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Laminar-flow immediate-overlay hepatocyte sandwich perfusion system for drug hepatotoxicity testing

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ABSTRACT

Drug hepatotoxicity testing requires *in vitro* hepatocyte culture to maintain the long-term and stable liver specific functions. We developed a drug testing platform based on laminar-flow immediate-overlay hepatocyte sandwich perfusion culture. The immediate-overlay sandwich (collagen-coated porous polymeric membrane as top overlay) protects the cells and integrity of the top collagen matrix from the impact of flow. A bioreactor was designed that allowed proper control of shear stress and mass transfer. The culture parameters such as the optimal perfusion initiation time and flow rate were systematically and mechanistically determined. The optimized system could re-establish hepatocyte polarity to support biliary excretion and to maintain other liver specific functions, such as the biotransformation enzyme activities, for two weeks that extended the usable *in vitro* hepatocyte-based drug testing window. When the perfusion cultured hepatocytes from days 7 or 14 were used for drug testing, the APAP-induced hepatotoxicity measurements were more sensitive and consistent over time than the static culture control, enabling further exploitations in large-scale drug testing applications.

1. Introduction

The assessment of drug toxicity to liver is crucial for new drug development. The use of isolated hepatocytes as an *in vitro* liver model facilitates the prediction of hepatotoxicity in the early phase of drug development [1,2]. Due to the scarcity of liver donors and the variation of liver functions among individual donors, hepatocytes from different batches of isolation are combined into a large cell pool so that batch-to-batch variation of cellular functions can be standardized [3]. The pooled hepatocytes are typically stored via cryopreservation; one aliquot is thawed for function validations and the rest of the lot is used for large-scale drug testing [4,5]. However, many cryopreserved cells are not plateable (low attachment efficiency) with the loss of some important liver specific functions such as transporter activities [6]. Ideally, the pooled hepatocytes should be cultured using long-term culture techniques to maintain stable cellular functions so that a small portion is used for functional validation while the rest is ready for drug testing at any time point during the culture period without freeze-and-thaw step. Long-term culture techniques can also extend the usable *in vitro* drug testing window so that drug treatment time can be long enough to yield cytotoxic effects [7]. Hepatocyte spheroid [8,9] and hepatocyte sandwich culture [10,11]

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are the two most promising long-term culture techniques exploited for drug testing. Hepatocyte spheroid culture suffers from mass transfer deficiency and uneven drug access because spheroid size varies making it difficult to control various parameters for drug testing applications [12]. Hepatocyte sandwich culture is widely used in drug metabolism and toxicity tests [13,14] since hepatocyte biotransformation enzyme functions [15] and transporter activities [16] are well maintained *in vitro*.

The hepatocyte sandwich perfusion culture can further improve long-term liver specific functions *in vitro*, due to the improved transport of oxygen and nutrients to the cell surface and effective waste removal from cellular local environment [17,18]. Perfusion bioreactors have been developed based on conventional sandwich culture which has a hepatocyte monolayer overlaid by a collagen gel layer 24 h after seeding [18–22]. However, the perfusion flow would introduce the hepatocyte culture to the effect of fluid-induced shear stress not typically encountered by the cells in natural liver where hepatocytes are shielded by a layer of sinusoidal endothelial cells from the direct shear of the blood flow. High shear stress in perfusion culture could be detrimental to hepatocyte viability and *in vitro* functions [23,24]. In addition, the integrity of the top collagen layer in direct contact with media flow may be compromised by the long-term perfusion that leads to the degeneration of sandwich matrix and, subsequently, the variation in mass transport of drug access during drug testing. We have previously developed an immediate-overlay sandwich culture in which the top gel layer of the conventional sandwich was replaced by a collagen-coated porous polymeric membrane to reinforce the sandwich structure [25]. Hepatocyte liver specific functions are greatly improved from the conventional sandwich culture due to the immediate presentation of top layer as repolarization cue. Here, we have developed a laminar-flow perfusion bioreactor for immediate-overlay sandwich culture that minimizes shear stress and preserves the mass transport consistency. The cultured hepatocytes exhibited restored cell polarity, biliary excretion and maintenance of metabolic functions for two weeks. Cells could produce consistent drug toxicity responses at different time points during the culture allowing further exploitations in large-scale drug testing applications.

2. Materials and methods

All reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise stated.

2.1. Collagen coating

80 μl of neutralized 1.5 mg/ml Type I Bovine dermal collagen (8 ml collagen, 1 ml 0.1 M NaOH, 1 ml 10 × PBS, 6 ml 1 × PBS) from Vitrogen, Angiotech BioMaterials Corp. (Palo Alto, CA) was spotted onto hydrophobic petri dishes and 12 mm polycarbonate membranes (Millipore Corp., Billerica, MA) were placed on the droplets for 20 min before transferred into a 37 °C incubator overnight for collagen gelation to occur.

2.2. Hepatocyte isolation and culture

Hepatocytes were harvested from male Wistar rats weighing 250–300 g by a two-step in situ collagenase perfusion method [26]. 200–300 million cells were isolated with viability above 90% as determined by Trypan Blue exclusion assay. Freshly isolated rat hepatocytes (0.18×10^6) were seeded on the collagen-coated membranes with an exposed diameter of 9 mm for 1.5 h. Hepatocytes were then immediately overlaid with collagen-coated inserts that were secured using the O-rings on the minusheet carriers (Minucells and Minutissue Vertriebs GmbH, Bad Abbach, Germany). Hepatocytes were cultured with Hepatozyme-SFM (Gibco Laboratories, Carlsbad, CA), supplemented with 39 ng/ml dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin. For inhibition of biliary excretion in hepatocytes, 2 μg/ml colchicine or 16 μg/ml anti-E-cadherin antibody, ECCD-1 (for adhesion inhibition) (Zymed Laboratories, CA) was added to the culture media after cell seeding.

2.3. Flow perfusion bioreactor

A schematic of the flow perfusion bioreactor is shown in Fig. 1. The bioreactor consisted of an acrylic body and top which was sealed with an O-ring. The culture chamber was 14.4 mm in diameter and had a channel height of 1 mm above each sandwich cassette in the cellular compartment. The bioreactor had an oxygen permeable membrane (Breathe-Easy®, Diversified Biotech, Boston, MA, USA) above the cellular compartment. Each bioreactor was connected by gas-permeable silicone tubing to a multi-channel peristaltic pump (Ismatec SA, Glattbrugg, Switzerland) and a recirculating media reservoir maintained at 37 °C.

