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Research Article

Using Mouse Kidney Organoid Model for Nephrotoxic Drug Screening

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Abstract

The kidneys often experience adverse effects and toxicity caused by exposure to foreign compounds, chemicals, and drugs. Early prediction of these influences is essential to facilitate the entry of new, safe drugs into the market. 3D organoids derived from mouse kidney tissue can be used to evaluate drug nephrotoxicity in a faster and more efficient manner compared to traditional methods. The establishment of a method to culture these kidney-like organs from mouse kidney tissue is an important step towards advancing our understanding of the mechanisms of nephrotoxicity. This study established mouse kidney organoid lines from mouse kidney tissue using an organoid culture system. The consistency of the organoids with the original kidney tissue was evaluated through histopathology, and the kidney-specific marker (NPHS1, PODXL, WT1, Calb1) expression levels were characterized using immunofluorescence (IF) and qPCR. The study also validated the use of the kidney organoids for evaluating nephrotoxicity by measuring ATP levels in response to nephrotoxic compounds (cisplatin, doxorubicin, and gemcitabine).This study reports the successful establishment of mouse kidney organoids within two weeks, with a maximum of seven passages. The expression of kidney-specific markers in the organoids was higher compared to the control group. The organoids also showed increased sensitivity to the drugs cisplatin and doxorubicin, with IC50 values 5 times lower than those of 2D cell lines. The mouse kidney organoids established in this study demonstrated the presence of kidney specific markers and were found to be an effective and biologically relevant model to study nephrotoxicity in vitro.

Keywords: mouse kidney, 3D organoids, drug screening, nephrotoxicity

Introduction

The kidney plays a crucial role in the metabolism and elimination of drugs and their toxic metabolites(1).

In recent times, drug-induced nephrotoxicity has become a major contributor to acute kidney injury due to the complexity of the disease and the use of multiple drugs(2, 3). Evaluating nephrotoxicity is a crucial aspect to consider in both pre-clinical research and clinical trials of new drugs.

The traditional method for evaluating drug nephrotoxicity relies on animal models, which measure changes in biomarkers such as urea nitrogen (BUN) and creatinine in the serum and urine of animals after long-term drug administration, and examine renal pathological morphology(4, 5). However, this method has limitations as the 2D renal cell line model does not accurately reflect drug toxicity in a physiological in vivo environment(6, 7). Modern drug toxicology has evolved to incorporate both in vivo and in vitro studies for improved screening efficiency of target compounds. A high-throughput in vitro nephrotoxicity evaluation model has the potential to enhance the screening process.

In 2009, the term "organoid" was formally proposed to refer to self-organizing 3D structures grown and differentiated from stem cells that mimic in vivo structures and the multilineage differentiation of original mammalian tissues. (8, 9) At the same time, organoids also have the characteristics of differentiation, proliferation, self-renewal, induced evolution, reproduction of tissue structure, and realization of physiological functions. (10, 11) The evaluation of drug nephrotoxicity based on 3D organoids can not only reduce the dosage of test substances, but also facilitate the early in-depth evaluation of the potential nephrotoxicity of compounds. It can quickly identify the specific sites of drug nephrotoxicity, and can complete the study of nephrotoxicity mechanism of target compounds in a short period of time, shorten the research period, and reduce the use of experimental animals. However, kidney organoids made from pluripotent stem cells have the potential to revolutionize how kidney development, disease, and injury are studied.(12) Current protocols are technically complex, suffer from poor reproducibility, and have high reagent costs that restrict scalability.(13)

At the same time, for drug nephrotoxicity, podocytes maintain the glomerular filtration barrier and are also the target of drug nephrotoxicity.(14) However, renal tubular epithelial cells express a wide range of transporters, many of which are unique to specific segments of renal tubules. The kidney also harbors a highly diverse population of endothelial cells and microvascular components. Endothelial cells in glomeruli and interrenal vessels are also sensitive to drug-induced injury.(15, 16) The mechanism of renal proximal nephron toxicity has been most extensively studied on renal proximal tubular epithelial cells (RPTECS).(17, 18) However, renal tubular epithelial cells express a wide range of transporters, many of which are unique to specific segments of the renal tubule. Therefore, the constructed organoids should have the presence of specific nephron components.

