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# Development of 3D Dynamic Flow Model of Human Liver and Its Application to Prediction of Metabolic Clearance of 7-Ethoxycoumarin

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Primary and cryopreserved hepatocytes and immortalized hepatic cell lines in static two-dimensional monolayer culture format have been widely used as *in vitro* liver models for studies of xenobiotic metabolism, enzyme induction, hepatocyte regeneration, and hepatotoxicity. However, the tissue structure and metabolic capacity in these liver models are often ill-defined and are not well preserved compared to *in vivo* liver-specific architecture and functions. For this reason, we developed a three-dimensional (3D) dynamic flow model with primary human hepatocytes, which was optimized for cell seeding density, medium composition, and extracellular matrix proteins. Human hepatocytes cultured in this system were maintained for up to 7 weeks and reproducibly recapitulated *in vivo* liver-like structure and important liver-specific functions, such as albumin/total protein production, glucose utilization, lactate production, and cytochrome P450 (CYP) 3A4 activity across multiple tissue donors. The *in vitro* intrinsic clearance ($CL_{int}$) of 7-ethoxycoumarin (7-EC) was determined from human hepatocytes cultured in the 3D dynamic flow model and compared to that in hepatocyte suspension. The 7-EC $CL_{int}$ values varied among individual batches and/or the two different *in vitro* liver models used in this study. The 3D flow model appeared to give more reproducible and stable estimates of clearance that is similar to previously published values. Overall, the results from these studies demonstrate that this culture system could be a valuable tool for making more accurate predictions of the metabolic clearance and long-term effects of chemicals and their metabolites in a complex 3D environment under dynamic flow.

## Introduction

The liver is the major organ for metabolism, detoxification, and elimination of xenobiotics and also plays an important role in the maintenance of physiological homeostasis, including gluconeogenesis, glycogenesis, triglyceride oxidation, urea synthesis, and plasma protein synthesis.[1,2] Liver metabolism is commonly involved in interspecies and intraspecies differences in toxic effects of environmental and industrial chemicals and also the overall therapeutic profiles of drugs.[3,4] As such, a large emphasis has been placed on the use of *in vitro* hepatic models to predict clearance, metabolite profiles, inhibition or induction of liver enzymes, and hepatoxicity. The most commonly used *in vitro* liver models are hepatic tissue fractions (microsomes, S9, and cytosol), recombinant drug metabolism enzymes (DMEs), isolated liver slices, primary and cryopreserved hepatocytes, and immortalized hepatic cell lines in various culture formats and conventional two-dimensional (2D) cultures.[4–6] Most *in vitro* studies are conducted under static culture conditions using short-term exposure periods to test compounds. However, the tissue structure and metabolic capacities in most current liver models are limited and less relevant compared to *in vivo* hepatic conditions.[4] Because hepatocytes are known to be anchorage-dependent and highly polarized via cell-to-cell interactions, their metabolic function in conventional monolayer culture rapidly decreased over time, especially phase I and phase II biotransformation reactions.[1,7] The static conditions of most conventional cell monolayers also limits the mass exchange of gases and essential nutrients as well as the removal of metabolic waste ultimately leading to cell stress and cholestasis.[1,5,8]

Due to their limited lifespan, conventional *in vitro* liver models under static conditions are not feasible to predict subchronic and/or chronic hepatotoxicity of parent compounds or their metabolites at physiologically relevant exposure levels or to estimate an accurate clearance of many chemicals, especially those with a low turnover rate.[2,9] Thus, it is of considerable importance to preserve *in vivo*-like hepatic-specific properties for an extended period of

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time for more accurate characterization of chemical toxicity and pharmacokinetics.[6,7] Several *in vitro* animal liver models with adequate liver function have been developed. These models include collagen sandwich culture of rat with dynamic flow conditions,[10] a perfusion bioreactor system of rat,[11] a stirred tank bioreactor of rat,[7] and a bioartificial liver (BAL) of pig.[12,13] For toxicological and pharmacological studies, human liver models have been developed in various culture systems, such as multicompartment perfusion bioreactors with HepaRG and human hepatocytes,[1,2,14,15] a micropatterned hepatocyte co-culture system,[16] and a static cell bioreactor of human small intestine-derived Int 407 cell line.[9]. However, there is still a lack of *in vitro* liver models reflecting physiological and biochemical responses similar to those occurring in the real-life human exposure to environmental contaminants, for example, pesticides,[17] a complex mixture of phthalates[18] and BPA in drinking water and consumer products,[19] and pharmaceuticals in tap water[20] that often involve low-level exposure for a very long time frames, allowing the exact prediction of the long-term safety and toxicology endpoints derived from the chemical–chemical interaction among the parent chemicals and/or their metabolites.

The aims of this study were to develop a three-dimensional (3D) dynamic flow model with primary human hepatocytes using the RealBio D$^{4TM}$ flow chamber and to evaluate basal metabolic functions (cytochrome P450 activity, glucose utilization, and lactate production), liver-specific functions (total protein and albumin synthesis), and the morphological integrity of the hepatocytes (alanine aminotransferases/aspartate aminotransferases [ALT/AST] levels, histochemical analysis) over an extended period of time ($>1$ month). Furthermore, this system was utilized to estimate the intrinsic clearance ($CL_{int}$) of 7-ethoxycoumarin (7-EC) and to compare its kinetics with that in the standard hepatocyte suspension cultures.

## Materials and Methods

### *Primary human hepatocytes*

Primary human hepatocytes in suspension were purchased from BD Biosciences (Woburn, MA), BioreclamationIVT (Baltimore, MD), Life Technologies (Frederick, MD), Triangle Research Labs (Research Triangle Park, NC), and XenoTech LLC (Lenexa, KS). The characteristics of human donors are given in Table 1.

