

# A Three-Layer Microfluidic Kidney Chip for Drug Nephrotoxicity Test

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**Abstract:** Nowadays, in the preclinical and clinical studies of drugs, cell culture in petri dishes and animal experiments still can't adequately reflect the effects of new drugs on human kidneys, which highlights a great need to develop a model with high accuracy to recapitulate organ function in vitro. Here, a three-layer microfluidic kidney chip that can be used to mimic the structure and function of kidneys by integrating polydimethyl siloxy (PDMS) microfluidic channels and porous membranes. We also developed a supporting microfluidic culture platform for the long-term culture of kidney cells. On this basis, two types of cell lines (renal proximal tubular epithelial cells, RPTECs, and peritubular capillary endothelial cells, PCECs) and three types of drugs (cisplatin, DDP, gentamycin, GM, and cyclosporine A, CsA) were used for accurate assessment of drug-induced nephrotoxicity. Considered of the effects of time, we also tested two dosing regimens of GM. Cells were under the fluidic shear stress of continuous flow to mimic the pharmacokinetics of single injection and continuous infusion. Unlike in petri dishes, cells cultured in the microfluidic kidney chips had a better performance on both the cell growth and the drug nephrotoxicity evaluation. The microfluidic devices presented here may be useful to mimic the renal tubular system in vitro for evaluating drug nephrotoxicity during preclinical studies.

**Key words:** Drugs, nephrotoxicity, pharmacokinetics, vitro.

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## 1. Introduction

Safety is the most important factor in the development of new drugs. Pharmaceutical companies spend more than a few decades and a great deal of money in the process of research and development [1], however, nephrotoxicity results nearly one third of new drugs failures during preclinical and clinical developmental stages [2]. The current mainly evaluation of drug nephrotoxicity is cell-based model and animal experiments [3], [4], they still can't reliably predict effects on the human organs. So there is a desirable need to predict drug-induced nephrotoxicity, as well as to reduce costs and time in the clinical stage.

Addressing the problem, the rapid development of microfluidic 'organ-on-chip' technology may provide a suitable way to simulate the in-vivo environment [5]-[7]. Organ on chip is a biomimetic system that uses micromachining techniques to create the part which can simulate the primary function of human organs on

a microfluidic chip. In particular, this study focuses on the kidney. The kidney is an important excretory organ, and its main function is to clear the body's metabolites and wastes by generating urine and to retain moisture and other nutrients through reabsorption [8]. The main component of the kidney is the nephron [9]; it consists of the glomerulus, renal capsule, and renal tubule.

However, in the process of culturing cells in past, cell differentiation was not promoted due to the lack of normal microenvironment. Co-culture of planar renal proximal tubular epithelial cells and peritubular capillary endothelial cells does not reproduce the 3D structure of the microvasculature in vitro well, as well as the interactions of these two cells through blood flow [10]. Therefore, we extended the microfluidic organ chip method to reproduce some renal functions in vitro by designing a three-layer microfluidic kidney chip.

With the aspiration to reconstruct the in vivo environment of the kidney with co-culture of the RPTECs and PCECs, we introduced a three-layer microfluidic kidney chip in this research: the RPTECs were cultured on a porous membrane between the upper and middle layers, and cells were exposed to a fluidic medium at low shear stress similar to that in the human micro-environment. Between the middle and lower layers were PCECs, which cultured on another membrane. The middle layer was filled with medium to simulate the interstitial space. On this basis, a microfluidic culture platform was designed, we can complete the automated and integrated function of cell culture and analysis.

Kidney toxicity is one of the most frequent adverse events reported during drug development, and the proximal tubules play a central role in eliminating foreign objects and reducing toxicity [11], [12]. We compared the microfluidic kidney chip and traditional cell culture model for evaluation of drug-induced nephrotoxicity via cisplatin (DDP), gentamycin (GM), and cyclosporin A (CsA). By cell morphology and the nephrotoxic specificity index—NAG, we compared the effect of concentrations of drugs on cell. And cell inhibition rate was calculated when cultured on the microfluidic kidney chips.

