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FISH-LIVER-ON-CHIP: A MICROFLUIDIC MODEL TO ASSESS BIOACCUMULATION OF ENVIRONMENTAL DRUG RESIDUES IN VITRO

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ABSTRACT

This paper reports first in its kind microfluidic, scaffold-free 3D culture of fish hepatocytes (RTH-149) and evaluates its feasibility for assessment of the toxicity of environmental drug residues in fish in vitro.

KEYWORDS: 3D cell cultures, environmental risk assessment, pharmaceuticals, ecotoxicity, bioaccumulation

INTRODUCTION

Pharmaceutical drug residues are ubiquitous in the aquatic environment [1]. Despite of their low environmental concentrations, many drugs are known to be intaken and bioaccumulate in wild fish [2]. The hepatic biotransformation (metabolism) reactions are in a key role in determining, whether the fish can eliminate the intaken drug residues or if they bioaccumulate in fish tissues (no metabolism). The hepatic enzymes in charge of these reactions are prone to inhibition by many environmental chemicals, which increases the risk of drug bioaccumulation and associated chronic effects in fish. Currently, the bioaccumulation risk is primarily evaluated based on in vivo assays (nearly hundred fish per drug compound), which is time-consuming and ethically-unsound. Therefore, recent efforts have focused on development of (static) cell-based assays to predict hepatic drug clearances in fish in vitro, whereas microfluidics has only rarely been exploited to setting up fish cell culture assays [3,4], with no prior equivalents of on-chip 3D fish hepatocyte cultures so far, to our knowledge.

THEORY

In static cell cultures, the threshold toxic concentration is typically determined by exposing the cells to different (fixed) amount of drug to derive the half-maximal inhibitory concentration (IC50). If the cells are not capable of eliminating the test compound (via metabolism or active efflux transport), the compound accumulates in the cells resulting in dose-dependent cell death. In microfluidic assays, the threshold toxic concentration can be much lower, however, as the feed solution continuously brings more drug to the cell culture. If the test compound is accumulative, this results in a time-dependent toxicity (increasing drug concentration inside the cells over-time). Thereby, the microfluidic setup better mimicks the continuous drug exposure as in environmental conditions, and could improve the *in vitro* predictions of the impacts of chronic exposure of fish to trace-level drug concentrations.

EXPERIMENTAL

We have previously developed polymer based microwell arrays for culturing scaffold-free 3D spheroids of human hepatocytes under microfluidic flow [5,6]. In this work, we adapted similar concept for culturing 3D fish hepatocytes on chip. The microwell arrays (total of ca. 1000 wells, id 200 μm, ca. 19 wells/mm^2) were prepared via a three-step replication process (Fig. 1a). The master mold (height ca. 180 μm) was made from organically modified ceramics, which enable fabrication of round-shaped microwells in a single lithographic step (here, 0.4 J/cm2, OAI 30/5) as in [5]. Next, the microwell array was replicated to PDMS (10:1) by soft lithography (90ºC, 2 h). The final microwell array was made from off-stoichiometric thiol-ene (OSTE) by UV-replicamolding (Dymax 5000-EC, 255 mW/cm^2, 10 min) using tetrathiol (PETMP) and triallyl (TATATO) monomers (Fig. 1b). Last, the OSTE microwell array was sealed with air-plasma (1 min, RF 30W) treated PDMS (15:1) microchannel (0.2×3×35 mm^3, $h \times w \times l$). The bulk OSTE composition featured 50 mol-% excess of the allyl functional groups, which enabled pegylation of the allyl ('ene')-rich surface with monofunctional PEG-thiol (1056 Da) via UV-initiated (LED 365 nm, 14 mW/cm^2) thiol-ene reaction in the presence of 0.1% (m/v) TPO-L photoinitator, as in [6]. After microchip sterilization (70% ethanol, aq), the fish hepatocytes (RTH-149, ATCC, 3.5 M/mL) were seeded in microwells (15 min, no flow), and thereafter cultured as 3D spheroids under microfluidic flow (0.9 μL/min, 0.0075 dyne/cm^2) in a medium containing MEM and HEPES (pH 7.4) and supplemented with 10% FBS, 1% NEAA, 1% Sodium pyruvate, 1% Pen-Strep. At 24h, the spheroids were exposed to the test pharmaceuticals, after which the culturing was continued for another 48h before cell staining in situ with standard live/dead cell stains.