2.4. Modeling fluid flow

A finite element approach was adopted in order to evaluate the velocity profile and the mean fluid shear stress. To simulate the flow, we used the commercial CFD package (ANSYS Inc, Canonsburg, PA, USA) to numerically solve the steady-state Navier-Stokes equations. Fluid properties were set to those of the culture media and the culture medium was modeled as an incompressible, isothermal, Newtonian fluid with a density of 1×10^3 kg/m^3 and a dynamic viscosity of 8.89×10^{-4} Pa s. Flow rates of 0.1, 0.06, 0.03 and 0.018 ml/min were simulated for comparison of mean shear stress, while flow rate of 0.03 ml/min was simulated for velocity profile. Outlet boundary condition was set at zero pressure outflow, and the no-slip walls boundary conditions were used along the walls of the model.

2.5. Release rate of dextrans

Measurement of the release rate of fluorescein isothiocyanate (FITC)-conjugated dextrans of 150 kDa, 70 kDa and 9.5 kDa under perfusion and static conditions through the collagen-coated membranes were achieved by sandwiching 15 μl of 2 mg/ml FITC-dextran and measuring the amount of FITC-dextran released into the surrounding media every 30 min from 2 to 4.5 h after initiation. The concentrations of FITC-dextran were measured at 490 nm (excitation)/525 nm (emission) against FITC-dextran standards using a microplate reader (Tecan Safire^2, Tecan Trading AG, Switzerland).

2.6. Immunofluorescence microscopy

Primary anti-CD147 mouse monoclonal antibody (mAb) was purchased from Serotec, Inc. (Raleigh, NC), primary anti-Mrp2 rabbit polyclonal antibody (pAb) from Sigma–Aldrich and secondary Pacific Blue goat anti-rabbit and Alexa Fluor 635 goat anti-mouse from Molecular Probes (Eugene, Oregon). 3.7% paraformaldehyde-fixed samples were blocked in 10% fetal calf serum (FCS) at room temperature for 1 h. Samples were incubated with the primary antibodies (1:10) overnight at 4 °C, before being rinsed with 1 × PBS thrice each lasting 5 min. Samples were then incubated with the secondary antibodies at room temperature for 1 h and rinsed with 1 × PBS before being mounted with FluorSave™ (Calbiochem, San Diego, CA). The samples were viewed with a confocal microscope (Fluoview 500, Olympus, Melville, NY) using 60× water lens.

2.7. Biliary excretion of fluorescein

For visualization of fluorescein excretion, 3 μg/ml fluorescein diacetate (Molecular Probes, Eugene, Oregon) in culture media was incubated with the cultures at 37 °C for 45 min. The cultures were then rinsed and fixed before viewing under a confocal microscope (Fluoview 300, Olympus, Melville, NY) using a 40× water lens.

2.8. Actin staining with SYTOX Green nuclear counter-stain

For visualization of actin, cells were first permeabilized with 0.1% Triton-X at room temperature for 10 min. The samples were then blocked with 2.5% FCS for 10 min and incubated with 0.2 μg/ml rhodamine–phalloidin at room temperature for 20 min. For nuclear counter-stain, cells were incubated with 200 nM SYTOX Green and 100 ug/ml RNase diluted in PBS at room temperature for 15 min.

2.9. Hepatocyte functional assays

All functional data were normalized to 10^6 cells based on the number of seeded cells. The daily albumin production was measured using the Rat Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, Texas). The urea production of the hepatocyte cultures incubated in culture media with 2 mM NH4Cl for 90 min was measured using the Urea Nitrogen Kit (Stanbio Laboratory, Boerne, Texas). The 7-ethoxyresorufin-O-deethylation (EROD) assay, which is a measure of the deethylation activity of cytochrome P450 (CYP) 1A-associated monooxygenase enzymes, was initiated by incubating the cultures with 39.2 μM 7-ethoxyresorufin in culture media at 37 °C for 4 h. The amount of resorufin converted by the enzymes was calculated by measuring the resorufin fluorescence in the incubation media at 543 nm (excitation)/570 nm (emission) against resorufin standards using the microplate reader (Tecan Trading AG, Switzerland).