Microfluidic techniques can generate various physiologic conditions to simulate the human microenvironments. And till now, researchers have made many achievements on kidneys, include kidney-on-a-chip, proximal tubule-on-a-chip [13], [14]. Although the existing microfluidic kidney chips have also been evaluated for toxicity of related drugs, studies on drug dosing regimens that conform to human pharmacokinetic characteristics are not yet mature. Pharmacokinetics can be very important in the initial dosage regimen of the drug. Therefore, in addition to considering the effect of concentration, time is also an important factor in drug toxicity testing. The three-layer microfluidic chip we constructed with RPTECs and PCECs that could be exposed to various pharmacokinetic profiles. By two different GM regimens: perfusing high to low concentrations of drug over time to mimic the single bolus injection; perfusing a constant concentration to mimic continuous drug infusion. The results demonstrated that the combination of shear stress and platform may be useful in drug screening and tissue engineering.

## **2. Materials and Methods**

### **2.1. Materials**

Materials for the chip were obtained from the following suppliers: polydimethyl siloxane (PDMS) (Dow Corning, Midland, MI, USA), polyester porous membrane (pore size; 0.4 mm, Whatman, Maidstone, UK). Materials for the cell culture: Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, phosphate-buffered saline (PBS) were obtained from (GIBCO BRL, Grand Island, NY); Collagenase B (Roche, Mannheim, Germany); dimethyl sulfoxide (DMSO) (Sigma, St.Louis, MO). DDP, GM and CsA were purchased from Sigma. Cell Counting Kit-8 was purchased from Dongren Chemical Technology (Shanghai) Co., Ltd. The Live/Dead reagents were obtained from Shanghai Enyi Biological Technology Co., Ltd. RPTECs and PCECs were purchased from Shanghai Saige Biological Co., Ltd.

## 2.2. Design of the Microfluidic Culture Platform

The automated culture device based on microfluidics are consisted of the microfluidic kidney chips and the microfluidic culture platform. The object of the culture platform is the cell system, so it is necessary to focus on the control of the environmental system to provide a suitable environment for cell growth. At the same time, the platform should provide an automated feed system to achieve the injection function.

The working principle of the entire platform is to connect the microfluidic kidney chip through the ports and control the peristaltic pump operation by the feed system. Here, the peristaltic pump system is composed of a stepper motor and a peristaltic pump head, and the speed of the peristaltic pump can be controlled by the computer through the Labview software. The temperature control system strictly controls the temperature of the platform which provides the most suitable environment for cell growth. As for heater, it is divided into two parts. First, the heater is attached to the chip to maintain the operating temperature in a preset state. Second, the culture liquid is heated to ensure the temperature of the entire liquid circulation. The temperature sensor detects the temperature value and then the temperature controller sends an instruction to control the heater to reach the desired temperature. The following Fig. 1 shows a complete schematic diagram of a microfluidic system-based culture platform.

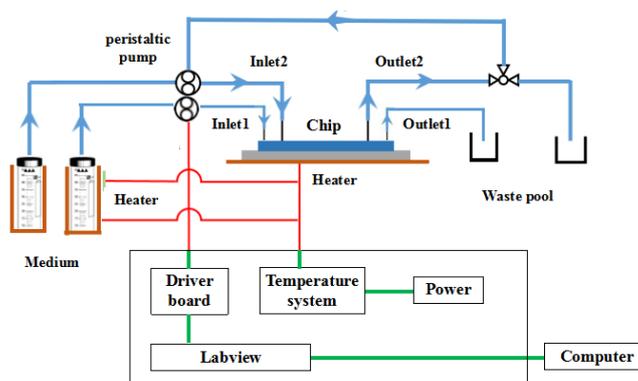


Fig. 1. The structure diagram of microfluidic system-based culture platform.