### *Hepatocyte culture in the liver bioreactor*

The RealBio D$^{4TM}$ Culture System, a 3D dynamic flow chamber, and the perfusion device were obtained from RealBio Technology, Inc. (Kalamazoo, MI). This system consists of multicompartmental configuration with 3D woven polyester scaffold ($\sim 10\,cm^2$ surface $\times 0.1$ cm depth) and decoupled nutrient and metabolic gas supplies (Fig. 1A). For preliminary assessing the optimal loading and culture conditions for primary human hepatocytes, various cell densities ($10–35 \times 10^6$ hepatocytes), extracellular matrix (ECM) coatings (gelatin [Sigma, St. Louis, MO] and matrigel [BD, Franklin Lakes, NJ]), culture media (HMM [Lonza, Rochester, NY], DMEM-HG, and DMEM-LG [Gibco, Grand Island, NY]), and fetal bovine serum (FBS) supplements were tested for the best attachment efficiency, cell counting and viability, and cellular uniformity, which resulted in the formation of a 3D tissue-like structure across the scaffold and sustainable biochemical endpoints over time. On the basis of the results for the histological cell reorganization and biochemical endpoints, including glucose, lactate, AST, ALT, albumin, and total protein (data not shown), a cell loading density of $15 \times 10^6$, ECM coating of 0.1% gelatin solution (1 mg mL$^{-1}$), FBS supplement in the HMM cell culture medium for 8 days, and serum-free HMM culture medium during the remaining culture period were chosen for subsequent long-term culture experiments.

Primary human hepatocytes ($15 \times 10^6$) with an average viability of $\sim 84\%$ were suspended in the HMM seeding medium supplemented with 10% (v/v) FBS (Gibco), 90 U mL$^{-1}$ penicillin G sodium, 90 U mL$^{-1}$ streptomycin sulfate (Gibco), 0.9 μM dexamethasone, and 3.6 μg mL$^{-1}$ insulin (Sigma). The suspended human hepatocytes were loaded uniformly into the gelatin-coated scaffold and allowed to settle under static conditions at 5% $CO_2$/95% humidified air and 37°C. After 24 h, continuous flow (0.25 mL min$^{-1}$) was initiated and continuously maintained until the end of the study. The medium was replaced with the HMM maintenance medium at day 8 followed by replenishment every 5–10 days to maintain the pH between 7.1 and 7.4. The HMM maintenance medium was supplemented with 98 U mL$^{-1}$ penicillin G sodium, 98 U mL$^{-1}$ streptomycin sulfate, ITS$^+$ (6.1 μg mL$^{-1}$ transferrin, 6.1 μg mL$^{-1}$ insulin, and 6.1 ng mL$^{-1}$ selenium-A) (Gibco), and 0.1 μM dexamethasone. Samples of the culture medium were collected every 1–3 days and subsequently analyzed for levels of glucose and lactate, pH change, and cell counting and viability. The

Table 1. Characteristics of Human Donors for Primary Hepatocytes

| *Donor ID* | *Gender* | *Age (years)* | *Race* | *Cause of death* | *Smoking status* | *Drinking status* | *Hepatocyte viability (%)* |
|---|---|---|---|---|---|---|---|
| HM10 | M | 21 | C | Head trauma | Smoker | Frequent drinker | 80 |
| HM11 | M | 8 | C | Anoxia | Nonsmoker | Nondrinker | 86 |
| HM12 | F | 31 | C | Stroke | Nonsmoker | Nondrinker | 85 |
| HM13 | F | 23 | C | Anoxia | Nonsmoker | Nondrinker | 82 |
| HM14 | M | 59 | B | ICH | Smoker | ND | 85 |
| HM15 | F | 52 | C | LB | Smoker | Nondrinker | 90 |
| HM16 | F | 48 | C | CVA | Nonsmoker | Nondrinker | 92 |
| HM17 | F | 56 | C | LB | Nonsmoker | Frequent drinker | 92 |

B, African American; C, Caucasian; CVA, cerebrovascular accident; F, female; ICH, intracerebral hemorrhage; LB, liver biopsy; M, male; ND, not determined.

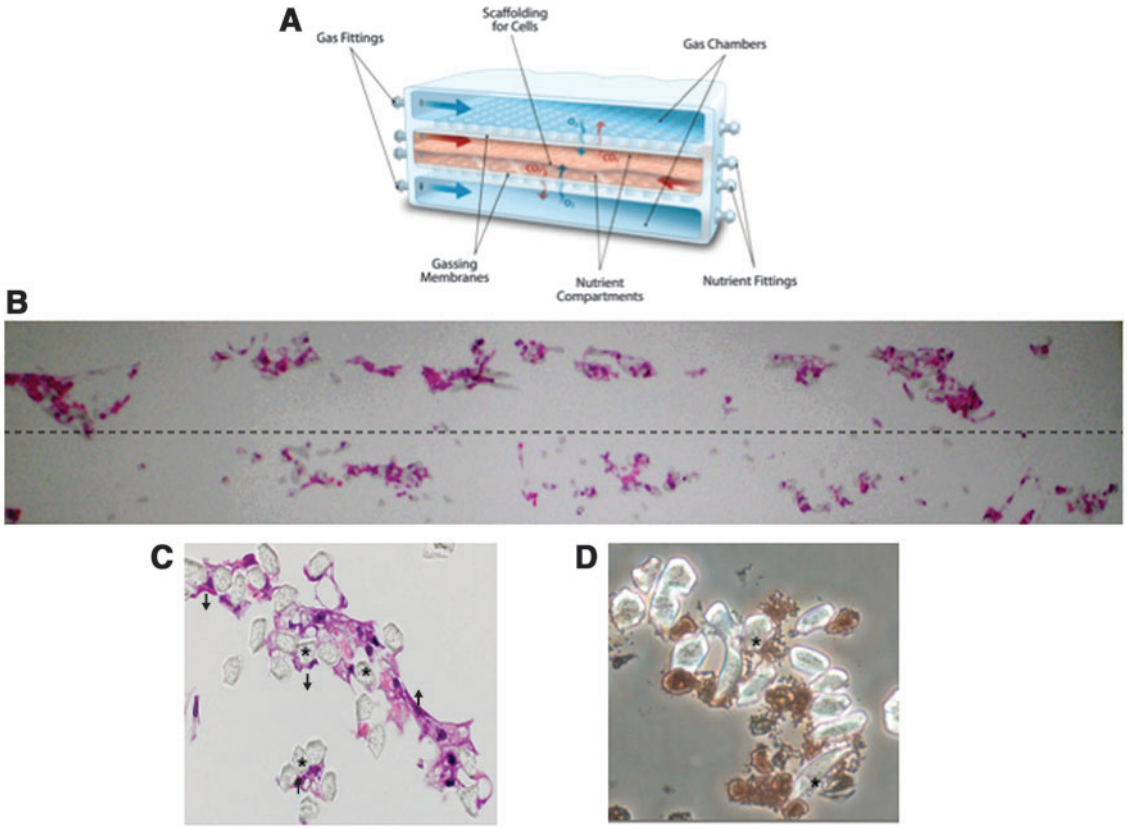


**FIG. 1.** Schematic view of the RealBio D$^{4}$™ culture chamber **(A)** and histological and immunohistochemical analysis of 37-day cultured human hepatocytes inside the liver bioreactor **(B–D)**. **(B)** Transverse histological sections with hematoxylin and eosin staining (×4); **(C)** formation of tissue-like structures of hepatocytes (×20); and **(D)** hepatocyte paraffin1 (Hep-Par 1) staining (×20). An asterisk and arrow represent a fiber from the scaffold and the elongated/flattened hepatocytes, respectively. The dashed line separates the scaffold sections taken from different location within the liver bioreactor.

