

Validation of a Novel Cell Culture System to Perform 3D *in vitro* Cytotoxicity Analyses using Primary Hepatocytes

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Abstract

Hepatocytes are the primary cell type of the liver providing the majority of the detoxification which increases the potential for cellular dysfunction and death. Though the source of the insult may be caused by several factors, exposure to drugs represents a significant concern warranting FDA guidance on drug-induced liver injury (DILI). *In vivo* studies are still the gold standard; however, *in vitro* screening has gained importance for reducing animal exposure, amendable to high-throughput platforms and better equipped to study cellular mechanisms of action.

Typically *in vitro* screening has incorporated primary hepatocytes cultured in a two dimensional (2D) format where the cells form a monolayer across the bottom of a well. However, when cultured and studied in this fashion they rapidly lose their key functions and de-differentiate over the course of only a few days. The ability to culture, characterize and challenge primary cells in a biomimetic 3D environment enables longer term studies.

Here we present data demonstrating the differences in response between human primary cells cultured in 2D and in the RAFT 3D cell culture system which has the benefits of a collagen hydrogel with tissue-like properties. Camptothecin and pycocyanin were tested for their ability to cause short-term oxidative stress, as well as long-term induction of apoptosis and necrosis. Kinetic live cell imaging was performed using multiple fluorescent non-perturbing probes to monitor the various effects in real time with incubations up to 48 hours which enabled a thorough assessment of the toxin profile. Image overlay allowed for discrete cellular analysis. Variations in cytotoxicity levels were observed after a 24 hour 800 nM camptothecin treatment (75%:2D; 28%:3D). ROS induction was less in 3D than 2D system. Overall, cells exhibited greater viability using the RAFT system, and were less sensitive to toxins than observed in traditional 2D culture for the endpoints which may indicate a more robust cell culture system.

Materials and Methods

2D and 3D Cell Culture Models: Cryopreserved plateable human hepatocytes (lot IZT) were provided by BioreclamationIVT (Baltimore, MD). The cells were prepared as instructed by vendor and cultured in 3D (100,000 cells/well) using the RAFT™ 3D Cell Culture System from TAP Biosystems (Hertfordshire, UK) and in 2D (50,000 cells/well) using BioCoat™ Collagen I 96-well black, clear bottom plates (Catalog No. 356649) from Corning Life Sciences (Corning, NY) using media provided by BioreclamationIVT.

Instrumentation: **MultiFlo™ FX Microplate Dispenser.** MultiFlo FX is a fully modular, automated reagent dispenser for 6- to 1536-well plates offering BioTek's unique Parallel Dispense™ technology – both peristaltic and syringe pump operation down to 500 nL. An available microplate wash module adds multi-purpose functionality. The instrument was used to dispense the cell/collagen mix for hydrogel creation, perform medium exchanges and test compound removal, and dispense assay components.

Cytation™ 3 Cell Imaging Multi-Mode Microplate Reader. Cytation 3 combines automated digital wide-field microscopy and conventional microplate detection. The instrument was used to perform all luminescent microplate reads, as well as kinetic and endpoint cellular imaging. Cell counting and quantification of the fluorescent signal from captured images was done using the Gen5™ Data Analysis Software.

Cell Viability and Activity Assays: CellTiter-Glo® (Catalog No. G7571) from Promega Corporation (Madison, WI) was used to assess viability by means of cellular ATP measurement. The P450-Glo™ CYP3A4 assay with Luciferin-IPA (Catalog No. V9002), also from Promega Corporation, was used to measure cellular function via cytochrome P450 (CYP450) enzyme activity analysis.

Cytotoxicity Assays: Cytotoxicity was determined with the CellTox™ Green Cytotoxicity Assay (Catalog No. G8731) from Promega Corporation. Kinetic measurements of reactive oxygen species (ROS) activity and external lipid membrane bilayer exposure of phosphatidylserine (early apoptosis indicator) were performed with the DCFDA Cellular ROS Detection Assay (Catalog No. ab112981) and Kinetic Apoptosis Kit (Catalog No. ab112981), respectively, from Abcam (Cambridge, MA), while endpoint assessments of ROS and caspase 3/7 activation were made by the CellROX® Deep Red Oxidative Stress Reagent (Catalog No. C10422) and CellEvent™ Caspase-3/7 Green detection Reagent (Catalog No. C10423). Both assay kits were purchased from Life Technologies (Carlsbad, CA).