## 2.3. Chip Fabrication

To recapitulate the human renal tubular micro-environment, we designed a three-layer compartmentalized microfluidic kidney chip. The three-layer microfluidic kidney chip mainly consisted of the upper, middle, and lower PDMS layers, each two layers are separated by a polyester membrane. The initial templates of each microchannel and chamber were designed using AutoCAD 2016 and produced by laser-cutting poly (methyl methacrylate) (PMMA) to create a fluidic channel mold (width: 2 mm, height: 1 mm, cell culture diameter: 14 mm). The PDMS prepolymer containing the silicone elastomer and the curing agent [Sylgard 184 A and B (10:1 w/w)] was cast on the PMMA mold, and placed in the freeze dryer to mix well until the bubble disappears. To cure the PDMS prepolymer, the mixture was then poured onto the master and heated at  $T = 70^{\circ}\text{C}$  on a heated stage for about 2 hours. After curing, the PDMS was peeled off from the masters and cut into narrow strips, and holes were punched for inlets and outlets by a needle. As shown in Fig. 2(A), the chip with multiple interfaces enabled the culture of different cells and assay of drug-induced toxicity. The upper and bottom layers of the chip simulated the environment of the renal tubules and perivascular capillaries, and the middle layer provided interstitial area spaces between them. To provide a supporting area for cell culture, two polyester membranes (pore size:  $0.4\ \mu\text{m}$ , thickness:  $10\ \mu\text{m}$ ) were sandwiched between two layers, as shown in Fig. 2(B).

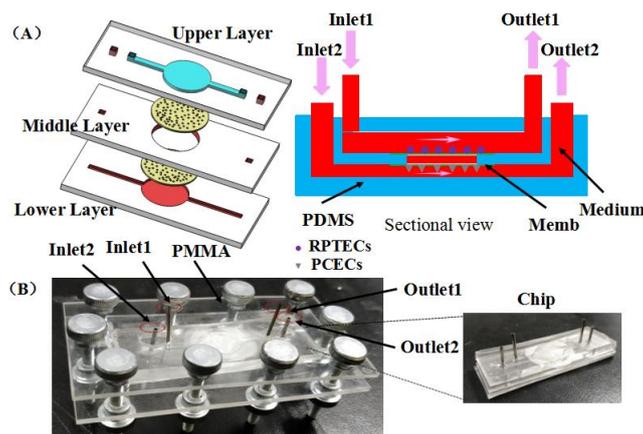


Fig. 2 Design of three-layer microfluidic kidney chip. (A) Schematic diagram of the chip and sectional view. The device is consisted by upper, middle and lower layers, and each two layers are separated by a porous membrane. (B)The real picture of the chip.

## 2.4. Culture Cells and Flow Experiment

Fig. 2 shows the structure of the chip. PCECs were loaded into the lower channel with cell density of  $5 \times 10^5$  cells/mL and cultured for 2 h. Next, as PCECs adhered on the membrane, the microfluidic kidney chip was placed upside down, and RPTECs with a cell density of  $5 \times 10^5$  cells were loaded into the upper channel. After cell culture for 2 h, inlets and outlets of the chip were respectively connected to centrifuge tubes containing culture medium. To perform microfluidic experiment, the chips were placed at 37 °C in humidified 5% CO<sub>2</sub> platform and connected to the culture platform for applying a shear stress. And the chip with multi-inetrfaces ensured the culture of different cells and assay of drug-induced toxicity.

After RPTECs and PCECs adhered on the membrane, both were exposed to a fluid shear stress of 0.2 dyne/cm<sup>2</sup>. The flow rate can be calculated using the equation:  $\tau = 6uQ/bh^2$ , where  $u$  is the medium viscosity at 37 °C (gm/cm/s),  $Q$  is the volumetric flow rate (cm<sup>3</sup>/s),  $b$  is the channel width and  $h$  is the channel height [15]. For the static group, cells remain in same media for the duration of the flow tests.

## 2.5. Immunofluorescence Staining

To ensure that the cell conditions were suitable for drug screening, it is important to determine whether cells are alive or dead as they are treated with the drug. Here, the Live/Dead reagents were used, which showed green (live cells) and red (dead cells). And cell counting kit-8 (CCK-8) assay was used to detect the cell viability qualitatively.

When time for detection, the CCK-8 solution (CCK-8 reagent:culture medium = 1:10) was added to memb 1 and incubated for 2 h. Then, the CCK-8 solution was aspirated into a 96-well plate (120-150 μL per well), and the wavelength at 450 nm can be measured using a microplate reader. The fluorescence image of RPTECs on the microfluidic kidney chip was also recorded and compared with the cells on the petri dish. These two indicators are used to determine the cell conditions in the kidney model.

## 2.6. The Group of Nephrotoxic Drug

Three nephrotoxic drugs—DDP, GM, and CsA were selected for verifying the functions on our platform. DDP, a yellow crystalline powder, and is one of the most effective drugs for lung and esophageal cancer, soft neoplasms and other solid tumors. Cisplatin-induced nephrotoxicity mechanisms include the following: DDP can make renal blood vessels shrink gradually, impacting the glomerular filtration rate and resulting in renal damage, proteinuria, and other symptoms [16].