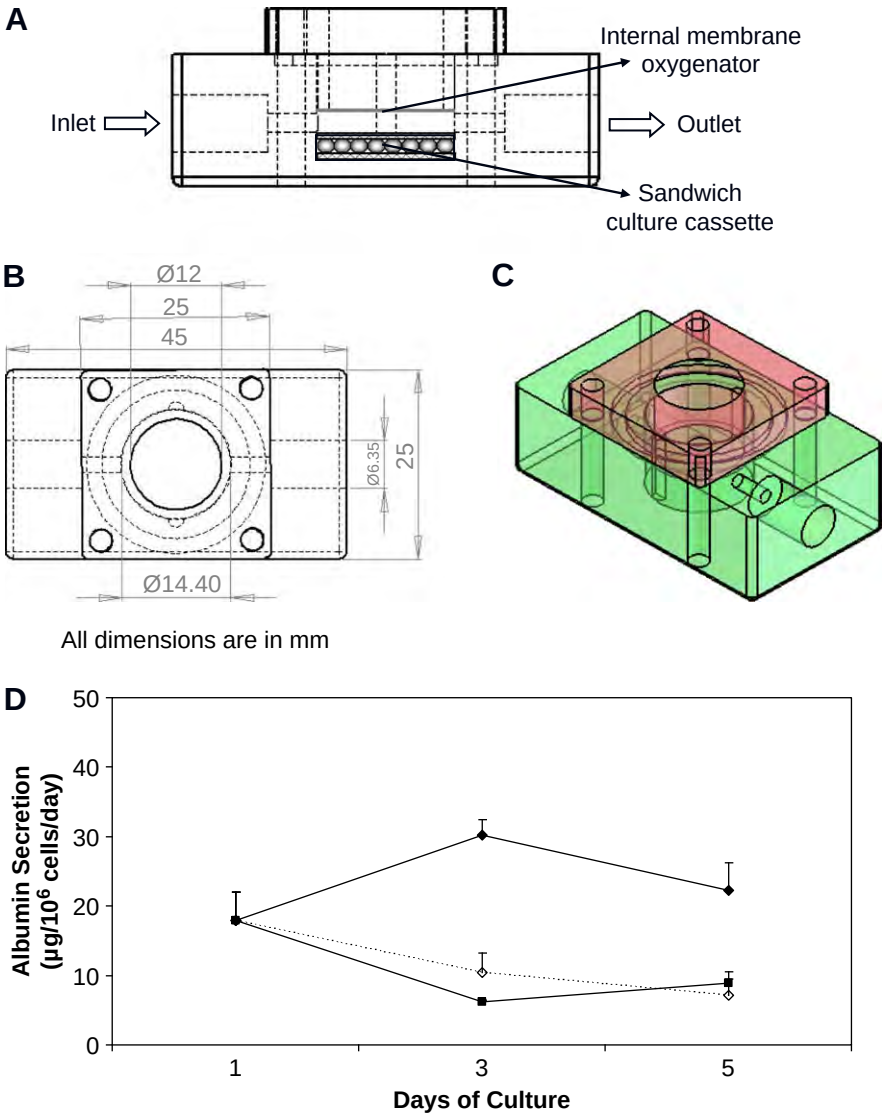

Fig. 1. Schematic of laminar-flow perfusion bioreactor. (A): Cross-sectional view; (B): top view, dimensions are in mm; (C): assembled bioreactor; (D): Influence of internal membrane oxygenator on the albumin secretion of hepatocytes. (◇) Static; (◆) perfusion with oxygenator; (■) perfusion without oxygenator.

2.10. Drug-induced hepatotoxicity testing

Hepatocyte cultures were used for drug testing on day 7 and day 14, and treated with 25 mM acetaminophen (APAP) for 24 h. 5 M of drug stock solution dissolved in DMSO was diluted with culture medium to the final concentration. The DMSO final concentration was less than 1%. Cell viability was measured by MTS assay using the CellTilter 96 Aqueous One Solution Reagent (Promega, USA).

2.11. Statistical method

The Student *t*-test was used to analyze the statistical significance of the data between two groups. One-way Analysis of Variance (ANOVA) was used when there were several groups for observation. Values with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Flow perfusion bioreactor

Fig. 1 shows the schematic of the perfusion bioreactor with the hepatocyte sandwich culture placed at the bottom of the culture chamber. The round shape of the bioreactor chamber is to ensure a uniform laminar-flow distribution during perfusion. Culture media was perfused through the top surface of the cell culture mimicking the *in vivo* blood flow configuration. Oxygen supply is an important limiting factor for hepatocytes under standard culture conditions [27]. In order to improve the delivery of oxygen to the surface of the cell culture, an oxygen permeable membrane was placed above the cellular compartment in the bioreactor (Fig. 1A). This internal oxygen supply configuration allowed the decoupling of oxygen supply from the fluidic perfusion. Albumin secretion was used as a representative liver specific function of hepatocytes in the perfusion optimizations. The albumin secretion of hepatocytes in perfusion culture with the internal membrane oxygenator was higher than that without oxygenator (Fig. 1D).

3.2. Initiation of perfusion culture

To optimize the perfusion initiation time, a comparison was made between the sandwich culture that was perfused immediately upon assembly and the sandwich culture that was perfused one day after assembly. Hepatocytes in the immediate perfusion culture exhibited irregular cell morphology (Fig. 2A) with large vacuoles (arrowheads, Fig. 2A–C) at 48 h of culture. This was