cell counting and viability of residual cells released from the scaffold and circulating in the medium were determined with acridine orange and propidium iodide staining and Cellometer automatic cell counter (Nexcelcom Bioscience, LLC, Lawrence, MA). Remaining cell numbers in the system were corrected with the results of cell counting during the culture. The collected media samples were also stored at −20°C until subsequent analysis of biochemical endpoints, including albumin, ALT, AST, and total protein.

### *The measurement of biochemical parameters*

Biochemical parameters for albumin, ALT (E.C. 2.6.1.2), AST (E.C. 2.6.1.1), and total protein were determined with an Olympus AU600 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN). The medium without cells was used as the background for the analysis of biochemical endpoints.

The levels of glucose and lactate in culture media were measured with the ACCU-CHEK Aviva meter (Roche Diagnostics) and Lactate Plus analyzer (Nova Biomedical, Waltham, MA) according to the manufacturer's instructions, respectively. The pH value of the media samples was measured with litmus paper.

### *The measurement of CYP3A4 activity*

CYP3A4 enzyme activity was measured using the P450-Glo™ CYP3A4 kit according to the manufacturer's instructions (Promega, Madison, WI). Human hepatocytes inside the liver bioreactor were incubated with luciferin-IPA (final concentration of 3 μM), under either the static or continuous flow condition (0.25 mL min$^{-1}$) and for various time periods (90–120 min) at 37°C. At the end of the incubation, media samples were collected (25 μL) and incubated with luciferin detection reagent for 20 min at room temperature. Relative luminescence units were measured with the SpectraMax M3 multimode microplate reader (Molecular Devices, Sunnyvale, CA). The quantitation of the luciferin produced was determined from a five-point calibration curve of D-luciferin over the concentration range from 0.002 to 20 nM.

### *Histology and immunohistochemistry*

Histological and immunohistochemical analyses of human hepatocytes inside the liver bioreactor were performed by the Histology Laboratory, Department of Veterinary Biosciences, North Carolina State University (Raleigh, NC). Upon culture termination, the chamber containing cells was fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer for 48 h, transferred to 70% (v/v) ethanol, and stored at 4°C for further processing. After dissembling, the chamber slices of the scaffold were placed in tissue cassettes followed by the process on a Sakura VIP 5 processor overnight on a routine 13-h cycle and embedded in paraffin. Complete transverse

5-μm sections were stained with hematoxylin and eosin to examine tissue architecture and organization.

Immunohistochemical staining was performed with a monoclonal mouse anti-human hepatocyte marker (Clone OCH1E5, HepPar-1, 1:200 dilution; Dako, Carpinteria, CA), followed by incubation with Dako Envision mouse polymer. The color development was completed by incubation with 3, 3′-diaminobenzidine (DAB)-chromogen (Dako), followed by counterstaining of nuclei with hematoxylin.

### In vitro *metabolism of 7-EC using human liver bioreactor and cell suspensions*

7-EC has been used extensively as a probe substrate for oxidative and conjugative metabolism as well as a marker to assess the metabolic competence for various *in vitro* systems, for example, isolated hepatocytes, precision-cut liver slices, recombinant DMEs, and microsomes.[21] In rats, *in vitro* characterization and *in vivo* pharmacokinetic performance of 7-EC have been well characterized.[21,22] Thus, *in vitro* prediction of *in vivo* clearance of 7-EC in human was examined using human liver bioreactor over the extended period of time.

7-EC (Sigma Aldrich, St. Louis, MO) stock solution was prepared in acetonitrile at 10 mM, and 40 μL of this stock solution was added to 40 mL of the HMM maintenance medium (final concentration of 10 μM, at 0.1% [v/v] acetonitrile in the culture medium). This substrate concentration for kinetic analysis was chosen from the range of steady-state plasma concentrations in rat to represent a concentration lower than the Michaelis constant ($K_m$).[21] Substrate incubations were initiated by exposing human hepatocytes of the liver bioreactor to 10 μM 7-EC delivered from the media reservoir bag at 0.25 mL $min^{-1}$. Aliquots of the medium were removed at 0.1, 0.2, 0.3, 0.5, 0.7, 1, 1.5, 2, 4, 6, 12, 24, 27, 30, 48, 51, 72, 75, 99, 120, 123, 144, and 147 h, followed by quenching with ice-cold acetonitrile (1:1, v/v). After centrifugation at 13,000 *g* for 10 min, the supernatant was stored at −20°C until further analysis. For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of 7-EC, 1 μM reserpine, as an internal standard, was added to the sample aliquot, followed by centrifugation at 2056 *g* for 10 min. The supernatants were transferred into a 96-well plate and subsequently analyzed by LC-MS/MS.

Primary human hepatocytes ($1 \times 10^6$ $mL^{-1}$) with an average viability of ∼91% suspended in the complete Williams' Medium E supplemented with 48 U $mL^{-1}$ penicillin G sodium, 48 U $min^{-1}$ streptomycin sulfate, ITS$^+$ (5 μg $mL^{-1}$ insulin, 5 μg $mL^{-1}$ transferrin, and 5 ng $mL^{-1}$ selenium-A), 1.9 mM glutamax, and 14.4 mM HEPES (Gibco) were preincubated in a reciprocating water bath at 37°C for 10 min. The reaction was initiated by the addition of 7-EC (final concentration of 10 μM) to hepatocyte suspension and further incubated for up to 2 h. The reaction was stopped by adding ice-cold acetonitrile (1:1, v/v). Samples were further prepared for LC-MS/MS analysis as described previously. At the end of the incubation period, the average viability of hepatocytes was ∼80%.

