Photograph of the assembled device, dimensions are 30×40 mm. (b) Device components. (c) Schematic illustration of a cross section of the microfluidic device. (d) An equivalent electrical circuit of the device with four electrodes.

into the devices, Renal Epithelial Cell Growth Basal Medium (REBM, CC-3191, Lonza) supplemented with the Renal Epithelial Cell SingleQuots Kit (CC-4127, Lonza) were used. Gentamicin and amphotericin, were replaced with 100 U mL^{-1} penicillin and $100 \mu\text{g mL}^{-1}$ streptomycin (Gibco).

Trypsinization was performed with trypsin (204-16935, Lonza) containing 0.05% EDTA concentration. RPTEC suspension was prepared at a concentration of $3 \times 10^6 \text{ mL}^{-1}$.

The channels were filled with FNC Coating Mix (0407, Funakoshi) for the top channel and REGM for the bottom channel, incubated for 1 minute. The suspension was introduced into the top channel (Figure 1c). Cell adhesion was confirmed after incubating for 1 h. Top and bottom channels were then filled with REGM. REGM was refreshed every two days by flushing with a pipette.

TEER measurement

TEER was measured in the microfluidic device, where RPTECs were cultured in the upper channel under static condition. Impedance of the RPTEC layer was measured using an LCR meter (ZM2731, NF). The TEER measurements were conducted using a four-probe impedance measurement (Figure 1d) by applying alternating voltage of $20 \text{ mV}_{\text{p-p}}$ over the frequency range 100 Hz to 1000 Hz at 10 points during the period [13]. Impedance was measured using electrodes 1-2, 3-4, 1-4, and 3-2. The TEER values were calculated to offset the effect of the electric double layers by the impedance results. Before seeding the RPTECs into the top channel in the microfluidic device, impedance was measured in both channels filled with only REGM without RPTECs to measure the resistance of the medium and device itself. In this study, the TEER value was defined as the value

obtained by subtracting the resistance value of the device from the measured resistance value and normalized by the culture area A. The TEER measurement was performed in an incubator.

Nephrotoxicity assessment

Cisplatin was introduced into the top or bottom channels after confirmation of the maturation of RPTEC tissue, and the TEER of the cultures was monitored. 1 mM cisplatin dissolves in Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS, 14040133, Gibco) was diluted with REGM to the 10 or 100 μM concentration. The medium in the top channel or bottom channel was replaced with 200 μL of 10 or 100 μM cisplatin in REGM, while the other channel was filled with 200 μL REGM.

Immunocytochemistry

RPTECs were fixed in the device by immersing them into a 4% paraformaldehyde solution (43368, PFA, ALF) for 15 minutes at room temperature. Samples were washed four times in PBS (20012027, Gibco), permeabilized in PBS containing 0.1% Triton-X (T8787-50ML, Sigma) for 15 minutes, washed four times in PBS, and blocked for 60 minutes in PBS containing 10% donkey serum (S30-100ML, Merck Millipore) for 90 minutes, before being incubated overnight at 4°C with a mouse primary antibody against ZO-1 (33-9100, Thermo Fisher Scientific). After the sample was rinsed six times in PBS, Donkey anti-mouse IgG conjugated with AlexaFluor 488 (A-21202, Thermo Fisher Scientific) was incubated in the devices for 2 hours at room temperature with a 1:2000 dilution of DAPI. After rinsing six times with PBS, RPTECs were mounted on a coverslip using anti-fade solution (S36937, Invitrogen) as a mounting medium. Microscopy was performed by using an Olympus laser confocal microscope (FV3000, Olympus).

LDH assay

Measurement of lactate dehydrogenase activity was performed on 96 well plates according to the protocol of LDH assay kit (347-91751, Dojindo).

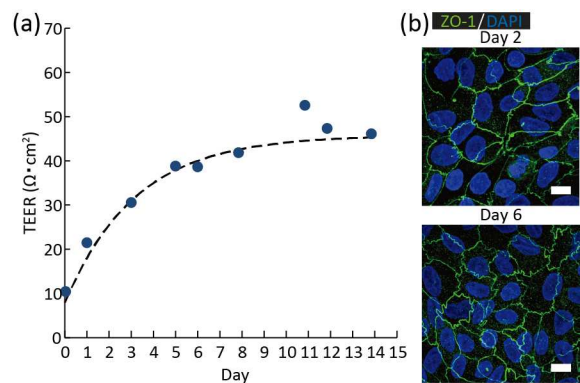


Figure 2: Time course of maturation of RPTEC. (a) TEER was measured from day 0 to day 14. A curve was fit to the data using an exponential formula in the form of $R(t) = 48 - 45 \times e^{-0.48t}$, where R is resistance, t is the time. (b) Fluorescent image of RPTEC tissue monolayers stained with ZO-1 (green) and DAPI (blue) on day 2 and 6. The complexity of the ZO-1 structure increased as the days passed. Scale bar = 10 μm .