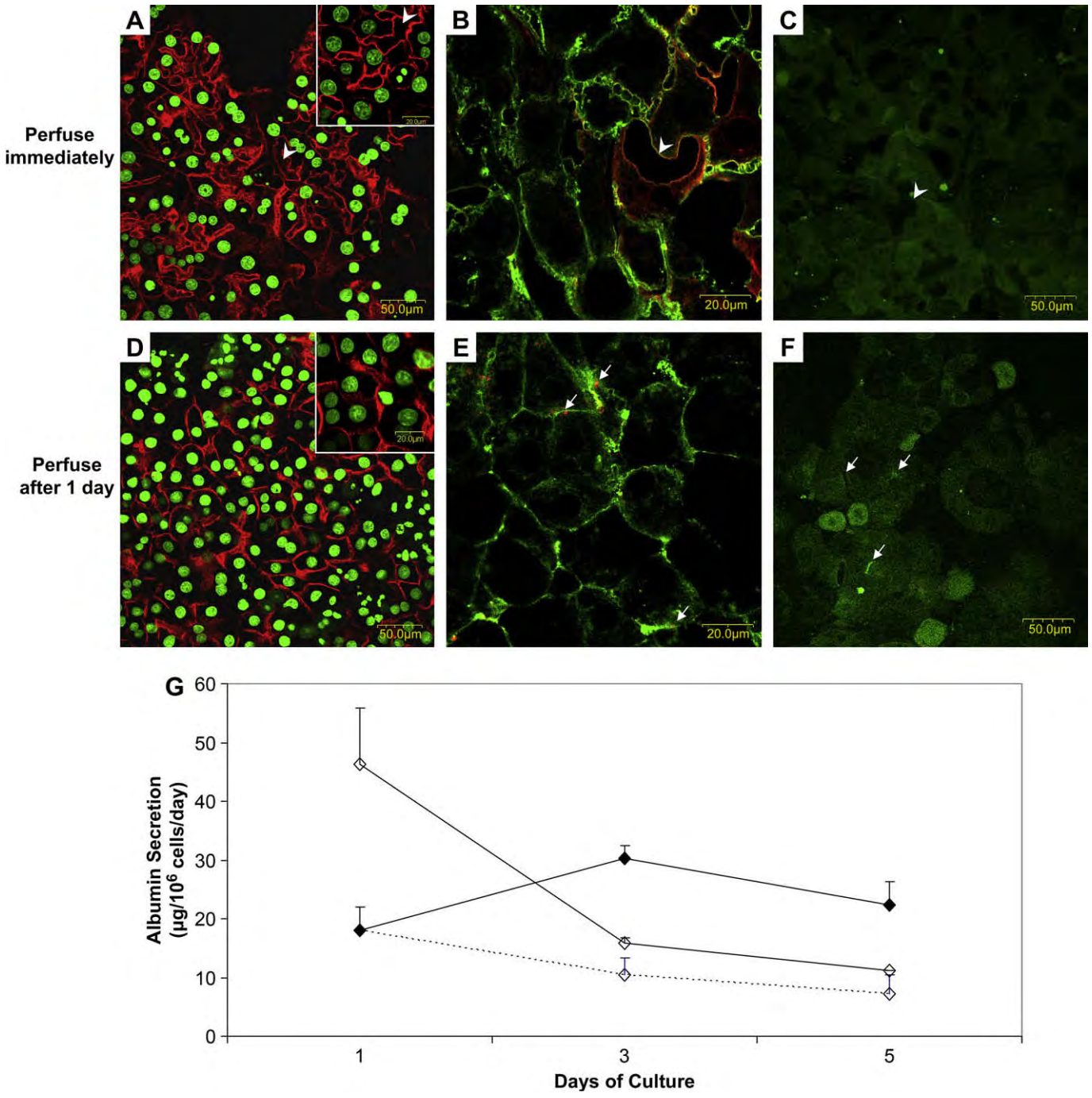

Fig. 2. Influence of static pre-stabilization before perfusion initiation on cell morphology and biliary excretion at 48 h of culture, and albumin secretion at the flow rate of 0.1 ml/min. (A–F): Top row: perfuse immediately; bottom row: perfuse after 1 day of static pre-stabilization. (A) and (D): Actin cytoskeleton (red) with nuclear counter-stain (green), inset shows a magnified view of a portion of the image; (B) and (E): immunostaining of apical transporter Mrp2 (red) with basolateral marker CD147 (green); (C) and (F): localization of fluorescein, a dye excreted by hepatocytes. Arrowheads point to vacuoles while full arrows point to bile canaliculi. (G): Influence of static pre-stabilization on the albumin secretion of hepatocytes. (-◇-) Static; (-◇-) Perfuse immediately; (-◆-) Perfuse after one day of static pre-stabilization. (H–P): Top row: cultures without ECCD-1 and colchicine treatment; Middle row: cultures with ECCD-1 and without colchicine treatment; Bottom row: cultures without ECCD-1 and with colchicine treatment. (H), (K) and (N): Actin cytoskeleton (red) with nuclear counter-stain (green). Arrows point to the bile canaliculi structure. (I), (L) and (O): immunostaining of apical transporter Mrp2 (red) with basolateral marker CD147 (green). Arrows point to Mrp2 localization. (J), (M) and (P): localization of fluorescein. Arrows point to fluorescein accumulating at bile canaliculi. [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

accompanied by disrupted polarity, particularly close to the vacuoles, shown by the diffuse staining of the apical transporter Mrp2; and loss of ability to excrete fluorescein (Fig. 2B and C). In contrast, hepatocytes perfused after one day of pre-stabilization in static culture maintained good cellular morphology (Fig. 2D) and polarity as seen by the localized apical distribution of Mrp2 (Fig. 2E); and regained the ability to excrete fluorescein (Fig. 2F). The improvement in albumin secretion of hepatocytes under perfusion was maintained only when they were perfused after one day of pre-stabilization in static culture (Fig. 2G). For immediate perfusion, although the albumin secretion was initially stimulated, it rapidly declined to the similar level as that of the static culture.