Assay Procedures
Cell Health Assessment: Medium was removed from wells and replaced with 50 µL of pre-warmed medium containing Luc-IPA substrate. The plate was then incubated at 37°C/5% CO₂ for one (2D) or four hours (3D). Following incubation, 50 µL of supernatant was transferred to a separate white 96-well plate, an equal volume of P450-Glo Luciferin Detection Reagent (LDR) was added to the same wells, the plate was shaken and incubated for 20 minutes at room temperature (RT) (2D) or at 37°C/5% CO₂ (3D), followed by luminescent signal detection. In the original cell plate, 50 µL of CellTiter-Glo reagent was added to the wells, the plate was shaken at RT for 1 minute (2D) or 5 minutes (3D), followed by an additional 25 minute RT incubation. The luminescent signal was once again quantified.

Kinetic Oxidative Stress and Apoptotic Activity Monitoring
DCFDA Assay: Medium was removed from wells, the cells were washed with 100 µL of 1X buffer, and then stained with DCFDA at a final 1X concentration of 25 µM for 60 minutes at 37°C/5% CO₂. Upon completion, the cells were washed with 1X buffer, followed by a 100 µL addition of test compound. The plate was placed immediately into the Cytation 3 and kinetic imaging was performed every 20 minutes.

Kinetic Apoptosis Assay: Compound dilutions were prepared in medium containing 10 µL/mL of pSIVA™ (Polarity Sensitive Indicator of Viability and Apoptosis) reagent. Medium was removed from wells, replaced directly with test compound, and the plate placed immediately into the Cytation 3 for kinetic imaging once per hour.

Long-term Cytotoxicity Detection: CellROX, CellEvent, and CellTox Green reagents were added to medium at 6X concentrations of 30 µM, 42 µM, and 6X, respectively. 20 µL volumes were then added to wells containing 100 µL of test compound to create final 1X concentrations of 5 µM, 7 µM, and 1X, and incubated at 37°C/5% CO₂ for 60 minutes. All wells were then washed 3X with PBS and imaged with the Cytation 3.

RAFT™ 3D Cell Culture System

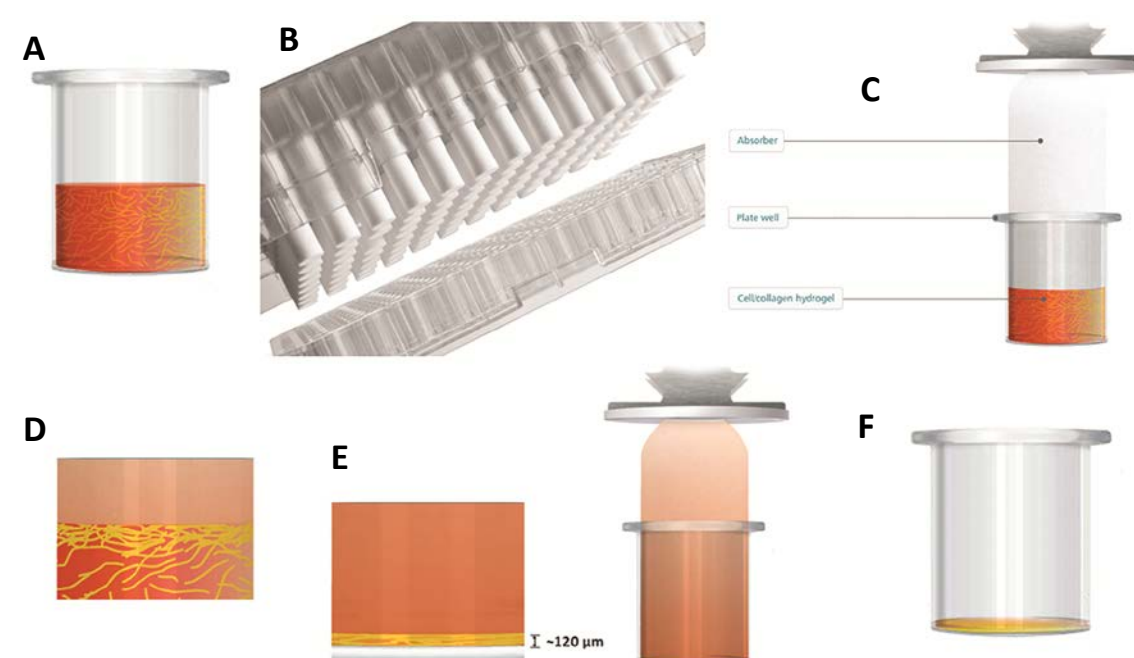


Figure 1 Creation of 3-Dimensional Hepatocyte/Collagen Hydrogel using RAFT System. (A) Cryopreserved hepatocytes were thawed and added to the prepared collagen solution. The mixture was then dispensed to the 96 well plate in a volume of 240 µL per well. The final cell concentration equaled 100,000 cells/well. The cell plate was then incubated at 37°C/5% CO₂ for 15 minutes. Absorption of medium, concentrating collagen and cells to in vivo strength (D) creates a 120 µm thick hydrogel (E). The absorbers were then removed and 100 µL of new medium was then added to the concentrated cell/collagen hydrogel (F). The plate was once again incubated at 37°C/5% CO₂ for three days with medium exchanges being performed on a daily basis.