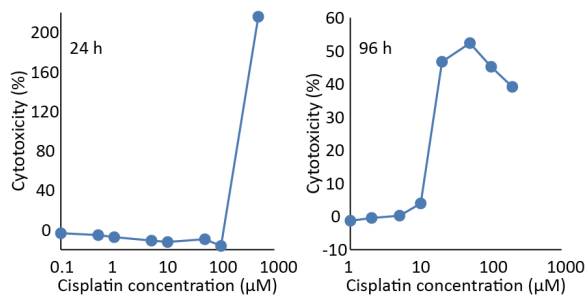


Figure 3: Results of LDH assay. Cisplatin toxicity was assessed in RPTECs after 24 h and 96 h at different cisplatin concentration, $n = 1$.

RESULTS AND DISCUSSION

The TEER values of the RPTEC monolayer gradually increased during the first several days and reached a plateau of about $48 \Omega \cdot \text{cm}^2$ after 9 days when the tolerance was set to $\pm 5\%$ (Figure 2a). The time constant was determined to be 2.1 day from the fitted curve. The complexity of the ZO-1 structure increased as the days passed (Figure 2b). These results show that the RPTEC epithelial monolayer reaches confluency and tissue matures in MPS after 9 days.

In the case of $100 \mu\text{M}$, cytotoxicity was not detected after 24 h, but detected at 96 h. In contrast, in the case of $10 \mu\text{M}$, cytotoxicity was not detected even after 96 h according to the LDH assay results (Figure 3). In the introduction of $10 \mu\text{M}$ cisplatin into the top and bottom channels, the TEER values at 120 h were higher than the initial values measured before the introduction of cisplatin. (Green dashed line in Figure 4a, Figure 4b). In contrast, the introduction of $100 \mu\text{M}$ into the top channel did not change the TEER values at first and, but after 24 h, caused a gradual drop over 96 h (blue line in Figure 5a). In the introduction of $100 \mu\text{M}$ into the bottom channel, the TEER values suddenly increased just after solution was introduced, but caused a gradual drop over 96 h (red line in Figure 5a). In the case of the bottom channel, the TEER values rapidly dropped and decreased by 52% at 24 h. In contrast, the TEER values maintained about 110% of its initial value at 24 h (green dashed line in Figure 5a, Figure 5b). Thus, the TEER values dropped sharply when the solution was introduced from the bottom channel as compared with the top channel.

In the introduction of $10 \mu\text{M}$ into the apical side, the

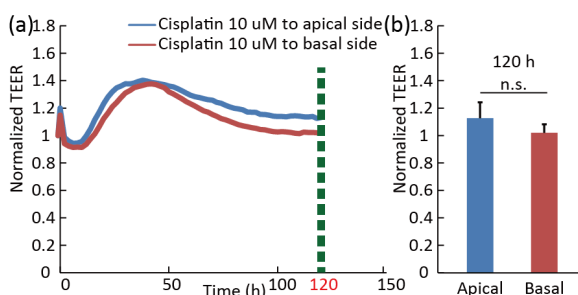


Figure 4: TEER recorded during introduction of cisplatin at the concentration of $10 \mu\text{M}$ to the apical side and to the basal side of RPTECs monolayer. (a) Time course. (b) TEER values at 120 h. The value is normalized by the initial value before the introduction of cisplatin. Error bar = S.D., $n = 3$ devices. n.s., t -test.

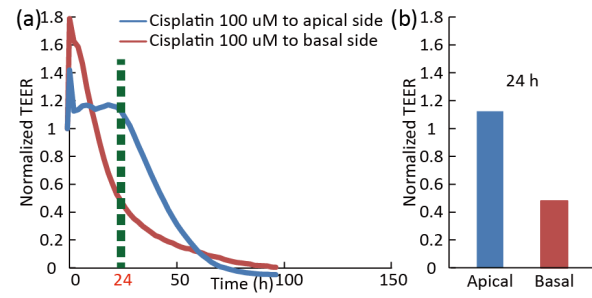


Figure 5: TEER recorded during introduction of cisplatin at a concentration of $100 \mu\text{M}$ to the apical and basal sides of RPTECs monolayers independently. (a) Time course. (b) TEER values at 24 h. The value is normalized by the initial value before the introduction of cisplatin, $n = 1$ device.