To overcome some of these issues, this study is the first time to construct kidney organoids by using mouse kidney tissue to improve the proliferation rate of kidney organoids, shorten the construction time and success rate of organoids. We further show that our protocol can robustly generate organoids of comparable quality from mouse kidney tissue without the need for organoid specific optimization. Taken together, the construction of a three-dimensional model of mouse kidney organoids with physiological functions similar to kidney can provide a model for the subsequent screening of nephrotoxic compounds.

Methods and Materials

Chemicals and reagents

Cisplatin(S1166), Gemcitabine(S1714) and Doxorubicin(S1208) were purchased from Selleck. MasterAim® mouse kidney organoid complete medium and tissue digestion solution were obtained from AimingMed Hangzhou. Anti- nephrin (NPHS1, ABT331) was purchased from sigma Aldrich. Corning Matrigel Matrix (356255) was obtained from Corning. Healthy KM male mice (8 weeks, weighing 43±2g) were purchased from Zhejiang Vital River Laboratory Animal Technology for use in this experiment.

Establishment of mouse kidney organoid cultures

Fresh mouse kidney tissue was cut into small pieces and transferred to a centrifuge tube. Digestion solution I was added and cut into small pieces of about 1 mm. All the tissue fragments were transferred to a 15 mL centrifuge tube, an appropriate amount of digestive solution I was added, and then shaken and digested in a 37℃ incubator. All the tissue fragments were transferred to a 15 mL centrifuge tube, and an appropriate amount of digestive solution I (MasterAim®) was added. After shaking and digestion, the tissue fragments were placed in an incubator at 37 °C on a shaker and centrifuged. Add digestive solution II(MasterAim®), continue digestion for 10 min, terminate digestion by adding DPBS, filter, the cells were resuspended, 1.5-2 times the volume of undiluted Matrigel was added, seeded into a 48-well plate, placed in a CO2 incubator at 37℃ for 5 min, and the gel drops were gently shaken and inverted carefully after no obvious flow was observed. After it was fully solidified, 500μL of medium (MasterAim®) rewarmed at room temperature was added and incubated at 37℃ in 5% CO2 for about 6-8 days for passage.

Quantitative -PCR

Total RNA was isolated from kidney, lung tissue, and kidney organoids using an RNA extraction kit. The extracted RNA was reverse-transcribed to cDAN using PrimeScript RT reagent kit containing gDNA Eraser (TaKaRa, RR047). The cDNA was amplified using PCR and subjected to Realtime PCR reaction (ABI7500). For each group, three replicate wells were prepared. The primer sequences used for Q-PCR were as follows:

NPSH1: GCCTGTGTCCGTGTCTGCTAAC (Forward) and AGGTTCAGTTCCTCCTCGTCTTCC (Reverse), Calb1: CGACGCTGACGGAAGTGGTTAC (Forward) and GATGAAGCCGCTGTGGTCAGTATC (Reverse), WT1: CAGCCTACCATCCGCAACCAAG (Forward) and CTCCCAGCAGCCATTCCCTTTAAG (Reverse), PODXL: GGCGGTGGCAGTGAAGAGAATC (Forward) and GCAGCAACAAGGAGCAGGAAGG (Reverse), Ki67: AGAGCCTTAGCAATAGCAACG (Forward) and GTCTCCCGCGATTCCTCTG (Reverse), VEGFR2: TTTGGCAAATACAACCCTTCAGA (Forward) and GCTCCAGTATCATTTCCAACCA (Reverse), GAPDH: GCATCTTCTTGTGCAGTGCC (Forward) and TACGGCCAAATCCGTTCACA (Reverse).

