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Assessment of hepatic metabolism-dependent nephrotoxicity on an organs-on-a-chip microdevice

Zhongyu Li[a,c,d], Lei Jiang[a], Yujuan Zhu[a,c], Wentao Su[a], Cong Xu[a,c], Tingting Tao[a,c], Yang Shi[a], Jianhua Qin[a,b,c,*]

[a] Division of Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China
[c] University of Chinese Academy of Sciences, Beijing 100049, China
[d] Key Laboratory of Separation Sciences for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, China

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ABSTRACT

Drug-induced nephrotoxicity is one of the most frequent adverse events in pharmacotherapy. It has resulted in numerous clinical trial failures and high drug development costs. The predictive capabilities of existing *in vitro* models are limited by their inability to recapitulate the complex process of drug metabolism at the multi-organ level *in vivo*. We present a novel integrated liver-kidney chip that allows the evaluation of drug-induced nephrotoxicity following liver metabolism *in vitro*. The liver-kidney chip consists of two polydimethylsiloxane layers with compartmentalized micro-channels separated by a porous membrane. Hepatic and renal cells were co-cultured in separate micro-chambers on a single chip. Ifosfamide and verapamil were used as model drugs, and their metabolites produced by hepatic metabolism were identified using mass spectrometry, respectively. The metabolites triggered significantly distinct nephrotoxic effects as assessed by cell viability, lactate dehydrogenase leakage and permeability of renal cells. This *in vitro* liver-kidney model facilitates the characterization of drug metabolism in the liver as well as the assessment of subsequent nephrotoxicity in a single assay. Obviously, this multi-organ platform is simple and scalable, and maybe widely applicable to the evaluation of drug metabolism and safety during the early phases of drug development.

1. Introduction

The kidney is critical to maintain the homeostasis of the body through its endocrine and metabolic activities, its excretion of waste products and its reabsorption of essential compounds. As such, the kidney is highly vulnerable to the toxic effects of drugs (Boroushaki et al., 2014; Perazella, 2009; Prozialeck and Edwards, 2007; Schetz et al., 2005). Drug-induced nephrotoxicity is one of the most frequent adverse events in pharmacotherapy, and causes 30% to 50% of severe acute renal failure in patients (Fuchs and Hewitt, 2011). Gaining insight into drug-induced nephrotoxicity may greatly contribute to the development of effective drugs with less adverse effects. *In vivo*, drugs go through a complex process of absorption, distribution, metabolism and exclusion, in which metabolism determines the pharmacological properties of drugs. All successful drug development efforts must carefully consider drug efficacy and safety, both of which are intimately relevant to drug metabolism.

As the major organ of drug metabolism, liver greatly alters the concentration and bioavailability of oral drugs. Many drugs exhibit altered renal toxicity after metabolism in the liver. For example, ifosfamide (IFO), a bifunctional alkylate agent used in cancer treatment, has low toxicity as a prodrug, but exhibits much higher nephrotoxicity in the body. Several studies have demonstrated that IFO's kidney toxicity may be due to its metabolites, acrolein (ACR) and isophosphoramide mustard (ISO), which are generated in the liver. Verapamil (VER), a calcium chamber antagonist in the treatment of cardiovascular disease is thought to be toxic to the kidney, but few studies have reported its nephrotoxicity. VER is actually extensively metabolized into norverapamil (NOR) in the liver, leading to low renal toxicity and greatly reducing its bioavailability. Considering the hepatic metabolism of drugs, there is a great need for an *in vitro* model that can better assess renal toxicity in a physiologically relevant manner.

Although liver metabolism plays an essential role in drug-induced nephrotoxicity, it is difficult to account for it in preclinical testing due to the lack of reliable *in vitro* models. Studies on drug-induced nephrotoxicity have mainly relied on experiments performed using cell

* Corresponding author at: Division of Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China.
E-mail address: jhqin@dicp.ac.cn (J. Qin).

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monolayers and animals (Astashkina et al., 2012; Gunness et al., 2010; Hoffmann et al., 2010; Yaman and Balikci, 2010). Specifically, two-dimensional cell-based *in vitro* models generally use renal cell lines, such as tubular cells, which are popular for their relative simplicity (Choucha Snouber et al., 2012; Frohlich et al., 2013; Kim et al., 2016; Zucco et al., 2004). However, cell growth and cellular functions are greatly limited *in vitro* due to the extreme variance from physiological conditions. Thus, cell-based models can only mimic an acute response and have difficulty recapitulating the biologically relevant responses of the human body. Although animal models can resemble drug-induced responses *in vivo* to some extent, their use is still restricted due to variance in species and metabolic features. Moreover, animal models are expensive, thereby limiting quantitative studies using target-based analyses or high-throughput assays.

Emerging organ-on-a-chip technology has proven to be advantageous in recapitulating *in vivo* environments to promote cell growth and maintain tissue or organ function (Bhushan et al., 2016; Esch et al., 2015; Ghaemmaghami et al., 2012; Xu et al., 2012; Young, 2013). A kidney proximal-tubule-on-a-chip has been developed, it has been shown to enhance cell polarization, rearrange the cytoskeleton and increase cell junctions by creating a chip-based *in vivo*-like environment (Jang et al., 2013). In addition, several kidney chips with distinct designs have been established and applied to assess nephrotoxicity in complex near-physiological conditions (Jellali et al., 2016; Leclerc et al., 2016; Maschmeyer et al., 2015). Most of these studies have mainly focused on constructing models of the renal tubular system. However, drug exposure has various nephrotoxic effects, such as glomerular dysfunction, which is one of the most characteristic nephrotoxic responses. It is thus highly desirable to develop an effective model related to glomerular filtration, which would further improve the predictive accuracy of drug-induced nephrotoxicity.