TEER values at 96 h was higher than the initial value. In the introduction of $100 \mu\text{M}$ into the apical side, the TEER value were higher than the initial value at 24 h, but were lower than the initial value at 96 h. These results correspond to the LDH assay results and indicate that cytotoxicity is not manifested when the TEER values are higher than the initial value. In addition, the results of Figure 5 show apical/basal susceptibility to $100 \mu\text{M}$ cisplatin monitored. The cytotoxicity was detected by the TEER measurements faster when the introduction into the basal side. Cisplatin uptake is dominantly mediated by Ctr 1 and OCT2 which are expressed in the basolateral membrane of the proximal tubule epithelial cells. Thus, the apical/basal susceptibility reflected that cisplatin introduced from the basal side was more accumulated in the cells to evoke the cytotoxicity. This MPS integrated with TEER monitoring detected the nephrotoxicity depending on the concentrations and the cell polarity. The observed differences matched the drug properties of cisplatin. This system contributes to the precise prediction of nephrotoxicity on different drugs in vivo.

CONCLUSION

A proximal tubule MPS with integrated ITO electrodes to measure TEER was developed. We demonstrated real-time monitoring of drug-induced nephrotoxicity by TEER in the MPS. The TEER measurements highlighted the differences of cisplatin-induced nephrotoxicity depending on the drug concentration. Moreover, apical/basal susceptibility to the nephrotoxicity was monitored as pharmacological actions. The proposed MPS displays a great potential in high-throughput drug screening systems and contributes to the precise prediction of nephrotoxicity on different drugs in vivo.

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REFERENCES

- [1] M. J. Wilmer, C. P. Ng, H. L. Lanz, P. Vulto, L. Suter-Dick, R. Masereeuw, “Kidney-on-a-Chip Technology for Drug-Induced Nephrotoxicity Screening”, *Trends Biotechnol.*, vol. 34, No. 2, pp. 156–170, 2016.
- [2] C. A. Naughton, “Drug-induced nephrotoxicity”, *Am. Fam. Physician.*, Vol. 78, No. 6, pp. 743–750, 2008.
- [3] A. E. Schultze, D. B. Walker, J. R. Turk, J. M. Tarrant, M. B. Brooks, S. D. Pettit, “Current Practices in Preclinical Drug Development: Gaps in Hemostasis Testing to Assess Risk of Thromboembolic Injury”, *Toxicol. Pathol.*, Vol. 41, No. 3, pp. 445–453, 2013.
- [4] T. Saio, C. Kurihara, “On extrapolation of animal experimentations to humans: An overview”, *Clin Eval*, Vol. 38, No.2, pp. 385–392, 2000.
- [5] H. Kimura, Y. Sakai, T. Fujii, “Organ/body-on-a-chip based on microfluidic technology for drug discovery”, *Drug Metab. Pharmacokinet.*, Vol. 33, No. 1, pp. 43–48, 2018.
- [6] M. A. Perazella, “Renal vulnerability to drug toxicity”, *Clin. J. Am. Soc. Nephrol.*, Vol. 4, No. 7, pp. 1275–1283, 2009.
- [7] J. Y. C. Soo, J. Jansen, R. Masereeuw, M. H. Little, “Advances in predictive in vitro models of drug-induced nephrotoxicity”, *Nat. Rev. Nephrol.*, Vol. 14, No. 6, pp. 378–393, 2018.
- [8] Y. Xu, X. Xie, Y. Duan, L. Wang, Z. Cheng, J. Cheng, “A review of impedance measurements of whole cells”, *Biosens. Bioelectron.*, Vol. 77, pp. 824–836, 2016.
- [9] B. Srinivasan, A. R. Kolli, M. B. Esch, H. E. Abaci, M. L. Shuler, J. J. Hickman, “TEER Measurement Techniques for In Vitro Barrier Model Systems”, *J. Lab. Autom.*, Vol. 20, No. 2, pp. 107–126, 2015.
- [10] R. P., Miller, R. K. Tadagavadi, G. Ramesh, W. B. Reeves, “Mechanisms of Cisplatin nephrotoxicity”, *Toxins*, Vol. 2, No. 11, pp. 2490–2518, 2010.
- [11] P. A. Reece, I. Stafford, J. Russell, M. Khan, P. G. Gill, “Creatinine clearance as a predictor of ultrafilterable platinum disposition in cancer patients treated with cisplatin: Relationship between peak ultrafilterable platinum plasma levels and nephrotoxicity”, *J. Clin. Oncol.* Vol. 5, No.2, pp. 304–309, 1987.
- [12] T. Ludwig, C. Riethmüller, M. Gekle, G. Schwerdt, H. Oberleithner, “Nephrotoxicity of platinum complexes is related to basolateral organic cation transport”, *Kidney Int.*, Vol. 66, No. 1, pp. 196–202, 2004.
- [13] Y. Takata, R. Ueno, R. B. Sadeghian, K. Naganuma, K. Tsuji, R. Yokokawa, “Evaluation of trans epithelial electrical resistance of proximal tubule tissue by inclusion and removal of extracellular calcium ion”, in *Digest Tech. Papers MEMS 2021 Conference*, On line, January 25-29, 2021, pp. 415-418.

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