Histological study and immunofluorescence

Mouse kidney organoids were fixed in 4% paraformaldehyde followed by a standard staining protocol concerning dehydration, paraffin embedding, sectioning and H&E staining. For immunofluorescence staining, the samples were incubated with primary antibodies, including claudin-1 and nephrin NPHS1. Then, primary antibodies were detected by incubating with Alexa Fluor 488 and Alexa Fluor 568.

Drug tests assays

After the organoids were dissociated into single cells, the collected cells were resuspended in complete medium containing 5% Matrigel and dispensed into 384-well white ultra-low adhesion plates at a concentration of 40 µL/ well using a multi-channel motorized pipette. Three days post-seeding, media was removed and replaced by media containing six concentrations of cisplatin, gemcitabine, doxorubicin. After 72 h, 50 µL CellTiter-Glo reagent (G7570, Promega) was added and the plates were shaken for 30 min at room temperature to lyse the cells. The luminescence value was read on the multifunctional enzyme marker (Tecan). the maximum half-inhibitory concentration (IC50) was determined to test drug nephrotoxicity.

Statistical analysis

IC50 calculations were performed by nonlinear regression using Prism (GraphPad Prism 9), and other data were also analyzed using GraphPad Prism software and presented as the mean ± SD of at least three independent experiments. One-way analysis of variance was used for comparison between groups. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered as statistically significant.

Results

Establishment and Enrichment of mouse kidney Organoids

As mentioned earlier, the culture of mouse kidney organoids is significantly challenging with a high initial failure rate. Using 3D organoid culture technology, mouse kidney organoids were successfully established from the collected mouse kidney tissue samples, and we modified the protocol to achieve a success rate of more than 80% Figure 1A. Under optimized conditions, 3D organoids generate circles within a few days. As shown in Figure 1B, organoids have morphological characteristics that are quite different from those of conventional 2D cultures. To evaluate the organoid growth process, a sequential observational study was performed. The results showed that more polycystic organoids could be observed after 3 days of culture, but more tissue debris interfered with the growth of organoids. After two passages, it was observed that tissue fragments were significantly reduced and more solid spherical organoids grew. After passage, mouse kidney organoids grew more. As observed by microscope imaging, with the extension of culture time, the volume of mouse kidney organoids increased significantly, and the mouse kidney organoids were successfully constructed. We also recorded the recovery of mouse kidney organoids from frozen stocks and showed that more than 80% of frozen mouse kidney organoids had 72% cell survival when thawed to room temperature. All established organoids were confirmed to be subcultured at least for more than 3 months and stored in liquid nitrogen for use as a renal organoid bank on demand.