### *Liquid chromatography/mass spectrometry*

The concentration of 7-EC in media samples was determined using a Shimadzu high-performance liquid chromatography system and a CTC Leap HTC autosampler coupled with an API 4000 triple quadrupole mass spectrometer. Analytes were eluted from an XSelect CSH $C_{18}$ column (2.1 × 30 mm, 2.5 μ; Waters) using gradient LC conditions with a mobile phase A of 5 mM ammonium formate in water/formic acid (100:0.1, v/v) and a mobile phase B of acetonitrile/formic acid (100:0.1, v/v). A gradient LC method was used at a flow rate of 0.5 mL $min^{-1}$. The gradient was 0–0.5 min A:B = 75:25 (v/v), 0.5–1.5 min A:B = 0:100 (v/v), 1.5–2.4 min A:B = 0:100 (v/v), and 2.4–2.5 min A:B = 75:25 (v/v). The MS conditions were as follows: ion source polarity electrospray positive, spray voltage of 5000 V, vaporizer temperature of 600°C, collision gas ($N_2$) of 7 psig, curtain gas ($N_2$) of 20 psig, ion source gas ($N_2$) of 60 psig, and dwell time of 50 ms. 7-EC and reserpine were detected using the multiple reaction monitoring of mass transition pairs (m/z), that is, 191.1 → 163.0 for 7-EC and 609.3 → 195.0 for reserpine. Collision energies were 25 and 41 eV and retention times were ∼1.1 and ∼1.1 min, respectively, for 7-EC and reserpine. The injection volume was 3 μL, and the column temperature was maintained at 45°C throughout the analysis. Peak area ratios of 7-EC to reserpine were used to quantify the samples. 7-EC was linear with $1/x^2$ weighting over the range of 0.02 to 2 μM.

### *Data analysis*

Depletion of 7-EC over time in hepatocyte suspension cultures and flow chambers was fitted to monoexponential and bioexponential decay model, respectively, using one-phase and two-phase exponential decay equations with Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Goodness of fit was appraised by VICc, visual analysis of the data, residual plot, and the accuracy of parameter estimation.[23]

*In vitro* $CL_{int}$ was calculated by dividing the initial amount of 7-EC with the area under the concentration–time curve, followed by the normalization with incubation volume and the number of hepatocytes used for the study.[24] *In vitro* $CL_{int}$ values were scaled to equivalent *in vivo* $CL_{int}$ using the hepatocellularity number for adult human liver ($120 \times 10^6$ hepatocytes per gram of human liver).[25] *In vitro* $CL_{int}$ values from hepatocyte suspension were adjusted with the unbound fraction in hepatocyte incubations ($f_{u\ inc}$). The $f_{u\ inc}$ value was determined using Equation 1 established previously[26]:

$$\mathrm{Log}((1 - f_{u\ inc})/f_{u\ inc}) = 0.4\ \log D/P - 1.38 \quad \text{Equation 1}$$

where logD/P is the logP of the basic molecule ($pK_a > 7.4$). The values of $pK_a$ and logP of 7-EC are 7.84 and 1.85 (the Human Metabolome Database in 2013 and the Department of Computing Science and Biological Sciences, University of Alberta, Canada, respectively). Three independent experiments were performed with the human liver bioreactor and hepatocyte suspension.

## Results

### *Morphological integrity of human hepatocytes*

Pilot studies for optimal culture conditions showed that the most consistent and uniform attachment of cells across the surface of the woven scaffold was obtained using (1) a

seeding density of $15 \times 10^6$ cells per chamber, (2) a precoating of 0.1% gelatin solution (1 mg $mL^{-1}$), and (3) supplemented HMM as the medium (data not shown). Under these conditions, representative cultures of primary human hepatocytes were fixed and prepared for histological and immunohistochemical analysis (Fig. 1B–D). Transverse sections of a representative scaffold cultured for 37 days showed that hepatocytes attach along and around the fibers of the scaffold and form loose 3D cellular clusters of various sizes throughout the liver bioreactor (Fig. 1B). Inside the woven scaffold, multiple contiguous layers of hepatocytes aggregates were observed to be aligned with more stretched cells around the fibers of the woven matrix (Fig. 1C). The larger 3D cellular clusters formed, the longer lifespan of the liver bioreactor became. Immunostaining with HepPar-1 showed HepPar1-positive cells inside and around the woven scaffold (Fig. 1D). HepPar-1 is a specific marker for normal hepatocytes with no zonal preference and does not stain nonparenchymal cell types.[27] Elongated and flattened hepatocytes found around the fibers of the scaffold were also observed in previous studies using a BAL device containing pig hepatocytes, which reported that polygonal hepatocytes inside the matrix became more elongated and flattened using the fibers as an anchor.[12,13] The results in this study confirmed that the tissue-like arrangement of hepatocytes inside the liver flow chamber accounted for the increase in the lifespan of the culture, further enabling the stable biochemical and metabolic functions tested in the current study, whereas human hepatocytes loaded onto noncoated scaffolds mainly formed limited cell aggregates without 3D tissue-like structures (data not shown).

### *Biochemical parameters*

The results for the analysis of various biochemical and metabolic parameters, including glucose, lactate, AST, ALT, albumin, and total protein, in media samples collected from human liver bioreactor are shown in Figures 2–4. Glucose utilization among the three different preparations of cells was explicitly correlated with the levels of lactate production throughout the culture period. In Figure 2A–C, during the 8 days of the acclimation phase, the corresponding range values of glucose/lactate were as follows: HM10 (Fig. 2A) 100.9–127.3/3.7–32.3; with HM11 (Fig. 2B) 99.2–110.5/13.9–29.3; with HM12 (Fig. 2C) 151.1–163.7/9.1–12.1 (mg $L^{-1}$ $10^6$ hepatocytes$^{-1}$/mg $L^{-1}$ $10^6$ hepatocytes$^{-1}$, respectively).