Due to its good thermal stability and reliability, GM is a widely used clinical aminoglycoside in the antibiotic category. It mainly treats the illness induced by Gram-negative bacteria, *Neisseria gonorrhoeae* and other infections. Although the efficacy of GM is good, but studies have shown that it has a strong nephrotoxicity [17], [18].

CsA is a potent immunosuppressant composed of cyclic peptides and it is mainly used for rejection due to organ transplantation such as heart, liver and kidney. Its working mechanism is that it can selectively act on T lymphocytes without inhibiting the growth of bone marrow, thus greatly reducing the infection rate during surgical transplantation. However, CsA has a severe nephrotoxicity, which greatly limits its use [19], [20].

RPTECs and PCECs were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM). To assess the toxicity, the cells were then divided into gradient concentrations over the following 24 h. Cells were divided into three groups based on their concentrations:

- 1) DDP group: DDP drugs with concentrations gradient of 0, 10, 20, 30, 40 mol/L;
- 2) GM group: GM drugs with concentrations gradient of 0, 10, 20, 30, 40 mmol/L;
- 3) CsA group: CsA drugs with concentrations gradient of 0, 10, 20, 30, 40 mol/L.

## **2.7. Drug Nephrotoxicity Experiments**

$\beta$ -N-acetylglucosaminidase (NAG) is an acidic enzyme in lysosomes and is widely found in various tissues and organs, platelets, red blood cells and white blood cells. When the proximal tubule is damaged, the NAG activity in the urine is significantly increased, and it is more sensitive than other enzymes. Therefore, NAG can be used as a specific indicator of nephrotoxicity in vitro. Early damage suffered. The substrate in the kit is hydrolyzed by the action of NAG and releases free p-nitrophenol. The addition of an alkaline solution allows color development of p-nitrophenol. The cells were deal with the above drug concentrations on 48-wells. Unit number of enzyme activity (U·L<sup>-1</sup>) = (measurement tube OD400-control tube OD400)/(standard tube OD400-blank tube OD400)×standard concentration× 1/15×1000.

Except for the cell morphology and nephrotoxic specificity index—NAG, we calculated the cell inhibition rate by CCK-8 kit, which showed a direct result. The cells were deal with the above drug concentrations on microfluidic kidney chips. After each group of data is averaged, the cell inhibition rate can be calculated based on the optical density value. Cell inhibition rate (%) = (control group OD450-experimental group OD450)/control group OD450×100%. The drug concentration or its logarithm as the abscissa and the corresponding cell inhibition rate as the ordinate, we can plot the curve of cell survival rate varying with the drug concentration.

In addition to considering the effect of concentration, time is also an important factor in drug toxicity testing. It was verified by the following two experiments. We compared GM-induced nephrotoxicity by two experiments, administered at the same total dose, but using different pharmacokinetic regimens.

For the static group: One is exposed with the 30 mmol/L GM for 4 h; The other is exposed with the 5 mmol/L GM for 24 h.

For the fluidic group: One is exposed with a short-term high concentrations (25 mmol/L, 4 h), then cultured with media for the next 20h; The other is exposed with a long-term short concentrations (3 mmol/L, 24 h)

For the static group, we obtained the preliminary results. Then, RPTECs were cultured under shear stress conditions and exposed to the GM by a pharmacokinetic regimen. Compared with static culture, dynamic culture with shear force group can improve the viability of cells. We tested two drug treatment regimens to give the same GM dose within 24 h. In regimen 1, we mimicked the drug clearance function of the human bolus injection, the cells were exposed to a concentration of 32 mmol/L GM, and the dose reduced by half every 2 h. In another, we continued to infuse the GM (5 mmol/L, 24 h). Cell death rate (%) = (control group

OD450-experimental group OD450)/control group OD450×100%.