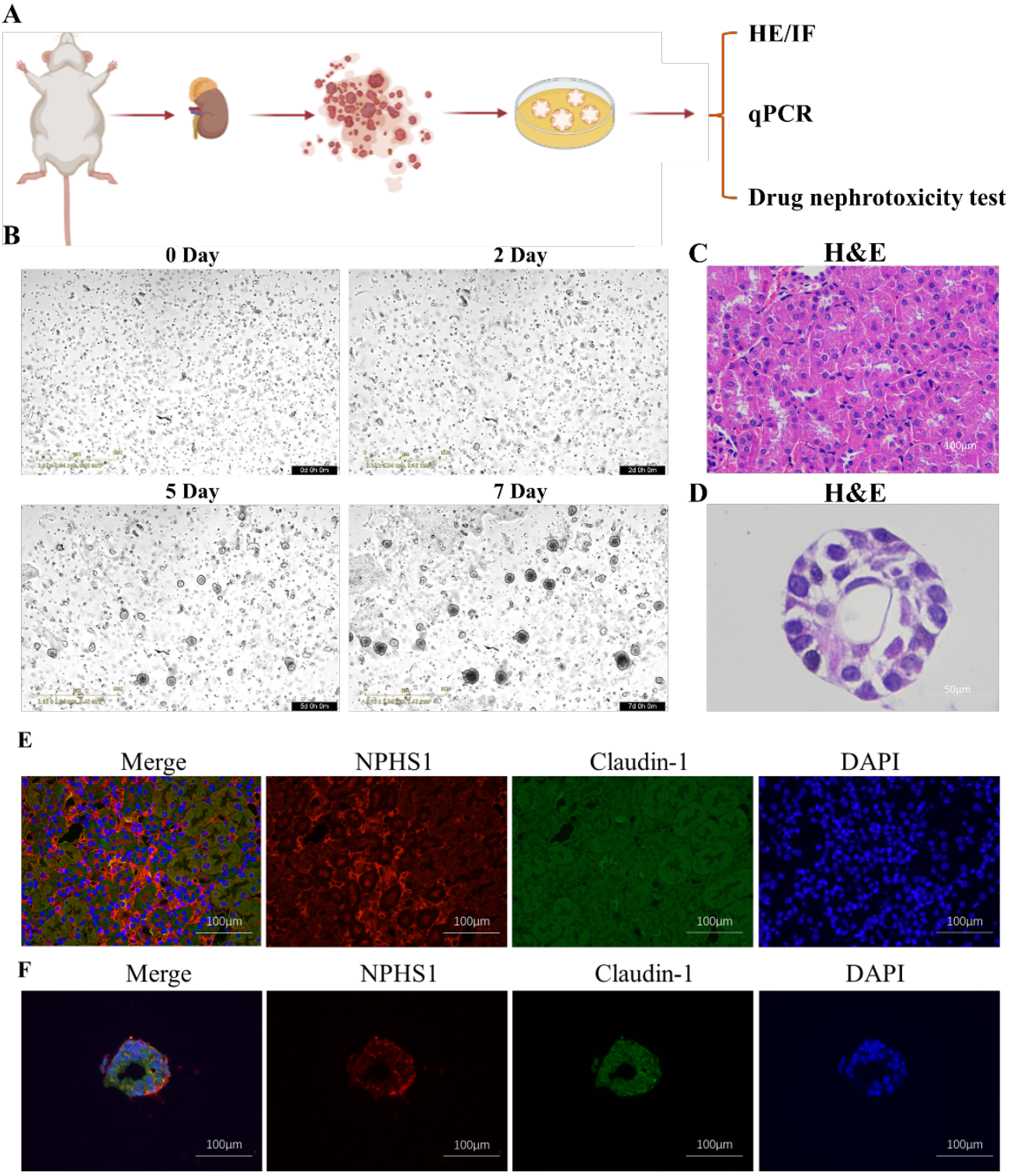

Figure 1. Representative images of mouse kidney tissue-derived organoids. (A). Culture procedure of mouse kidney-derived organoids; (B). Diagram of changes in mouse kidney organoid cultures after passage. Stereomicroscope; (C). HE staining of mouse kidney tissues; (D). HE staining of mouse kidney organoids. Immunofluorescence of organoids of NPHS1 and Claudin1 kidney markers. (E). Immunofluorescence of mouse kidney tissues; (F). Immunofluorescence of mouse kidney organoids.

Histological features of mouse kidney organoids

HE (Figure 1C-D) and IF analyses were performed on mouse kidney organoids. As shown in Figure 1E-F. The results showed that claudin-1 was expressed in all mouse kidney organoids. Markers of mouse mesonephros such as NPHS1 were also detected in organoids by IF staining. Thus, mouse organoids obtained by in vitro 3D culture can recapitulate the morphological and histological features of mouse kidney.

Expression of renal markers in mouse kidney organoids

To further characterize the mouse kidney organoids, mouse kidney tissue (positive control), mouse liver tissue (negative control), and cultured mouse kidney organoids were collected to detect the expression of kidney specific maker - NPHS1 (a specific marker of renal podocytes), wilm tumor gene1 (WT1), podocalyxin (PODXL, a specific marker of renal podocytes) and calbindin-1 (calb1) by qPCR.