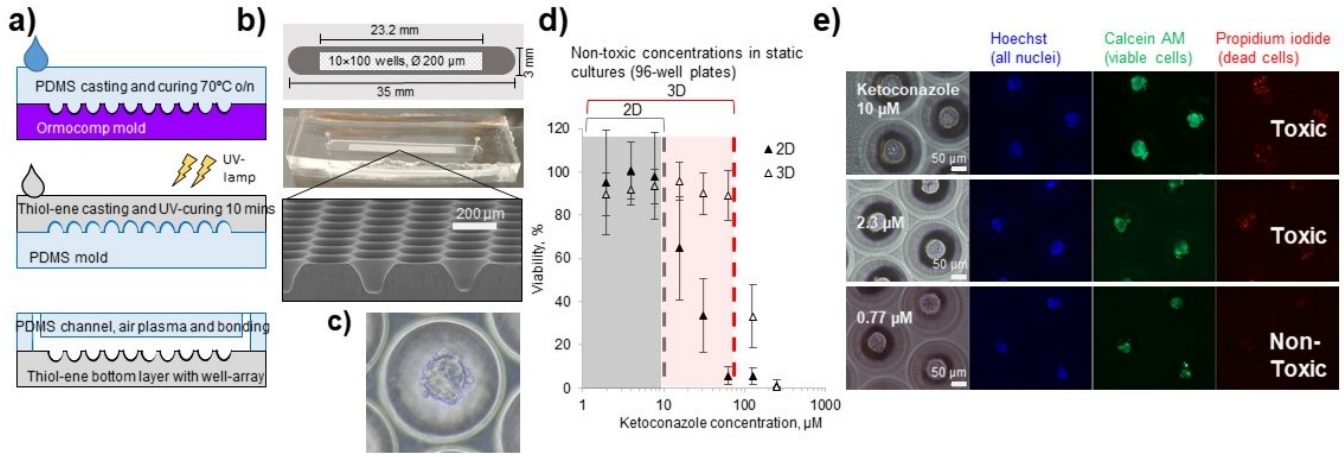

Figure 1: a) Schematic view of the replica molding protocol for OSTE microwell array and PDMS bonding. b) Schematic of channel dimensions, a photograph of the assembled chip and scanning electron micrograph (SEM) of the concave microwells. c) Compact RTH-149 spheroid in situ inside a well. d) Dose dependent toxicity of ketoconazole in 96-well plates. RTH-149 grown as static monolayers (2D, 10 000 cells/well) or spheroids (3D, 350 cells/well). Viability determined with CellTiterFluor (2D) and CellTiterGlo3D (3D) kits (Promega). e) Lowered toxicity profile of ketoconazole in through-flow conditions on chip.

RESULTS AND DISCUSSION

In this work, we optimized the previously developed microwell fabrication (well shape and size) and surface functionalization (prevention of cell adhesion) protocols to enable culturing of fish hepatocytes as scaffold-free 3D spheroids under microfluidic flow. Compared with human hepatocytes, the fish cells adhered to chip surface more strongly, which necessitated careful optimization of the pegylation process to support forced floating and aggregation of the cells seeded in the microwells (Fig. 1c). In optimized conditions, the fish hepatocytes formed compact 3D spheroids. As expected, the threshold toxic concentration of, for instance, ketoconazole (an antifungal that is known to bioaccumulate in fish tissues in vivo) was much lower under microfluidic flow compared with that of static 2D or 3D fish hepatocyte cultures. In static assays, the 10 µM concentration (48h exposure) was well below toxic threshold of ketoconazole in both 2D and 3D cultures (Fig. 1d), but under flow conditions already 2.3 µM concentration resulted in substantial toxicity (Fig. 1e), likely resulting from accumulation of ketoconzale in the cells over time. In total, continuous feed of 10 µM ketoconazole for 48h (0.9 µL/min) corresponds to ca. 26 nmol of ketoconazole, which is equivalent to ca. 130 µM concentration in the static assays (200 µL). The result was further confirmed by exposing the spheroids to two more ketoconazole concentrations, 0.8 and 2.3 µM on chip, that yielded total loads equivalent to 10 and 30 µM ketoconazole in the static assays, respectively. These concentrations resulted in negligible and ca. 50% inhibition in cell viability, similarly in both flow conditions and static assays.

CONCLUSION

The results suggest that the microfluidic 3D fish hepatocyte culture platform developed in this study could help predict the time-dependent toxicity associated with chronic drug exposure in fish *in vitro*.

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