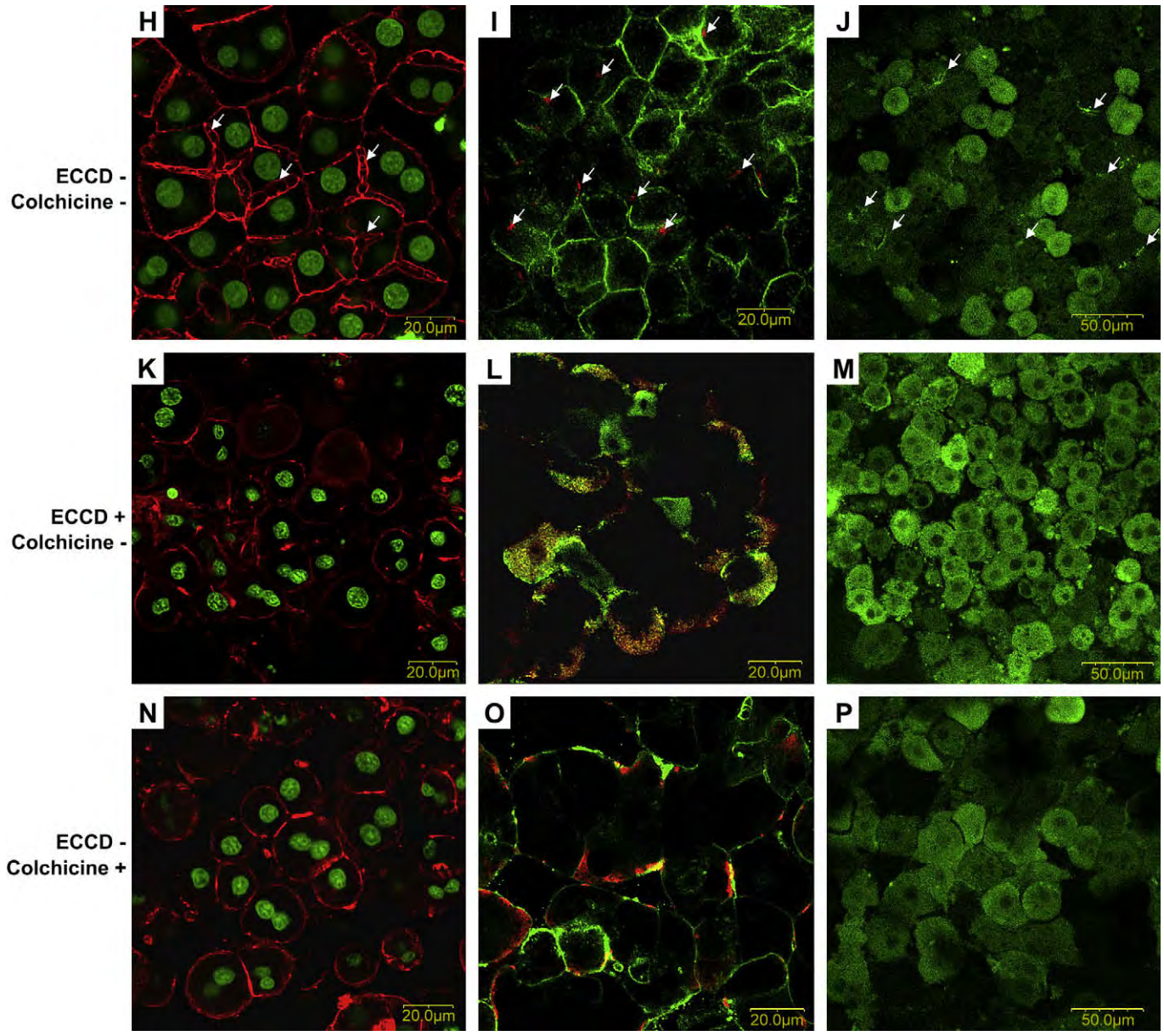

Fig. 2. *Continued*

The above observation may imply that extra-cellular cues in the immediate-overlay hepatocyte sandwich culture could be disrupted by immediate initiation of perfusion culture upon sandwich assembly prior to stabilization of the cell polarization essential for biliary excretion. To investigate this observation, two different reagents, ECCD-1 and colchicine, were used to inhibit polarity and biliary excretion at the different stages of polarity regeneration [28,29]. ECCD-1, the adhesion-inhibitory anti-E-cadherin antibody may disturb cell-cell interaction [30,31] to disrupt tight junction formation [28] and inhibit actin filament anchorage [32]. A low concentration of colchicine (2 μg/ml) [33–35] may prevent the reorganization of microtubules at the apical domain by inhibiting microtubule polymerization [36,37] which is important for the trafficking of apical transporters during bile canaliculi formation [38] and secretory vesicles after the bile canaliculi is formed [39,40]. Our study demonstrated that polarity inhibition of the ECCD-1 and colchicine treated cultures were similar to those of immediate perfusion cultures. When ECCD-1 was added, well-connected bile canaliculi (Fig. 2K) and formation of the apical domain (Fig. 2L) were inhibited in the static conditions at 48 h of culture. The excretion of fluorescein was greatly reduced in ECCD-1 treated culture compared with non-treated cultures (Fig. 2J and M, arrows). The addition of colchicine prevented the formation of the apical domain, well-connected bile canaliculi and fluorescein excretion (Fig. 2N–P) similar to ECCD-1 treated cultures. Therefore, static pre-stabilization before perfusion initiation is important in stabilizing the formation of cell-cell contacts, biliary excretion and metabolic functions. We have thus performed all perfusion cultures after stabilization in static culture for one day.

3.3. Optimization of perfusion flow rate

Higher perfusion flow rate leads to improved mass transfer in terms of nutrients supply and wastes removal whereas it also results in elevated shear stress which can be detrimental to hepatocytes. We optimized the perfusion flow rate for maximal mass

