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The Effect of Cell Culture Method on Long-Term Primary Hepatocyte Cell Health

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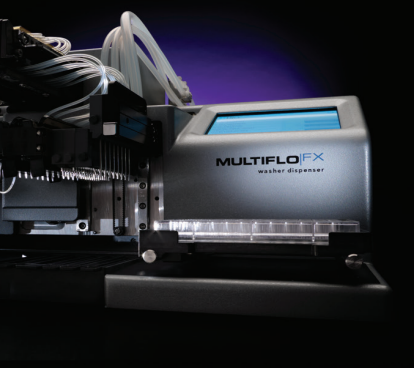

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Introduction

Hepatotoxicity studies are an important part of the drug discovery process following lead molecule generation. Whereas the initial screening process identifies those molecules with target efficacy, hepatotoxicity studies uncover whether a potential drug causes drug-induced liver injury (DILI) in spite of any therapeutic effect.

While *in vivo* studies are still the gold standard; *in vitro* screening using primary hepatocytes, the cells predominantly responsible for the liver's mass and metabolic functions, has gained importance for reducing animal exposure, is amendable to high-throughput platforms and better equipped to determine toxicity mechanisms of action. Typically, this testing involves repeatedly dosing the hepatocytes with the potential drug over multiple days to assess cell viability, metabolism and toxicity. However, the challenge remains in that hepatocytes cultured in traditional two-dimensional (2D) formats undergo rapid de-differentiation and loss of key functions[1,2,3]. Additionally, 2D cultured cells are less able to form the cell-cell and cell-matrix communication networks seen *in vivo*[4]. Therefore, hepatotoxicity studies are usually limited in duration, and results may not provide a complete understanding of the drug's cumulative and long-term *in vivo* effects.

Newer three-dimensional cell culture models exist, allowing cells to grow as aggregates thereby better resembling the functionalities and communication networks found *in vivo*[4]. Two such models will be discussed here. One model uses a standard-sized 96-well microplate with a small cavity at the bottom of each tapered well. When media and cells are added to the wells, the cells self-aggregate into microtissue spheres in the hanging media drop formed by the hole. The other uses a collagen hydrogel scaffold with tissue-like properties that again encourages cells to aggregate into three-dimensional structures (3D). Both methods offer a higher degree of biomimicry, and encourage the re-establishment of cell-cell and cell-ECM communication. The ability to culture, characterize and challenge cells over longer periods is of key importance during dosing and safety research. Here, we use two common screening tests to demonstrate differences between primary hepatocytes cultured using traditional 2D culture methods and those cultured using two novel 3D culture methods. Cell function was measured via cytochrome P450 isoform 3A4 (CYP3A4) enzyme activity analysis using the P450-Glo™ CYP3A4 Assay with Luciferin-IPA, while cell viability was assessed by means of cellular ATP measurement using the CellTiter-Glo® Assay or CellTiter-Glo 3D Assay. All assays are manufactured by Promega Corporation.

Materials and Methods

Materials

Cells

Cryopreserved plateable human hepatocytes (Lot IZT) were provided by BioreclamationIVT (Baltimore, MD). 3D liver microtissues from the aforementioned human hepatocyte lot, and created using proprietary hanging drop technology, were purchased from InSphero, Inc. (Cambridge, MA) and supplied in ready-to-use 96-well GravityTRAP™ plates.

Reagents, Kits, Consumables

P450-Glo CYP3A4 Assay with Luciferin-IPA (Catalog No. V9002), CellTiter-Glo Assay (Catalog No. G7571), and CellTiter-Glo 3D Assay were donated by Promega Corporation (Madison, WI). BioCoat™ Collagen I 384-well black, clear bottom plates (Catalog No. 354667) and BioCoat Collagen I 96-well black, clear bottom plates (Catalog No. 356649) were donated by Corning Life Sciences (Corning, NY). The collagen hydrogel RAFT™ 3D Cell Culture System was donated by TAP Biosystems (Hertfordshire, UK).

Instrumentation

MultiFlo™ FX Microplate Dispenser

MultiFlo FX Microplate Dispenser was used to dispense the cell/collagen mix for RAFT hydrogel creation, perform medium exchanges and test compound removal, and dispense assay components.

Cytation™ 3 Cell Imaging Multi-Mode Microplate Reader

Cytation 3 Cell Imaging Multi-Mode Microplate Reader was used to perform all luminescence microplate reads using a 0.25 second integration time.

Methods

Cell Culture and Propagation

RAFT 3D Cultured Hepatocytes

Cryopreserved hepatocytes were thawed and added to the prepared collagen solution provided in the RAFT 3D Cell Culture System. The mixture was then dispensed to a 96-well microplate in a volume of 240 µL per well at a final concentration of 100,000 cells/well. The cell plate was then incubated at 37 °C/5% CO_2 for 15 minutes. After incubation, a 96-well RAFT plate containing individual sterile absorbers was inserted into the cell plate wells and the combined system was incubated at 37 °C/5% CO_2 for 15 minutes to allow media absorption and collagen concentration. After incubation, the absorbing plate was removed and 100 µL of new medium was added to the 120 µm thick cell/collagen hydrogel. The cell plate was incubated at 37 °C/5% CO_2 for three days with daily medium exchanges.