NPHS1 is the gene responsible for encoding nephrin, and WT1 is highly expressed in early embryonic tissues, while its expression is only limited to hematopoietic tissues and kidney after embryonic development, and specifically expressed in podocytes in mature kidney. PODXL, also known as PODXL-like protein 1 which is synthesized by glomerular endothelial cells and podocytes and is anchored to the apical membrane of glomerular podocytes during podocyte maturation. Calb1 is localized in the distal convoluted tubules, connecting tubules, and cortical collecting ducts of the distal nephron. All of the above four makers were specifically expressed in the kidney, and the levels of kidney-specific maker in different tissues were detected by qPCR. The results (Figure 2A-D) showed that the levels of kidney-specific maker in mouse kidney organoids and renal tissues were significantly higher than those in the control group ($p<0.001$), indicating that the constructed mouse kidney organoids were consistent with the characteristics of the kidney.

Determination of the composition of mouse kidney organoids

The kidney organoid components were classified by detecting Ki67 and Vascular endothelial growth factor 2 (VEGFR2) markers. The qPCR results (Figure 2E-F) showed that the expression of VEGFR2 in mouse liver tissues was significantly higher than that in mouse kidney tissues and organoids ($p<0.001$), which may be caused by the rich blood vessels in liver tissue. As a maker used to label cells in the proliferation cycle, the qPCR results of Ki67 gene showed that the expression of mouse kidney organoids was higher than that of mouse kidney tissue and mouse liver tissue ($p<0.001$), indicating that the successfully constructed mouse kidney organoids had good activity and strong proliferation ability.