During the remaining culture period, the corresponding range values between feedings were as follows: HM10 63.7–113.1/11.6–57.4; with HM11 56.7–125.7/11.0–80.9; and with HM12 137.4–183.9/3.2–22.5 (mg $L^{-1}$ $10^6$ hepatocytes$^{-1}$/mg $L^{-1}$ $10^6$ hepatocytes$^{-1}$, respectively). When assessing the rates of glucose consumption and lactate production, after the 24 h, the rates of glucose consumption and lactate production reduced from day 2 onward. After 8 days of acclimation, the rates of glucose consumption and lactate production by HM10 were parallelly increased up to 22 days, for HM11 on day 21, and for HM12 on day 24 with corresponding values of 17.9/10.6 (HM10), 20.7/15.1 (HM11), and 6.1/2.7 (HM12) (mg $day^{-1}$ $10^6$ hepatocytes$^{-1}$/mg $day^{-1}$ $10^6$ hepatocytes$^{-1}$, respectively). Subsequently, they reached the stable values during the remainder of the culture period, whereas temporary increase in the rates of glucose consumption and lactate production in HM10 was found on day 32 and day 32, by HM11 on day 45 and day 41, respectively, and by HM12 only the former on day 29. These results give evidence of the aerobic and anaerobic metabolic capacities by hepatocytes inside the liver bioreactor.

The production of total protein and albumin was measured in media samples (Fig. 3A–C). For the 8 days, total protein/albumin production among three donors varied

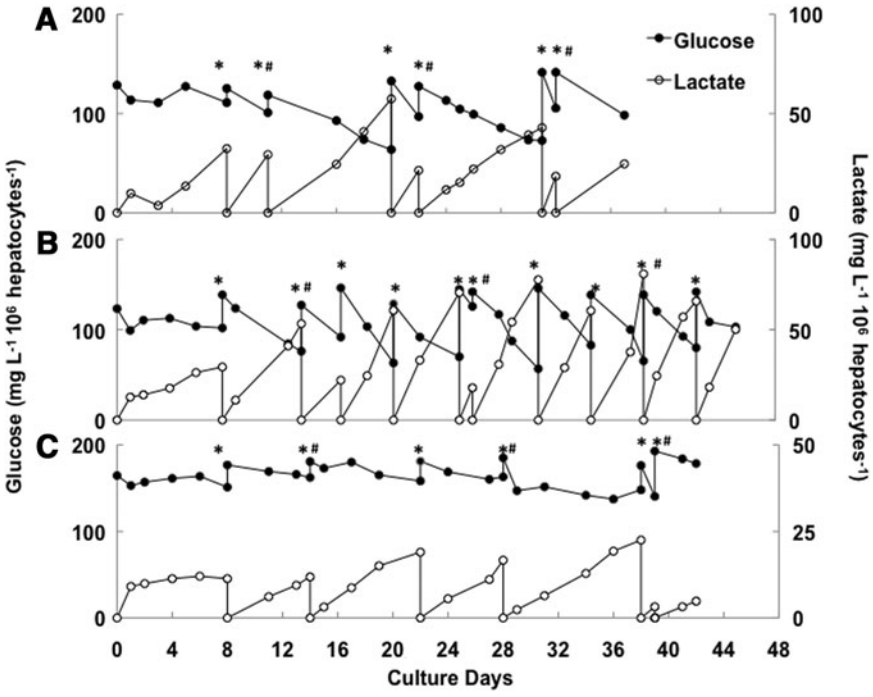


**FIG. 2.** Glucose utilization and lactate production of human hepatocytes from the donors of HM10 **(A)**, HM11 **(B)**, and HM12 **(C)** cultured in the liver bioreactor for 37, 42, and 47 days, respectively. *Media replacements. #Treatment with IPA-luciferin.

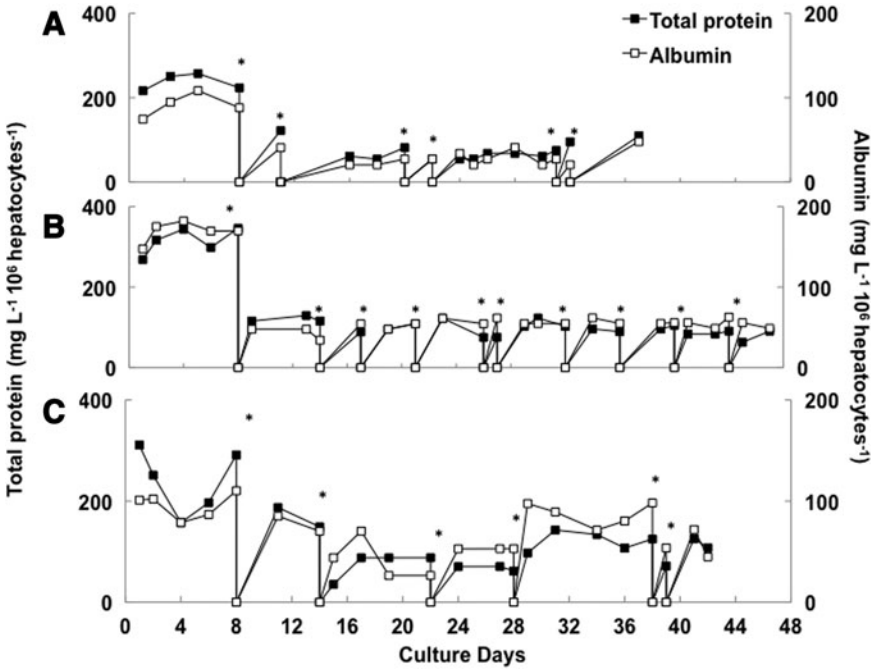


**FIG. 3.** Albumin and total protein production of human hepatocytes from the donors of HM10 **(A)**, HM11 **(B)**, and HM12 **(C)** cultured in the liver bioreactor for 37, 42, and 47 days, respectively. *Media replacements.

with the corresponding range values of 153.3–300/73.3–180 (mg $L^{-1}$ $10^6$ hepatocytes$^{-1}$/mg $L^{-1}$ $10^6$ hepatocytes$^{-1}$, respectively). After 8 days, total protein/albumin production reached the stable levels between media exchanges with the corresponding range values of 54.2–122.0/20.3–47.8 (HM10, 3A), 62.6–129.2/34.0–62.6 (HM11, 3B), and 35.1–187.2/26.3–97.5 (HM12, 3C) (mg $L^{-1}$ $10^6$ hepatocytes$^{-1}$/mg $L^{-1}$ $10^6$ hepatocytes$^{-1}$, respectively).

The circulating levels of AST and ALT that were associated with potential release of damaged and dead cells were also measured in media samples (Fig. 4A–C). After the 24 h static seeding, AST and ALT peaked initially but were significantly reduced from day 2 onward. After 8 days, the values of AST/ALT reached stable levels with the corresponding range values of 0.58–2.56/0.12–0.46 (HM10, Fig. 4A), 0.46–0.85/0.18–0.29 (HM11, Fig. 4B), and 0.18–0.85/0.09–0.28 (HM12, Fig. 4C) (U $L^{-1}$ $10^6$ hepatocytes$^{-1}$/U $L^{-1}$ $10^6$ hepatocytes$^{-1}$), respectively.