We present a novel liver-kidney chip to assess drug metabolism in liver cells and the resulting toxicity in kidney cells simultaneously on a single device. The multilayer design of the chip incorporates cells from two organs, contributing to test drug toxicity at the multi-organ level. HepG2 cells cultured on the upper layer simulate liver tissue during drug metabolism, while glomerular endothelial cells (GECs) cultured on the bottom layer allowing the assessment of drug-induced toxic effects on kidney filtration. We adopt IFO and VER as model drugs to investigate metabolism-dependent nephrotoxicity in an integrated liver-kidney chip. We suggested the utility of this *in vitro* model in testing hepatic metabolism-dependent kidney toxicity at the multi-organ level, as it closely mimics *in vivo*-like drug responses.

2. Experimental

2.1. Materials

An SU-8 3035 negative photoresist was purchased from MicroChem Corp. A polydimethylsiloxane (PDMS) prepolymer and curing agent and a porous polycarbonate membrane (0.4 μm pore size, Whatman Corp.) were purchased from Dow Corning Corp. and Casmart Mall (Beijing, China), respectively, to fabricate the microfluidic devices. Dulbecco's modified eagle medium (DMEM, Gibco), an endothelial cell medium (Gibco), fetal bovine serum (FBS, Gibco), trypsinEDTA (Gibco), Matrigel (BD), live/dead kit (BD), cell counting kit-8 (CCK-8, Dojindo, CK04), ZO-1 (Abcam), DAPI, fluorescein immunoglobulin G (IgG), an albumin assay kit, lactate dehydrogenase(LDH) assay kit, IFO and VER were purchased from Casmart Mall (Beijing, China) for cell-related experiments. All of the chemical reagents used in this experiment were analytical reagent grade.

2.2. Device design and fabrication

As shown in Fig. 1, the device consisted of two PDMS layers separated by a porous polycarbonate membrane. The top layer of the chip had one cell culture chamber that was 500 μm in height, 2 mm in width and 11 mm in length. While the bottom layer had two higher chambers in parallel separated by the lower chamber with extracellular matrix (ECM). One higher chamber on the left was 300 μm in height, 2 mm in width and 15 mm in length, another one on the right, named as the collection unit was 300 μm in height, 2 mm in width and 11 mm in length. The lower collagen chamber was 100 μm in height, 900 μm in width and 11 mm in length. The interface between higher and lower chamber allowed cells to attach and grow to confluence. ECM (Matrigel) in the lower chamber supported the complex interactions of cells with matrix, mimicking physiological 3D microenvironments. A porous polycarbonate membrane with a 0.4 μm pore size was sandwiched between the two PDMS layers, allowing for the penetration of drugs and their metabolites. All of the chambers shared one inlet and one outlet for sampling in and out.

The liver-kidney chip was fabricated by soft lithography and micromolding as previously reported (Li et al., 2016). To prepare the template, the SU-8 photoresist was spin-coated onto two clean glass wafers and then selectively cured under an ultraviolet light source with different masks. The master of the bottom layer was exposed to UV-light twice, thus, the microstructures were fabricated with different heights. A mixture of the PDMS monomer and the curing agent at a ratio of 6:1 by mass was used to generate two layers of PDMS replicas. The porous membrane was then sandwiched between these two PDMS replicas (Aran et al., 2010; Chueh et al., 2007). Specifically, the top PDMS layer and the porous membrane were bonded together after oxygen plasma treatment, before assembly with the bottom layer using a glue, a mixture of the PDMS monomer and the curing agent at a ratio of 20:1 by mass. The sealed three-layer device was finally placed in an oven at 80 °C for 1 h to cure the glue.

2.3. Co-culture of liver and renal cells on a chip

HepG2 cells (liver tumor cells) were cultured with a DMEM medium supplemented with 10% FBS and 100 U/mL of penicillin and 100 U/mL of streptomycin in the cell incubator with 5% CO_2 at 37 °C. The cells were passaged weekly at confluence. The culture medium was changed every 48 h. Primary glomeruli were extracted from rats. GECs were purified from glomerular micro-tissues by differential adhesion. The cells were grown in an endothelial cell medium supplemented with 10% FBS and 100 U/mL of penicillin and 100 U/mL of streptomycin in the cell incubator with 5% CO_2 at 37 °C.

After fabricating the multilayer PDMS device, chilled liquid Matrigel was aseptically pumped into the ECM lower chamber with the final concentration of 6 mg/mL using cold pipette tips, and finally gelled at 37 °C for 30 min. The micro-chambers were precoated with 8 μg/mL collagen for 12 h to maintain a biocompatible surface for cell seeding and culture. GECs were harvested by centrifuging, and re-suspended in cell culture medium to the final density of 1×10^4 cells/mL before loading into the lower cell chamber from the inlets. The microchip was then side cultured to enable cell adhesion to the surface of gelled matrix. After incubation in a humidified incubator at 37 °C for 72 h, GECs finally proliferated to form a glomerular endothelial barrier on the chip.

By then, HepG2 cells were injected into the top chamber at a density of 1×10^6 cells/mL following cell harvest, and were finally incubated in the incubator in a humid atmosphere with 5% CO_2 at 37 °C.

2.4. Immunostaining assay

After drug treatment, cells were quickly rinsed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Samples were permeabilized with 0.2% Triton-X100 for 10 min and washed with PBS three more times before incubation with primary antibodies (ZO-1) overnight at 4 °C. After washing with PBS, samples were then incubated with fluorescence-labeled secondary antibodies for 1 h at room