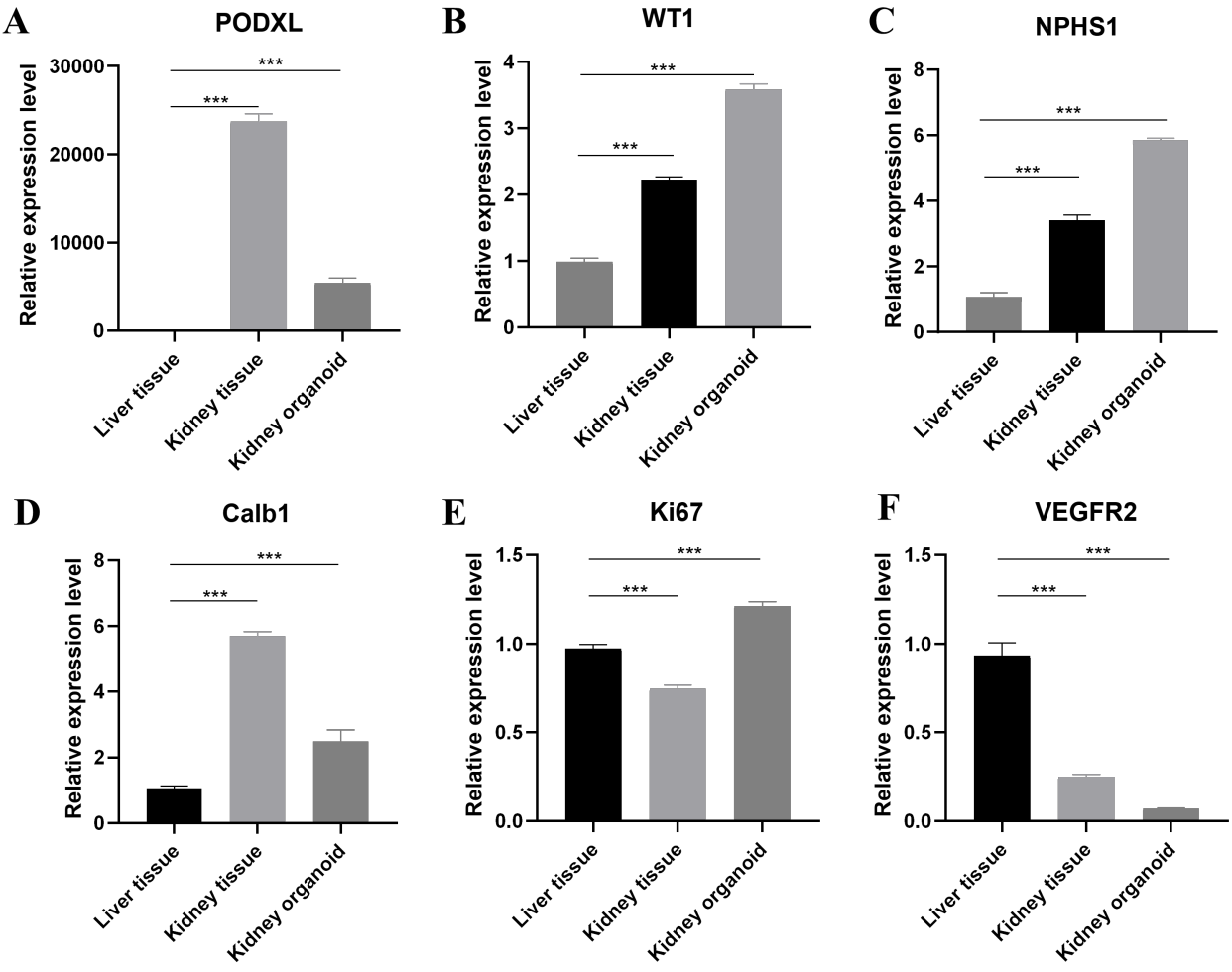

Figure 2. Expression of renal markers in mouse kidney organoids. The relative expression level of (A) PODXL, (B) WT1, (C) NPHS1, (D) Calb1, (E) Ki67, (F) VEGFR2. ***p < 0.001. PODXL: podocalyxin; WT1: wilm tumor gene1; NPHS1: nephrin; Calb1: calbindin 1; VEGFR2: Vascular endothelial growth factor 2.

Mouse kidney organoids for drug toxicity testing in vitro

Here, mouse kidney organoids were used to preliminarily assess for drug sensitivity testing in vitro. Three commonly nephrotoxic drugs were used for drug toxicity screening: cisplatin, gemcitabine and doxorubicin. Six different concentrations of each drug were used to test drug sensitivity. As shown in Figure 3B-D, the IC50 values of mouse kidney organoids for cisplatin was 0.04932mM, these for gemcitabine was 1.94mM; those for doxorubicin was 0.5367μM, respectively; As shown in Figure 3A, the results showed that after 3 days of treatment with the maximum drug concentration, compared with the control group, obvious apoptosis could be observed in the kidney organoids of the experimental group, indicating that the high drug concentration had stronger cytotoxicity. The IC50 curve was drawn by the viability of cells at different concentrations. The results showed that the drug toxicity of doxorubicin was significantly stronger than that of cisplatin and gemcitabine. Overall, the mouse kidney organoid lines showed drug sensitive responses in the drug susceptibility screening assays.