The pH values of the media samples collected from flow chambers ranged from 7.1 to 7.4 during the acclimation phase and remained within these ranges between subsequent media changes (data not shown). Additionally, the percentage of

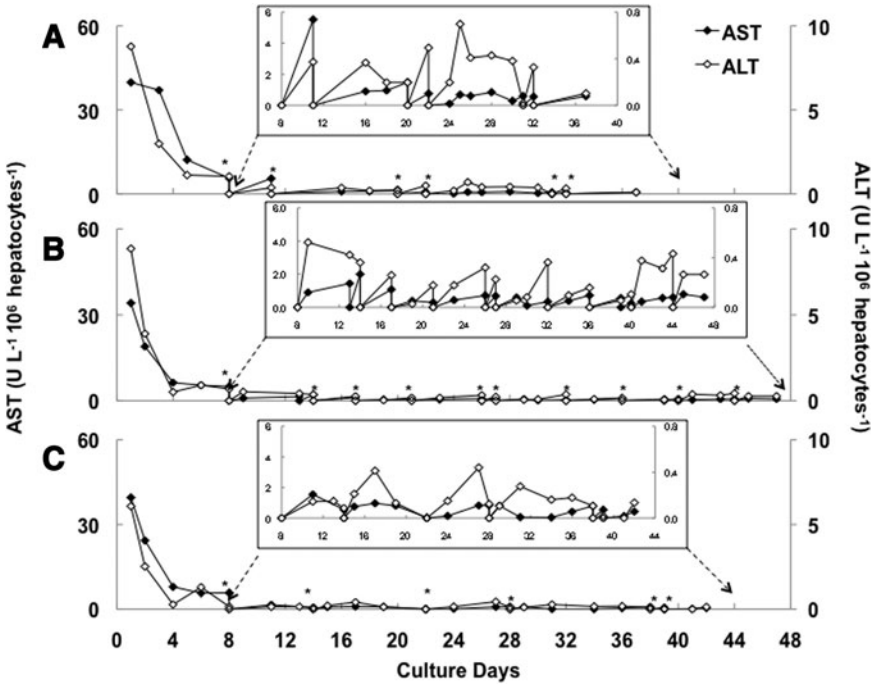


**FIG. 4.** The releases of alanine aminotransferases (ALT) and aspartate aminotransferases (AST) of human hepatocytes from the donors of HM10 **(A)**, HM11 **(B)**, and HM12 **(C)** cultured in the liver bioreactor for up to 37, 42, and 47 days, respectively. *Media replacements.

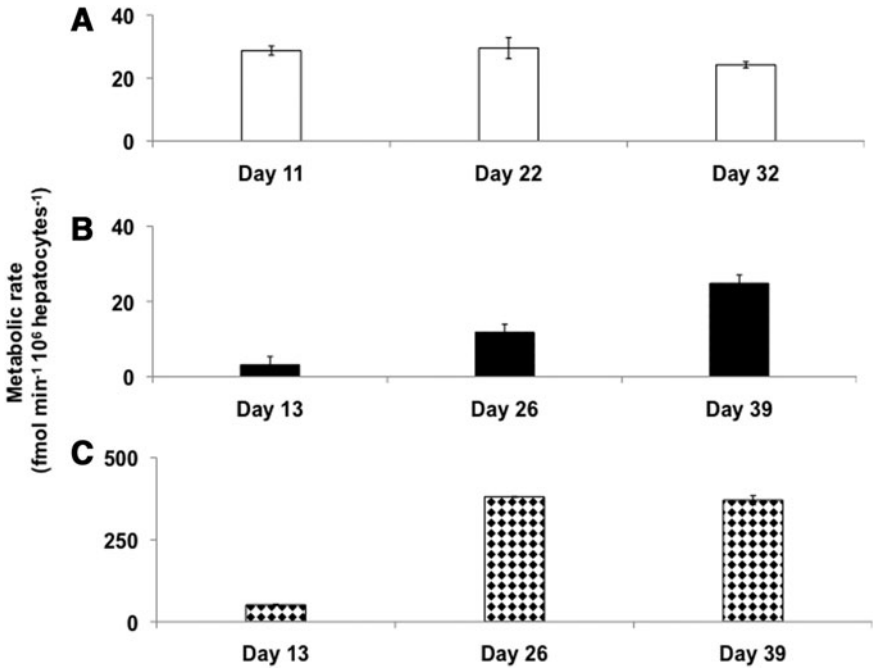


**FIG. 5.** The time profile of the CYP3A4 activity of human hepatocytes from the donors of HM10 **(A)**, HM11 **(B)** and HM12 **(C)** cultured in the liver bioreactor and measured at day 11, 22, and 33, at day 13, 26, and 39, and at day 13, 26, and 39, respectively. Values are mean ± standard deviation of technical replicates.

total hepatocyte loss from human liver bioreactor containing cells from HM10 and HM12 (1.5% and 9.7%) were approximately twofold higher during the 8-day acclimation phase than those (0.8% and 4.7%, respectively) during the remaining culture period. The percentage loss from the flow chamber with cells from HM11 (1.9%) during the acclimation phase was comparable to the loss after that period (2.3%, respectively) (data not shown).

### *The measurement of CYP3A4 activity*

The CYP3A4 activity of human hepatocytes inside the liver bioreactor was determined by measuring the production of luciferin from luciferin-IPA (Fig. 5A–C). Differences in time-dependent profiles of the CYP3A4 activity were observed among the 3 different batches of cells. The metabolic rates for the production of luciferin by cells from HM10 (Fig. 5A) were consistent for up to 32 days with the corresponding values of 28.7, 29.5, and 24.2 (fmol $min^{-1}$ $10^6$ $hepatocytes^{-1}$) at day 11, 22 and 32, respectively. HM11-derived hepatocytes (Fig. 5B) showed an increase in the CYP3A4 activity over time with the corresponding values of 3.1, 11.7, and 24.8 (fmol $min^{-1}$ $10^6$ $hepatocytes^{-1}$) at 13, 26, and 39 days in the culture, respectively. The CYP3A4 activity of hepatocytes from HM12 (Fig. 5C) increased for up to 26 days and reached a stable level during the remainder of the culture period with corresponding values of 51.5, 380.5, and 370.6 (fmol $min^{-1}$ $10^6$ $hepatocytes^{-1}$) at day 13, 26 and 39, respectively.