3D Liver Microtissues

Medium was exchanged in 96-well GravityTRAP plates containing 3D liver microtissues from the supplier, and the plate was incubated overnight at 37 °C/5% CO_2.

2D Hepatocytes

Cryopreserved hepatocytes were thawed, diluted with media provided by BioreclamationIVT, and plated in 384-well black, clear bottom plates at concentrations of 2000, 5000, and 10,000 cells/well or in 96-well black, clear bottom plates at a concentration of 50,000 cells/well and incubated overnight 37 °C/5% CO_2.

CYP3A4 Activity

RAFT 3D Cultured Hepatocytes

Medium was removed from the wells and replaced with 50 µL of 37 °C pre-warmed medium containing 3 µM Luc-IPA substrate. The cell plate was then incubated at 37 °C/5% CO_2 for four hours.

Following incubation, 50 µL of supernatant was transferred to a separate white 96-well assay plate, and an equal volume of P450-Glo Luciferin Detection Reagent (LDR) was added to the same wells. The assay plate was then shaken for 60 seconds and incubated for 20 minutes at 37 °C/5% CO_2, followed by luminescent signal measurement.

3D Liver Microtissues

The aforementioned procedure was repeated using 30 µL of pre-warmed medium containing Luc-IPA substrate on selected wells. 50 µL of LDR was then added to those wells, and the wells were mixed five times by aspirating/dispensing. The contents of those selected wells, including microtissue, were then transferred to a white, 96-well assay plate and incubated and read as previously described.

2D Hepatocytes

The aforementioned procedure was repeated using 25 µL or 50 µL of pre-warmed medium for the 384-well and 96-well culture plates, respectively. Following incubation, 12.5 µL of supernatant from the 384-well cell plate was transferred to a separate white 384-well assay plate along with an equal amount of LDR, while 50 µL of supernatant from the 96-well cell plate was transferred to a separate white 96-well assay plate along with an equal amount of LDR. The plates were shaken for 60 seconds and incubated for 20 minutes at room temperature followed by luminescent signal detection.

Cell Viability

RAFT 3D Cultured Hepatocytes

Using the original cell plate, 50 µL of CellTiter-Glo reagent was added, the plate was shaken at room temperature for 5 minutes, then incubated at room temperature for 25 minutes. After incubation, the luminescent signal was quantified.

3D Liver Microtissues

Using the original cell plate, 35 µL of CellTiter-Glo 3D reagent was added to selected wells. The wells were mixed five times by aspirating/dispensing, and the contents of those selected wells, including microtissue, were transferred to a white, 96-well assay plate. The plate was shaken for 5 minutes at room temperature, followed by a 25 minute room temperature incubation and luminescent signal detection.

2D Hepatocytes

After cell activity was measured, 12.5 µL of CellTiter-Glo reagent was added to the original 384-well cell plate, and 50 µL of CellTiter-Glo reagent was added to the original 96-well cell plate. The plates were shaken at room temperature for one minute, followed by an additional 25-minute room temperature incubation and subsequent luminescent signal detection.

Results and Discussion

CYP3A4 Activity

CYP3A4 activity in 3D and 2D cultured hepatocytes was assessed for approximately two weeks, with daily media exchanges, and calculated as normalized percentages from RLU values using the following formula:

$$RLU_{(Day\ X)}/RLU_{(Day\ 1)}*100$$

When comparing the liver microtissues to the 2D cultured cells in 2000, 5000 and 10,000 cells/well concentrations (Figure 1A), 3D CYP activity remains unaffected throughout the incubation period, while the 2D culture activity decreases significantly across all three cell seeding densities, with the lowest activity being seen with the 2000 cells/well concentration. Enzyme activity in the RAFT 3D cultured cells also remains steady over the culture period, while the enzyme activity in 2D cultured cells decreases starting by the third day and continuing to the tenth day (Figure 1B).