### 3. Results and Discussion

#### 3.1. Cell Morphology and Detection of NAG

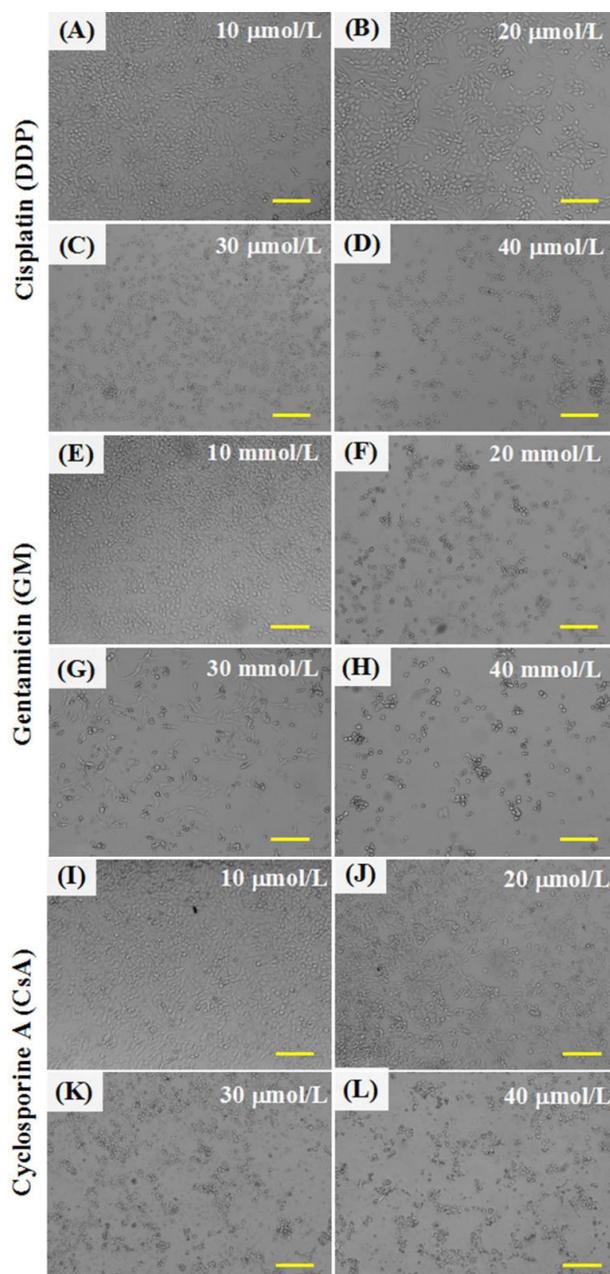


Fig. 3. The effect of different concentrations of drugs on RPTECs. (A)–(D) DDP (10, 20, 30, and 40  $\mu\text{mol/L}$ ) on cell morphology. (E)–(H) GM (10, 20, 30, and 40  $\text{mmol/L}$ ) on cell morphology. (I)–(L) CsA (10, 20, 30, and 40  $\mu\text{mol/L}$ ) on cell morphology. Scale bar = 200  $\mu\text{m}$ .

In addition to CCK-8 assay, cell morphologies with different drug concentrations are another indicator to determine the drug effect. RPTECs cultured in a normal medium without the drug were defined as the control group. As shown in Fig. 3, they were almost adherent cells and showed a typical “paving stone” shape. Cells in low-concentration groups began to shrink, and some were even shed. However, most cells in high-concentration groups died, and the remaining adherent cells gradually became small and round. As the

drug concentration increased, the cells were damaged severely, and their morphology became almost spherical. The results indicated that RPTECs showed different degrees of toxicity under drug treatment, suggesting that the nephrotoxicity of these drugs can be evaluated and screened.

The enzyme activity unit can be calculated by measuring the absorbance at 400 nm. As shown in Fig. 4, the NAG activity in the cell supernatant was positively correlated with the concentrations of DDP, GM, and CsA, indicating that the damage was gradually increased as the drug concentration increased. What's more, from the result, the toxicity from large to small is: DDP, GM, CsA. Nephrotoxic specificity index—NAG can be used to quantify renal proximal tubules.