### *Determination of $CL_{int}$ of 7-EC*

*In vitro* $CL_{int}$ of 7-EC was determined by a substrate depletion over time in primary human hepatocytes in suspension culture and the human liver bioreactor. The values of *in vitro* $CL_{int}$, predicted *in vivo* $CL_{int}$, and half-life ($t_{½}$) are shown in Table 2. The values for *in vitro* $CL_{int}$ derived from the liver bioreactor varied among the three different

TABLE 2. *IN VITRO* $CL_{int}$ OF 7-ETHOXYCOUMARIN USING HUMAN HEPATOCYTE SUSPENSION FROM THE DONORS OF HM15, HM16, AND HM17 AND THE LIVER BIOREACTOR WITH THE DONORS OF HM13, HM14, AND HM15, RESPECTIVELY, A HALF-LIFE ($t_{½}$) AND THE PREDICTION OF *IN VIVO* $CL_{int}$

| *Human liver bioreactor* | | | | *Human hepatocyte suspension* | | | |
|---|---|---|---|---|---|---|---|
| *Donor ID* | $t_{½}$ *(h)* | In vitro $CL_{int}$ *(mL* $min^{-1}$ $10^6$ $HC^{-1}$*)* | *Predicted* in vivo $CL_{int}$ *(mL* $h^{-1}$ *g of* $liver^{-1}$*)* | *Donor ID* | $t_{½}$ *(h)* | In vitro $CL_{int}$ *(mL* $min^{-1}$ $10^6$ $HC^{-1}$*)* | *Predicted* in vivo $CL_{int}$ *(mL* $h^{-1}$ *g of* $liver^{-1}$*)* |
| HM13 | 20.9 | 5.1 | 10.3 | HM15 | 2.8 | 100.4 | 200.9 |
| HM14 | 18.1 | 26.0 | 52.1 | HM16 | 0.16 | 203.4 | 406.9 |
| HM15 | 27.9 | 14.1 | 28.2 | HM17 | 0.23 | 176.5 | 353.0 |
| | | 15.1 ± 10.4 (average ± SD) | 30.2 ± 20.9 (average ± SD) | | | 160.1 ± 53.4 (average ± SD) | 320.3 ± 106.8 (average ± SD) |

Values are mean ± SD.
$CL_{int}$, intrinsic clearance; HC, human hepatocytes; SD, standard deviation.

donors ranging from 5.1 to 26.0 mL $min^{-1}$ $10^6$ hepatocytes$^{-1}$, respectively. The values obtained from the liver bioreactor with HM14 (26.0 mL $min^{-1}$ $10^6$ hepatocytes$^{-1}$) and HM15 (14.1 mL $min^{-1}$ $10^6$ hepatocytes$^{-1}$) were 5.0- and 2.7-fold higher than that exhibited by HM13 (5.1 mL $min^{-1}$ $10^6$ hepatocytes$^{-1}$) with the corresponding half-lives of 18.1 (HM13), 27.9 (HM14), and 20.9 h (HM15). Predicted *in vivo* $CL_{int}$ of 7-EC was 10.3, 52.1, and 28.2 mL $h^{-1}$ g of liver$^{-1}$ for flow chambers containing cells from HM13, HM14, and HM15, respectively.

Additionally, the effect of 7-EC on the biochemical parameters of human hepatocytes inside the flow chamber was monitored during 6 days of treatment. The levels of glucose utilization, lactate production, albumin/total protein production, and the release of AST/ALT were comparable in a control and 7-EC treated liver bioreactors (data not shown).

The values of *in vitro* $CL_{int}$ obtained from hepatocyte suspensions from HM16 and HM17 (203.4 and 176.5 mL $min^{-1}$ $10^6$ hepatocytes$^{-1}$, respectively) were 2.0- and 1.7-fold higher than that from HM15 (100.4 mL $min^{-1}$ $10^6$ hepatocytes$^{-1}$), with corresponding half-lives of 2.84 (HM15), 0.16 (HM16), and 0.23 h (HM17). Predicted *in vivo* $CL_{int}$ was 200.9, 406.9, and 353.0 mL $h^{-1}$ g of liver$^{-1}$ for suspension cultures containing cells from HM15, HM16, and HM17, respectively. Additionally, the accuracy of prediction of 7-EC $CL_{int}$, given as the ratio of *in vitro* $CL_{int}$ (predicted) and *in vivo* $CL_{int}$ (observed),[24] cannot be assessed further because there are no data in the literature reporting on *in vivo* $CL_{int}$ of 7-EC in human.

## Discussion

Recently, a focus has been placed on using more physiologically relevant and metabolically competent hepatic model systems for pharmaceutical and environmental chemical safety testing and risk assessment.[28] In this study, a 3D dynamic flow model with primary human hepatocytes was established and characterized for liver-specific properties and cellular reorganization over an extended period of time. Furthermore, the quantitative metabolic activity of the system was assessed and compared with hepatocyte suspension culture using *in vitro* to *in vivo* extrapolation (IVIVE) of 7-EC clearance. The results observed represent the most relevant human liver bioreactor described to date with *in vivo*-like cell organization, hepatocyte viability, and liver-specific properties maintained for ~7 weeks.