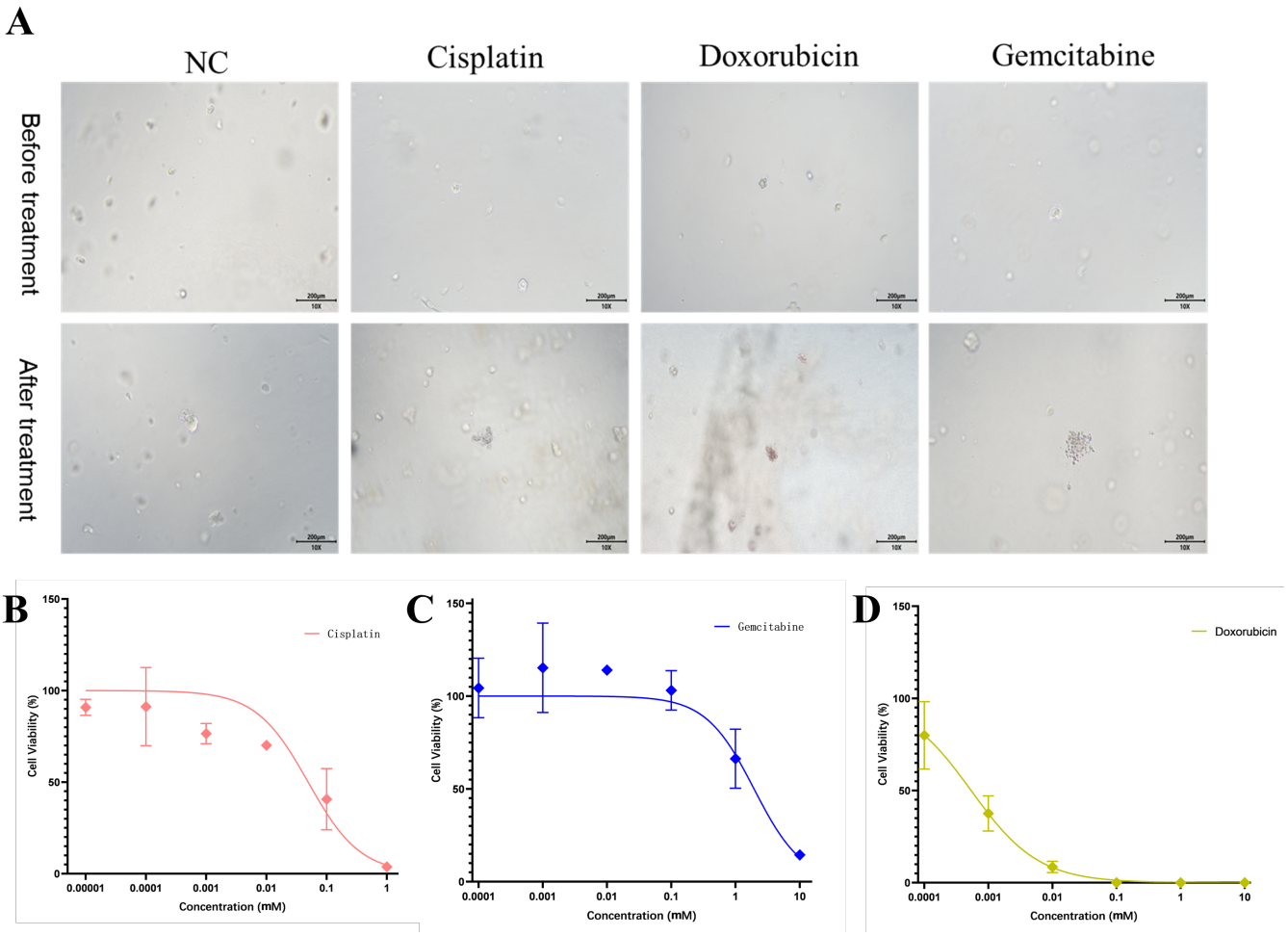

Figure 3. Mouse kidney organoids for drug toxicity testing. (A) Morphological changes of organoids before and after drug treatment. Dose-response curves of the (B) cisplatin, (C) gemcitabine and (D) doxorubicin assessed by CTG assay. Mouse kidney organoids were cultured with several concentrations of drug for 72h.

Discussion

Organoids have proven to be useful biomarker tools to guide and tailor therapy. (19, 20) For establishment, organoid lines that could be extended for at least five generations were considered successful. The organoid treatment response test was completed in less than four weeks, indicating that the model can generate treatment recommendations in a clinically meaningful time frame. Multiple approaches for differentiating pluripotent stem cells into kidney organoids have been reported and adopted. (21-24) However, the protocols are technically complex, suffer from poor reproducibility, and have high reagent costs that restrict scalability

In this study, we successfully established mouse-derived kidney organoids to overcome these problems. Mouse kidney organoids can still maintain good growth activity up to the seventh generation, but it should be noted that the primary samples have many tissue fragments, which can be removed by multiple passages. At the same time, we examined the effect of cryopreservation on mouse kidney organoids.