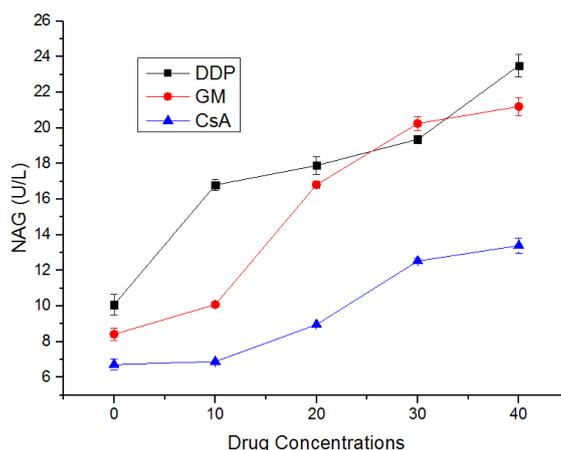


Fig. 4. Secretion of NAG caused by different concentrations of DDP ( $\mu\text{mol/L}$ ), GM ( $\text{mmol/L}$ ), CSA ( $\mu\text{mol/L}$ ).

### 3.2. The Effect of Drugs on Cell Inhibition Rate

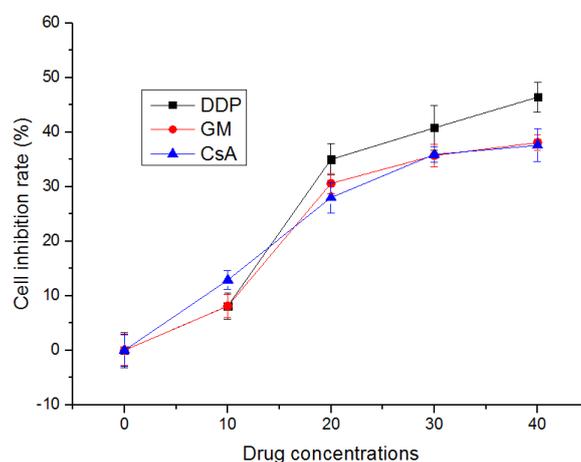


Fig. 5. The effects of different concentrations of DDP ( $\mu\text{mol/L}$ ), GM ( $\text{mmol/L}$ ), CSA ( $\mu\text{mol/L}$ ) on cell inhibition rate.

The renal tubules have a rich transport function and are very sensitive to the exogenous nephrotoxic drugs. This time we selected three typical nephrotoxic drugs to detect the toxicity of renal tubular cells. We can see from the Fig. 5, it is the different concentrations of DPP, GM and CSA on cell inhibition rate. The results show that renal tubular cells also exhibit degrees of toxicity under three drug concentration gradients, which indicates that using of these drugs for evaluation and screening on drug nephrotoxicity is possible.

### 3.3. Pharmacokinetic Test of GM

For the static group, we obtained preliminary results. Cell death rate was calculated from the above formula. As can be seen from Fig. 6(A), the cell death rate of the GM (5mmol/L, 24 h) group was about 13%, and the cell death rate of the GM (30mmol/L, 4 h) group was about 35%. The death rate of the former group is much lower than that of the latter one. This is also the same as our understanding of drugs. The cytotoxicity of short-term high concentrations is much greater than the long-term low concentration. Fig. 6(B) shows the images of life and death staining of two groups. Green indicates living cells and red indicates dead cells. It can also be seen from the figure that the survival rate of the latter cells is much lower than that of the former.

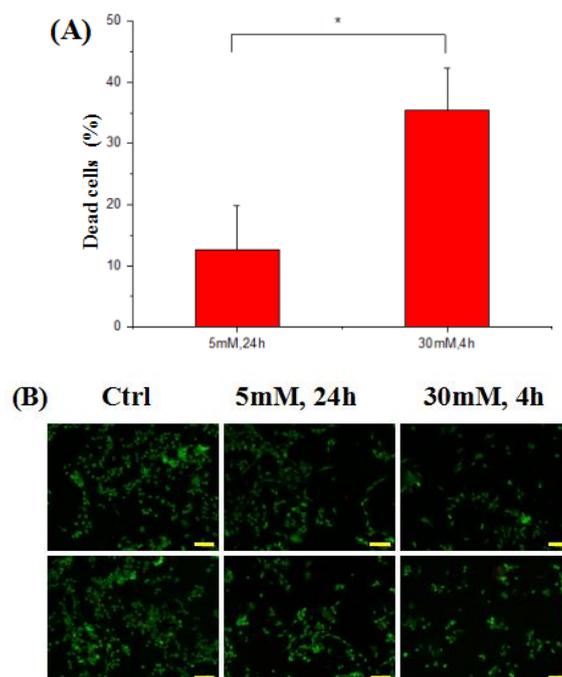


Fig. 6. Static culture results of two different GM regimens. (A) Cell viability. (B) Live/dead fluorescence images. Scale bar = 200  $\mu$ m. (\* $P$ <0.05.).