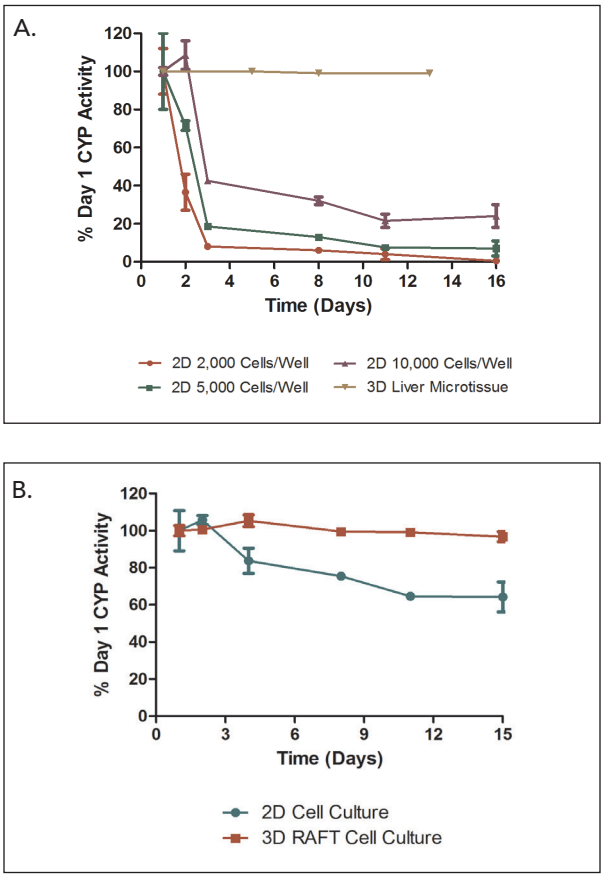

Figure 1. CYP450 Enzyme Activity Assessment in Long-Term 2D and 3D Hepatocyte Cell Cultures. **(A)** 3D liver microtissues and 2D cultured cells in 2000, 5000, 10,000 cells/well concentrations. **(B)** Collagen hydrogel cultured 3D cells and 2D cultured cells at a concentration of 50,000 cells/well.

These findings demonstrate that enzyme activity remains constant in both hepatocyte 3D culture models over the entire culture period, thus confirming that 3D cell cultures provide a superior cellular model for maintaining long term CYP enzyme activity relative to conventional microplate cell seeding.

It is also interesting to note the effect of cell seeding density in 2D cell culture evident in 96-well plates, (Figure 1B) 50,000 cells/well is sufficient cell density to ensure cell:cell contact, which appears to better maintain long term CYP activity relative to that demonstrated at lower cell densities in 384-well plates (Figure 1A).

Cell Viability

Cell viability, as measured by ATP activity, in 3D and 2D cultured hepatocytes was also assessed for the same time period, with daily media exchanges, and calculated using the aforementioned formula from RLU values.

Comparing the normalized ATP activity percentages in the liver microtissues versus the 2D cultured cells in varying concentrations (Figure 2A), it can be observed that 3D cultured cell ATP levels remain constant. ATP levels for the 2D cultured 2000 and 5000 cells/well fall to 50% or lower by the end of the culture period, while the ATP levels of the highest 2D cell culture concentration remain similar to those seen in the 3D hanging drop cultured cells. When comparing the RAFT 3D cultured cells to 2D cells (Figure 2B), the 3D cultured cell viability is again constant, while a loss of viability is seen starting at day 7 in the 2D cultured cells.

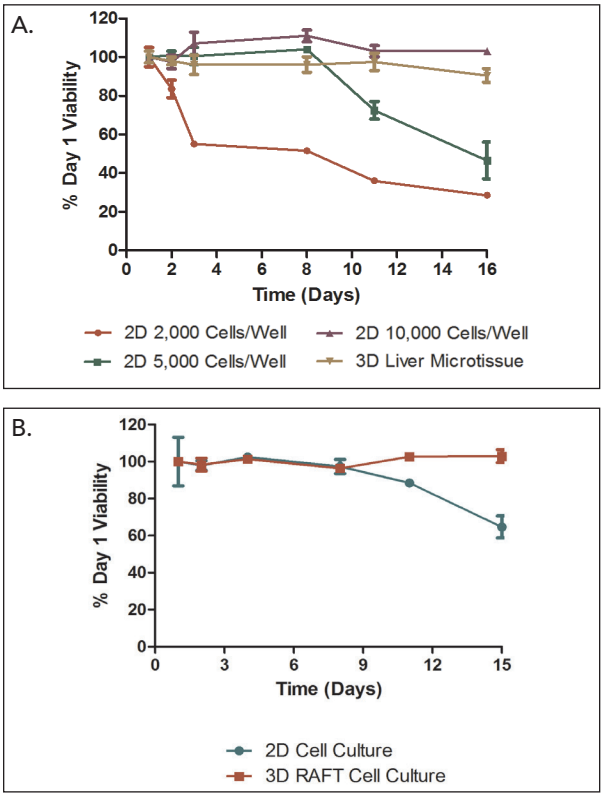

Figure 2. Cell Viability in Long-Term 2D and 3D Hepatocyte Cell Cultures as calculated from ATP levels. **(A)** 3D liver microtissues and 2D cultured cells in 2000, 5000, 10,000 cells/well concentrations. **(B)** Collagen hydrogel cultured 3D cells and 2D cultured cells at a concentration of 50,000 cells/well.

The viability data also confirms that 3D cultured cells retain plasma membrane integrity better relative.

Also, similar to the CYP3A4 activity data, cell density has a demonstrable effect on cell viability.

Conclusions

Both the liver microtissue and collagen hydrogel 3D cell culture models retained viability and normal function, as witnessed by CYP3A4 activity and cell viability data, over extended culturing conditions. The variability between 2D and 3D cultured hepatocytes highlights the necessity to incorporate relevant 3D cell models when performing long term toxicity studies that incorporate multiple week dosing periods in order to fully understand any cumulative hepatotoxic effects of a drug.

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