In the bioreactor, human hepatocytes reproducibly recapitulated 3D liver-like structures and liver-specific functions regardless of the inherent interindividual variability in the donor cells. The defined culture conditions, including gelatin coating of the polyester scaffold, addition of FBS supplement, and use of serum-free maintenance medium, allowed consistent cellular attachment and reorganization into 3D tissue-like structures, which was correlated with the stabilized liver functions, including albumin/total protein production, glucose utilization and lactate production, and CYP3A4 activity for extended period of time. These results are in agreement with earlier studies that found culturing hepatocytes as 3D structures promotes an *in vivo*-like culture environment, enabling the retention of important hepatic functions without the addition of ECM components.[7,29] It was also reported that serum-free media after cell aggregation supports the reproducible expression of liver-specific properties and phenotypes because serum affects both albumin synthesis and CYP activity of primary human hepatocytes.[30]

Noninvasive measurements of biochemical parameters from the liver bioreactor were utilized to help monitor cell health and functionality over an extended period of time. After the initial acclimation phase (~1 week), a consistent time course of glucose utilization and lactate production was observed for over 1 month (up to 47 days of culture with some individual chambers) (Fig. 2A–C). The initial high peak of AST and ALT releases and the corresponding low levels of glucose metabolism were observed during the acclimation phase, which was in agreement with earlier studies, which reported that human hepatocytes inside hollow fiber liver bioreactors showed high releases of AST and low rates of glucose metabolism at the beginning of the culture.[1,15] The substantial production of albumin (Fig. 3A–C) was proportionally correlated with the stabilized levels of ALT and AST (Fig. 4A–C). After 8 days, the stable levels of albumin production among three individual donors (HM10, 20–46.6; HM11, 33.3–60; and HM12, 26.6–93.3, mg $L^{-1}$ $10^6$ cells$^{-1}$, respectively) were considerably higher than that in a previous study with human liver cell spheroid (0.005–0.0215 mg $dL^{-1}$ $10^6$ cells$^{-1}$).[31]

The time profile of CYP3A4 activities for HM10, HM11, and HM12 showed substantial interindividual variability (Fig. 5A–C). The initial level of CYP3A4 toward luciferin-IPA in chambers with cells from HM10 was stably maintained for up to 22 days and then declined slightly to 84% of the initial level on day 32 of the culture. However, time-dependent increases in the CYP3A4 activity were observed with chambers containing cells from HM11 and HM12, with the latter reaching a plateau on day 39. The observed difference in the metabolic rate toward luciferin-IPA, a substrate for CYP3A4, among the three different individuals for an extended period of time might be explained by the frequency of media change[11] and an adequate oxygenation[6] before and after treatments, the duration of the treatment, and cell preparation from different donors with heterogeneity in phenotypes and genotypes.[2] The time-dependent increases in the CYP activity seen in the current study are similar to previous reports in which the activities of 7-ethoxyresorufin O-deethylation (EROD) and 7-EC O-deethylation increased in primary human and rat hepatocytes cultured in a hollow fiber liver bioreactor or a perfused 3D-encapsulated cell system, respectively, for 8 days.[11,15] On the other hand, it also has been reported that overall activities of CYP isoforms of human hepatocytes inside a hollow fiber bioreactor were decreased over time, especially CYP3A4 (~30% loss of initial levels) at day $10^1$ and CYP2C9 activity (~65% loss of initial levels) at 10–23 days of the culture.[2] The results of this study suggest that an increased durability of hepatocyte integrity and functionality is achieved under dynamic culture conditions employed for these experiments.

*In vitro* $CL_{int}$ of 7-EC was compared between human hepatocyte suspension cultures and cells in the liver bioreactor (Table 2). A substantial difference in *in vitro* $CL_{int}$ of 7-EC was observed between individual batches of cells and across two different *in vitro* liver models used. Individual variations in *in vitro* $CL_{int}$ values from the human liver

bioreactor differed approximately 5-fold among three different donors and a twofold variation was observed in hepatocyte suspensions. The variability in $CL_{int}$ values most likely reflects human variations in 7-EC metabolism due to the genotypes and phenotypes of CYP isoforms and UDP-glucuronosyltransferases and sulfotransferases involved in its metabolism.[31–33] As shown in Table 2, an average value of *in vitro* $CL_{int}$ in human liver bioreactor (15.1) was approximately 10-fold less than that in hepatocyte suspension (160.1 mL $min^{-1}$ $10^6$ hepatocytes$^{-1}$, respectively). Also, a 10-fold difference was observed in the HM15 donor that was used in both the bioreactor and suspension measurements. The obvious explanation of this difference is the expression of metabolic enzymes in the two different systems. Previous studies have also shown differences in clearance estimates between suspension, 2D, and 3D culture systems.[1,34–36] The *in vitro* $CL_{int}$ of propranolol using rat hepatocyte suspension for a 2 h incubation was considerably higher than that in hepatocyte monolayer culture in accordance to initial higher activity of CYP2C9.[34] In addition, Hoffmann *et al.*[1] also reported that the rates of diclofenac disappearance and its metabolites production in human hepatocyte monolayer culture were initially higher than that in perfused hollow fiber bioreactor on day 1 of the culture, followed by the rapid decrease in its metabolic rate over time.

In comparison with previous studies, the average predicted *in vivo* $CL_{int}$ of 7-EC obtained using the liver bioreactor (30.2 mL $h^{-1}$ g of liver$^{-1}$) is comparable to the value from the recent study by Lu *et al.*[35] using primary human hepatocyte suspension (53.3 mL $h^{-1}$ g of liver$^{-1}$), but less than the study by Somers *et al.*[36] (81.9 mL $h^{-1}$ g of liver$^{-1}$, respectively). In contrast, the average *in vivo* $CL_{int}$ value obtained from human hepatocyte suspension in this study (320.3 mL $h^{-1}$ g of liver$^{-1}$) was higher than those in the recent studies with hepatocyte suspension,[35,36] which may reflect individual variations in the metabolic capacity of 7-EC and expression of the metabolic enzymes in the various culture models. In our hands, the 3D flow model described in this study appears to give more reproducible and stable values for 7-EC clearance that is similar to other *in vitro* data.[35,36]

In conclusion, the human liver bioreactor used in this study reproducibly achieved the long-term maintenance of liver-specific features and sustained functional properties, such as CYP3A4 activity, albumin/total protein synthesis, and basal metabolism for $\sim$7 weeks. IVIVE of 7-EC clearance and monitoring its effect on liver-specific properties over prolonged culture periods support the use of this model for obtaining more accurate predictions of *in vivo* clearance of chemicals, especially chemicals that exhibit low hepatic clearance. In addition, the capability to perform long-term cultures allows the evaluation of repeat-dose hepatocellular injury, adaptive recovery, and ultimate hepatotoxicity. Further studies with this human liver bioreactor are required to explore the most relevant conditions, such as exposure time and concentration profiles for toxicological/pharmacological studies of chemicals and their major metabolites. This system could also be utilized to investigate the impact of 3D tissue organization and flow dynamics on hepatic properties under physiological and pathological conditions supported by heterotypic cell interactions, for example, hepatocyte co-culture with endothelial cells, monocytes, or Kupffer cells.[15,31,37]

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## Disclosure Statement

No competing financial interests exist.

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