For the fluidic group, we also tested two drug regimens, which exposed to a fluid shear force of 0.2 dyn/cm<sup>2</sup>. The half-life of GM in humans is approximately 2 h [21], [22]. Regimen 1 mimicked the single GM bolus injection and the drug concentration was reduced by half every 2 h. After 8 h, the blood-free medium was perfused for next 16 h. In contrast, regimen 2 mimicked continuous perfusion of GM for 24 h.

As shown in Fig. 7(A), the cells cultured under fluidic conditions had a death rate of less than 5%. Compared to static culture, dynamic culture can significantly increase the viability of cells. Compared to regimen 1, regimen 2 showed a higher cell death rate. The cytotoxicity was lower than the continuous injection regimen in the single-injection treatment regimen simulated on the kidney chip. As a result of the previous static culture, the death rate of cells exposed to short-term high concentrations was higher, which is exactly the opposite of our results in the fluidic group. This suggests that the kidney chip provides a more favorable environment for cells to grow. This may be due to the fact that the narrow channel width changes the shape of the cell and enhances the autocrine retention effect, suggesting that fluid shear stress may help to restore damaged cell. Fig. 7(B) is the image of the dead and live staining of the cells, which is consistent with our previous analysis.

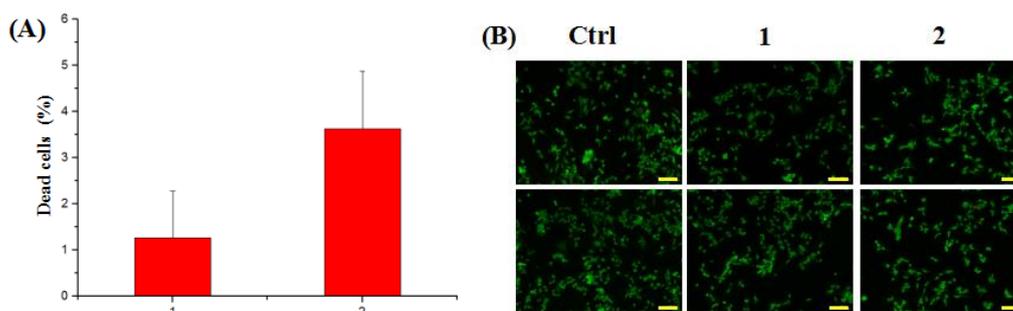


Fig. 7. Fluidic culture results of two different GM regimens. (A) Cell viability: the regimen 1 mimics the change of drug dose in the human kidney after a single injection of GM. Since GM half-life in the human body is 2 h, the drug concentrations is halved every 2 h for 8h. The blood-free medium was further cultured for 16 h. The regimen 2 simulates the continuous infusion of GM for 24 h in vitro. Both cells were exposed to shear forces of 0.2 dyne/cm<sup>2</sup>. (C) Live/dead fluorescence image. Scale bar = 200  $\mu$ m.

#### 4. Conclusion

In this research, a three-layer microfluidic kidney chip was presented, which can provide a biomimetic microfluidic platform to form a micro-environment similar to that in vivo for efficient culture and analysis of the renal tubular cells. The shear stress of 0.2 dyne /cm<sup>2</sup> was applied on the cells, which favorably promoted cellular polarization. RPTECs grew better and had a higher survival rates when cultured in the microfluidic kidney chip. And the microfluidic culture platform we designed provided an automated way to culture and analyze the cells. On this basis, we tested three common nephrotoxic drugs: DDP, GM and CsA. With the cell morphology and the nephrotoxic specificity index—NAG, we compared the drug-induced nephrotoxicity. The higher drug concentration caused lower cell viability. And except for the concentrations, we compared GM-induced nephrotoxicity through different regimens. The results showed that toxicity measured on chip more closely mimicked the in vivo responses than the cells maintained under static conditions. The in vivo-like pathophysiology observed would be useful for evaluating human-relevant renal toxicity. And the microfluidic kidney chip described here can be used in various aspects including disease model systems such as diabetic nephropathy (NDI) or edema status, drug screening.

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