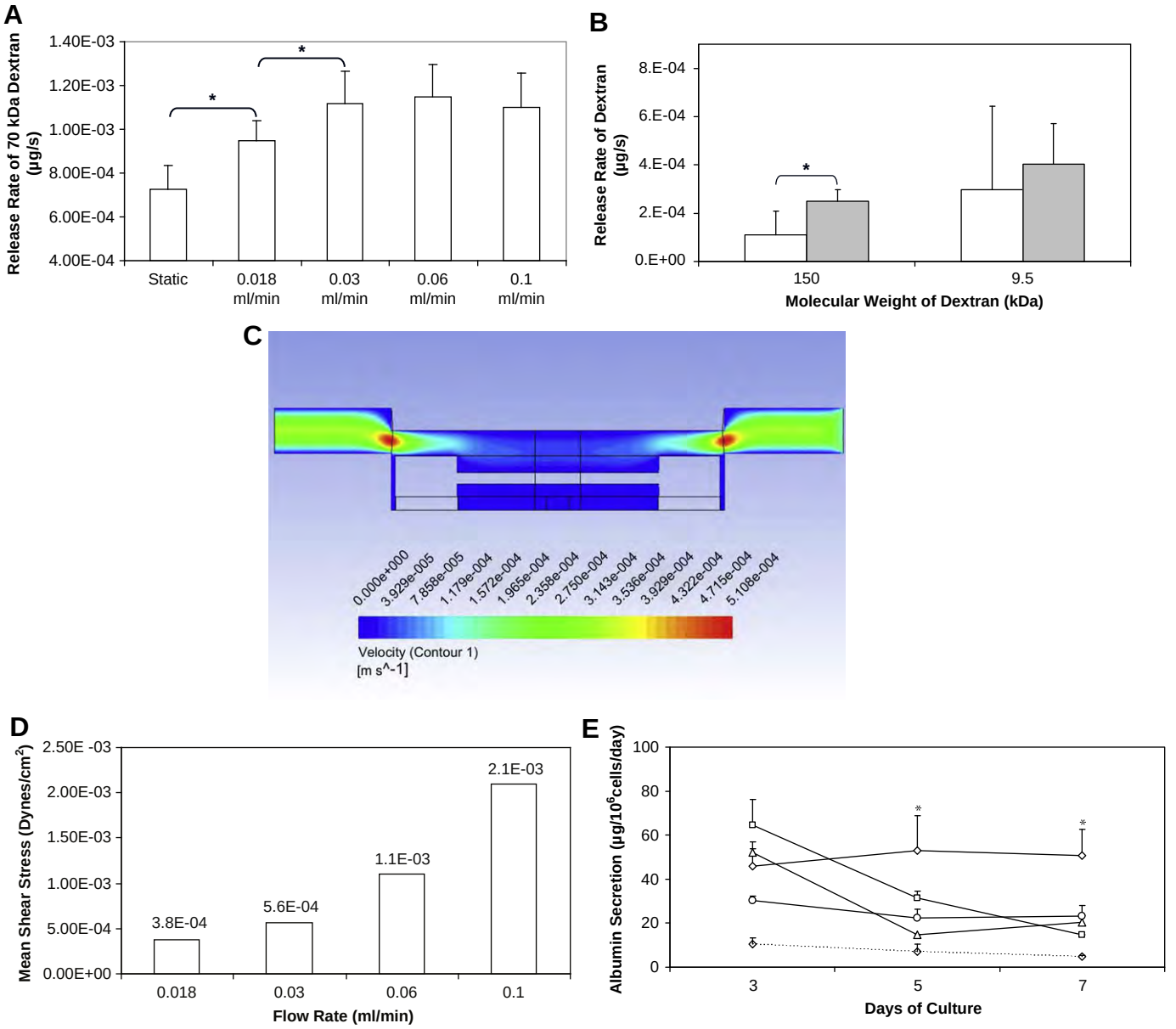

Fig. 3. Flow rate optimization based on mass transport across the collagen-coated membrane (as measured by the release rate of model dextran molecules), shear stress and albumin secretion at various flow rates. (A): Influence of perfusion flow rate on the release rate of model 70 kDa dextran from the sandwich compartment. (B): Release rate of 150 kDa and 9.5 kDa dextrans from the sandwich compartment at 0.03 ml/min. (□) Static; (■) perfusion. Asterisk indicates statistical significance at $p < 0.5$ (t-test). (C): Velocity profile across the bioreactor chamber at 0.03 ml/min. (D): Mean shear stress experienced by cell culture at various flow rates. (E): Albumin secretion of hepatocytes at different flow rates. (◇) Static; (□) 0.018 ml/min; (◇) 0.03 ml/min; (△) 0.06 ml/min; (○) 0.1 ml/min. 0.03 ml/min yielded statistically improved albumin secretion over other flow rates on days 5 and 7 at $p < 0.5$ (ANOVA).

transfer with minimal shear stress on cell surface. 70 kDa dextran were chosen as a model molecule to study mass transfer across the sandwich compartment since albumin, an important carrier for removing metabolic wastes and toxins such as bilirubin and mercaptans [41], is approximately 70 kDa. There was significant increase in the transport of the 70 kDa dextran in perfusion conditions up to 0.03 ml/min, beyond which the mass transport efficiency saturates (Fig. 3A). This saturation suggests that mass transport may be limited by diffusion across the collagen-coated membrane. At 0.03 ml/min, the transport of 150 kDa and 70 kDa dextran molecules was 1.5 and 2.3 folds higher than the static condition respectively but there was no significant difference between the perfusion and static conditions for transport of 9.5 kDa dextran molecules (Fig. 3B). This suggests that perfusion exhibits the greatest advantage for transport of molecules at or larger than 70 kDa (especially the complexes formed by binding of excreted metabolic wastes to albumin).

The flow velocity was uniform across the bioreactor chamber (Fig. 3C). The flow is laminar in nature with a Reynolds number of 0.045 (Equation: $\mathrm{Re} = \rho V h/\mu$, where density ρ is 1×10^3 kg/m^3, mean velocity V is 4×10^{-5} m/s, chamber height h is 1×10^{-3} m and dynamic viscosity μ is 8.89×10^{-4} Pa s). To calculate the fluid-induced shear stress, we built a computational fluid dynamics (CFD) model using commercial software ANSYS to solve the steady-state Navier-Stokes equations. Though mass transfer at 0.06 and 0.1 ml/min was similar to that at the flow rate of 0.03 ml/min, the shear stress was increased by ~2 and ~4 folds, respectively, from that at 0.03 ml/min (Fig. 3D). The albumin secretion of the