The results showed that the survival rate of mouse kidney organoids after cryopreservation for 2 weeks was better, and more organoids could be grown after 5 days of cryopreservation. However, the survival rate decreased after 3 months of cryopreservation, and only a small number of organoids grew after 2 weeks of culture, indicating that prolonged cryopreservation may have a greater impact on the resuscitation efficiency. Therefore, optimizing the culture system and other methods to improve the success rate of renal organoid resuscitation still requires further study.

NPHS1 and PODXL are both renal podocyte markers. NPHS1 is specifically expressed in the slit diaphragm of podocytes, and PODXL regulates foot process structure and function.(25, 26) Podocytes maintain the filtration barrier in the glomerulus and are also the target of drug-induced nephrotoxicity. (14) Certain drugs have a direct toxic effect on podocytes. For example, puromycin is taken up by podocytes through the plasma membrane monoamine transporter (PMAT; also known as ENT4, encoded by SLC29A4), in contrast to bisphosphonates that cause podocyte injury by destroying the cytoskeleton.(27) Once injured, the podocytes undergo a dedifferentiation process, destroying the glomerular filtration barrier and leading to nephrotic syndrome. The resulting proteinuria can cause secondary renal tubular damage.(28) Thus, podocytes are one of the most important markers of the kidney. Interestingly, HE staining and immunofluorescence results showed that the mouse kidney organoids obtained by 3D culture in vitro could reproduce the morphological and histological characteristics of the primary kidney. Simultaneously, the qpcr results showed that the expression of NPHS1 in mouse kidney tissues and mouse kidney organoids was higher than that in the control group. And the higher expression of NPHS1 in mouse kidney organoids may be due to the regulation of NPHS1 expression by Wilms tumor protein. This indicates that the mouse kidney organoids we constructed conform to the relevant characteristics of kidney.

To gain a deeper understanding of the composition of mouse kidney organoids, we conducted an analysis of Ki67 and VEGFR2 expression. The results showed that ki67 expression was the highest in mouse renal organoids. As a marker of cells in the proliferative cycle, higher expression of Ki67 indicates active growth rate of mouse renal organoids. This suggests that the established mouse kidney organoids have robust growth potential. However, it is unclear on the specific cell type compositions or which type of cells contributing the organoids' proliferation, which can be further assessed via single-cell multi-omics approaches.(29) Besides, functional assay for kidney organoid or further-differentiation in vitro to build more physiological in vitro kidney function models can be further studied.

Previous work demonstrated that animal and 2D-cell experiment provide modest contributions toward human healthcare. In this study, we utilized three widely adopted nephrotoxic drugs to explore the different response in organoid and 2D cell lines. Combined with the previous studies and relevant data, the IC50 values of cisplatin and doxorubicin in 2D cell lines were 2.0-2.7 mM and 1.9-2.3mM, respectively, which were higher than those of organoids, indicating that mouse kidney organoids were more sensitive to drugs.(30, 31) Interestingly, the IC50 values of doxorubicin and cisplatin were not significantly different in 2D cell lines, but were notably different in mouse kidney organoids, which is consistent with the clinical higher toxicity of doxorubicin than cisplatin. (32-34) The IC50 value of gemcitabine was found to be much higher in 3D organoids compared to 2D cell lines. This could be due to the fact that gemcitabine is mainly

excreted by the kidneys, but the main observed toxicities are myelosuppression, paresthesia, and severe rash. This highlights that 3D organoids may be more physiologically relevant in evaluating drug nephrotoxicity.

In our study, we were able to construct mouse kidney organoids that displayed specific markers representative of the kidney. This offers a cost-effective and practical alternative for in vitro studies of nephrotoxicity. The results of our study suggest that mouse kidney organoids can effectively mimic the functional characteristics of the primitive kidney and can therefore be used as a valuable tool in testing the nephrotoxicity of new drugs.

Conclusion

Organoids are commonly employed in evaluating drug-induced nephrotoxicity due to their ability to accurately reflect the molecular and morphological features of the source tissue. This research shows that mouse kidney organoids can be effectively generated from mouse kidney tissue and used to assess drug-induced nephrotoxicity.

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