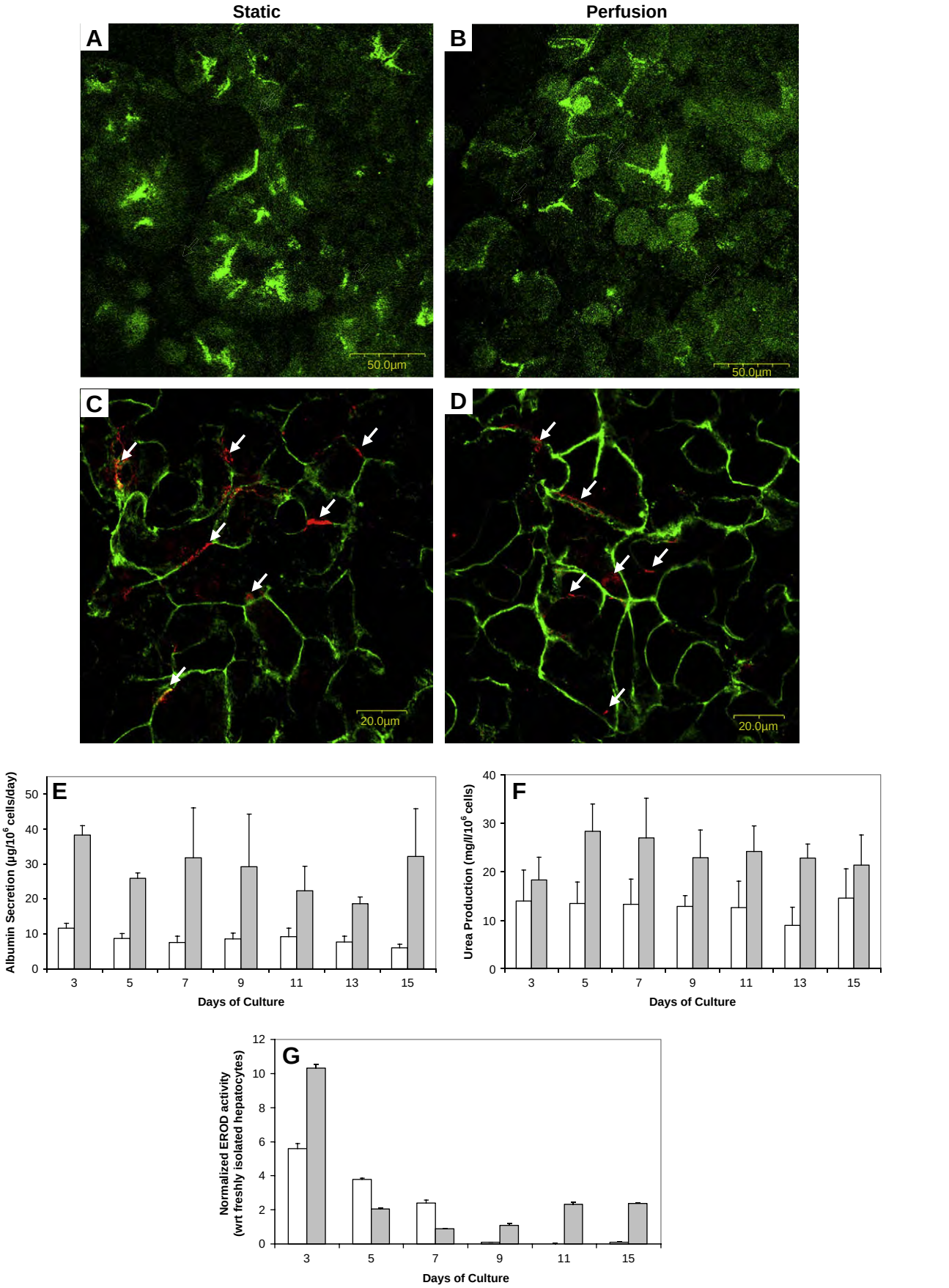

Fig. 4. Hepatocyte repolarization and functional maintenance under optimized perfusion conditions. (A–D): Left column: static; right column: perfusion. (A) and (B): Localization of fluorescein, a dye excreted by hepatocytes. Arrows point to fluorescein excretion. (C) and (D): Immunostaining of apical transporter Mrp2 (red) with basolateral marker CD147 (green). Arrows point to the Mrp2 localized at the bile canaliculi. (E): Albumin secretion from day 3 to day 15; (F): urea production from day 3 to day 15; (G): normalized 7-ethoxyresorufin-O-deethylase (EROD) cytochrome P450 activity relative to freshly isolated hepatocytes from day 3 to day 15. (□) static; (▣) perfusion. There were statistically significant differences between the two conditions at all time points at $p < 0.5$ (t-test). [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

hepatocytes was also well maintained at 0.03 ml/min (Fig. 4E). Albumin secretion was initially higher at 0.06 and 0.018 ml/min but dropped significantly on day 5, whereas albumin level was consistently lower for static culture and perfusion at the high flow rate of 0.1 ml/min. Therefore, we conducted all our perfusion culture at the flow rate of 0.03 ml/min.

3.4. Maintenance of excretory and metabolic functions

We investigated the biliary excretory as well as metabolic functions in the optimized immediate-overlay hepatocyte sandwich perfusion culture conditions. The biliary excretion of fluorescein was intensively observed in both static and perfusion culture (Fig. 4A and B). The apical repolarization and the Mrp2 localization in the perfusion culture were similar to those in static conditions 36 h after perfusion initiation (Fig. 4C and D). It suggested that hepatocyte polarity and excretory functions were well maintained in our perfusion system.

We monitored the functional maintenance of hepatocytes during a period of fifteen days. The albumin secretion and urea production of the hepatocytes in the perfusion culture were amplified ~3 and 2 folds over those in the static culture throughout the culture period (Fig. 4E and F). The 7-ethoxyresorufin-O-deethylation cytochrome P450 activity (normalized as folds relative to freshly isolated hepatocytes) of hepatocytes in the perfusion culture recovered to ~2 times of the freshly isolated levels after 1 week; while that in the static culture dropped steadily to zero (Fig. 4G). These results indicated that high levels of liver specific functions of the hepatocytes in the perfusion culture could be maintained over the 15-day period.

3.5. Consistent drug testing result at different time points during the two-week perfusion culture

APAP was used as a model drug to investigate the optimized immediate-overlay hepatocyte sandwich perfusion system for drug toxicity testing. Hepatocyte cultures on day 7 or day 14 were treated with APAP for 24 h and cell viability assessed by MTS assay. Both cell viability of the perfusion culture after APAP treatment on day 7 (45 ± 4%) and day 14 (38 ± 9%) were lower than that of the static culture on day 7 (70 ± 14%) and day 14 (43 ± 14%), indicating that sandwich perfusion culture was more sensitive to the APAP-induced toxicity than the static culture. There was no statistically significant difference of cell viability after APAP treatment at days 7 and 14 of perfusion culture (Fig. 5). In contrast, cell viability after APAP treatment varied greatly between days 7 and 14 of static culture. It demonstrated the consistency of drug response in the perfusion culture at different time points during the two-week culture period.

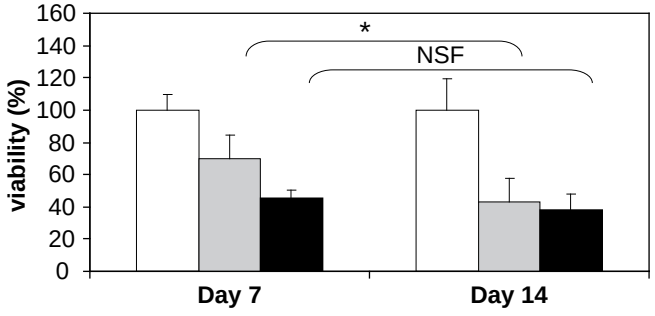

Fig. 5. Cell viability after 25 mM APAP treatment for 24 h at days 7 and 14 of culture. (□) Perfusion sandwich culture without APAP treatment; (▨) static sandwich culture with APAP treatment; (■) perfusion sandwich culture with APAP treatment. % viability represents the cell viability normalized to the values without APAP treatment. Asterisk indicates statistically significant difference at $p < 0.5$ (t-test) between the two groups. NSF indicates no statistically significant difference.

4. Discussion

Our results demonstrated that the combination of immediate-overlay hepatocyte sandwich assembly with perfusion culture was important for long-term maintenance of hepatocyte viability and liver specific functions *in vitro*. The immediate-overlay sandwich assembly facilitates the re-establishment of hepatocyte polarity *in vitro*, characterized by the localization of hepatobiliary transporters on their respective membrane domain for hepatic uptake and biliary excretion, and the re-arrangement of cytoskeleton for cytoplasmic trafficking and biliary contraction. Endogenous metabolites, such as bile acids and bilirubin, are conjugated to binding proteins or packed in vesicles in the cytoplasm and guided along microtubule to the apical domain after metabolism [42–44]. Bsep and Mrp2, the apical transporters, are responsible for the efflux of bile acids and bilirubin, respectively [45,46]. The contractility of bile canaliculi, requiring the reorganization of cytoskeleton around bile canaliculi, further expels the metabolites to the surrounding [47]. Herein, metabolism and transport of endogenous substances highly depend on hepatocyte polarity. Malfunction in either cytoplasmic trafficking or biliary excretion would cause the intra-cellular accumulation of these toxic metabolites which may disrupt cytoplasmic organelles or functional enzymes and lead to functional impairment or cell death [48,49]. On the other hand, the excreted metabolites tend to accumulate in the extra-cellular local environment in the static culture. It was found that such drugs as progesterone can trans-inhibit Bsep activity after excretion into bile canaliculi so that further excretion via Bsep is prohibited and toxic substance would start to build up intracellularly [50]. This may explain why long-term exposure of small amounts of toxic metabolites to cultured hepatocyte affected cellular functions [51]. Perfusion culture works better possibly by removing the wastes from the extra-cellular local environment after secretion from the cells. Our results support this possibility since the liver specific functions were maintained for up to 15 days only in the sandwich perfusion culture where wastes in both intra-cellular and extra-cellular environment were effectively removed (Fig. 4E–G).

This study has highlighted several important issues pertaining to the optimization of hepatocyte sandwich perfusion culture. Firstly, the intrinsic design of the bioreactor ensured laminar-flow and minimized shear stress. The bioreactor chamber was round in shape, which eliminated dead volume so that flow was uniformly distributed through the cell culture during perfusion. The direct mounting of oxygen permeable membrane on the top of the chamber enhanced oxygen supply to the cells. Many other sandwich perfusion bioreactor were based on full-scale square flat membrane configuration for application in bio-artificial liver [18–20]. Modified tissue culture flasks were used in sandwich perfusion culture for other applications such as cell oxygen uptake rate [21] or acidification rate [22] measurement. Our bioreactor was designed specifically for drug testing, and hepatocyte sandwich can be easily transferred from the bioreactor to a 24-well plate for drug incubation. The assembly of bioreactor was also made to be simple in handling. Secondly, we observed that static pre-stabilization before perfusion initiation plays a significant role in the stabilization of cellular morphology, biliary excretion and liver specific functions under perfusion conditions. Either blocking the cell junction formation by ECCD-1 or inhibiting the cytoskeleton reorganization by colchicine will disrupt apical transporter distribution and biliary excretion since cell junction formation and cytoskeleton reorganization are upstream of polarity regeneration [44]. In the culture without pre-stabilization,

the failure of apical transporter localization and loss of excretory function were observed (Fig. 2B and C) because the immediate flow impact would disturb extra-cellular cues for hepatocyte repolarization and lead to the similar outcome as that of ECCD-1 and colchicines treated cultures. This is consistent with reports that hepatocytes pre-aggregated for up to 3 days before seeding into bioreactors exhibited greater morphological stability and higher functional activity than those of direct seeding of single cells [52,53]. Thirdly, the porous polymeric membrane overlay to shield the cells also greatly reduced the shear stress on the cell surface. The shear stress in our system at 0.03 ml/min (5.6×10^{-4} dynes/cm^2) was much lower than in other systems [23]. We also found that the perfusion flow rate warranted careful control to balance mass transport and shear stress for the maintenance of hepatocyte functions. For flow rate below 0.03 ml/min, mass transport was dominant in promoting hepatocyte functions; while above 0.03 ml/min, shear stress was exerting deleterious effects on hepatocyte functions. In addition to protecting cells from direct shear, the top membrane was able to further reinforce the sandwich matrix because the flow shear tended to disintegrate the top collagen layer of the conventional sandwich culture in long-term perfusion. The uneven top collagen gel layer of the conventional sandwich after perfusion could lead to variation in drug access to the cell surface during drug treatment. Therefore, the pre-stabilization of cellular morphology and biliary excretion, and avoidance of fluid-mediated forces are important considerations in perfusion optimization.

Liver specific functions of hepatocytes, such as the phase I drug metabolic function (e.g. EROD activity) could be maintained in our sandwich perfusion system for up to 15 days. APAP was used as a model drug to test the sensitivity and feasibility of the system in drug testing application. Both phase I (CYP2E1) and phase II (glucuronide, sulfate or glutathione conjugation) drug metabolic pathways are involved in APAP metabolism to a non-toxic form, and the intermediate, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), produced after phase I metabolism, is toxic [54]. The perfusion culture exhibited a higher sensitivity to APAP-induced toxicity than the static culture on both day 7 and day 14 (Fig. 5), attributable to the well-maintained drug metabolic functions of the sandwich perfusion culture. Approximately 60% of cell death was observed in the perfusion culture treated with 25 mM of APAP for 24 h. This observation was similar to the finding that Wang et al. and Mingoia et al. reported the APAP IC_{50} value (the drug concentration causing 50% of cell death) obtained from their hepatocyte culture system to be 14.01 mM and 20.42 mM, respectively [55,56]. Furthermore, the cell viability in the perfusion culture after APAP treatment on day 7 and day 14 was similar (Fig. 5). In contrast, static culture treated with APAP on day 7 and day 14 produced highly variable cell viability results (Fig. 5). Hepatocytes with overall low metabolic functions in the static sandwich culture on day 14 seemed more vulnerable to the drug-induced toxicity than the cells cultured for 7 days. Static hepatocyte sandwich cultures for 3–5 days were typically used by many groups for toxicological studies to avoid functional drop beyond that period [57–59]. Our sandwich perfusion system extended the usable period of *in vitro* hepatocyte culture up to 15 days with consistent and reliable drug response at different time points during the culture period. Instead of using cells from different batches of isolation which may cause batch-to-batch variation, we could combine a large number of isolated hepatocytes for long-term culture in the immediate-overlay sandwich perfusion system. One aliquot of the cultured cells can be used for functional validation while the rest of the combined lot can be ready for drug testing at any required time points. This would enable the industry-scale drug testing with improved consistency without the limitations of the current pooled and freeze protocols [6].

5. Conclusion

We have developed a laminar-flow perfusion bioreactor that integrates immediate-overlay sandwich culture of hepatocytes. We have optimized the parameters of the perfusion condition for immediate-overlay hepatocyte sandwich culture, such as static pre-stabilization and the perfusion flow rate. The cultured hepatocytes exhibited restored hepatocyte polarity and biliary excretion, and maintained liver specific functions for two weeks. Cells could produce sensitive and consistent drug toxicity responses at different time points during culture allowing further exploitations in industry-scale drug testing.

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