Kinetics of Early Cytotoxicity Markers

Mechanism of action studies were conducted with the DNA topoisomerase I inhibitor camptothecin, and redox-active phenazine pycocyanin to determine whether oxidative stress and apoptosis play a role in potential hepatotoxic effects that the compounds may exhibit. Since both are early markers of cytotoxicity, and ROS induction has been shown to occur rapidly following addition of camptothecin (Sen *et al.*, 2004), kinetic imaging was performed immediately following compound addition using the procedures previously explained. Total image capture time ranged from 4-9 hours to 24 hours for oxidative stress and apoptosis monitoring, respectively. Cells manifesting a fluorescence value above 5000 RFU from the DCFDA or pSIVA probes were considered to exhibit evidence of the respective marker. The Hoechst 33342 nuclear stain was also incorporated to determine total cell number and subsequently to compute % effected cell number per image.

The results shown in Figure 2 and 3 confirm that camptothecin and pycocyanin cause oxidative stress and apoptosis, as previously reported by Sen *et al.*, 2004, Gómez-Lechón *et al.*, 2002, and Muller *et al.*, 2002 and 2006. What is also demonstrated, however, is the lack of response within the treatment times examined for hepatocytes cultured in 3D. Therefore, it was decided that long-term dosing studies should be performed to determine whether responses would be seen from 3D cultured cells following extended exposure to each compound.

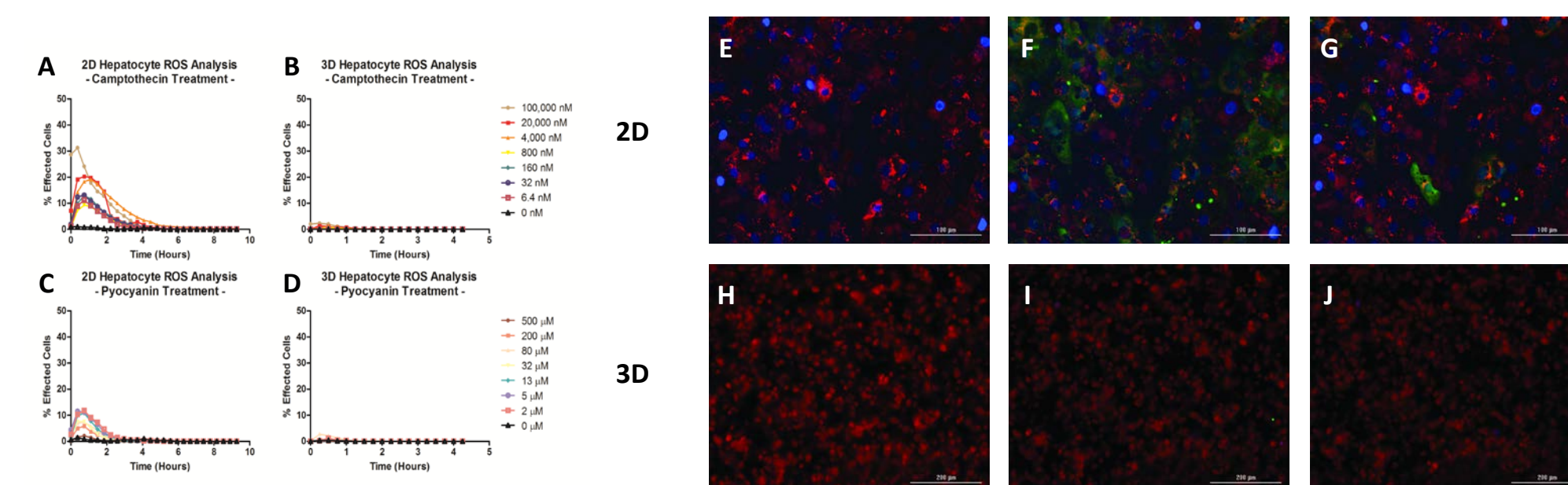


Figure 2. Kinetic ROS Activation Analysis. (A-D) Total cell percentage exhibiting oxidative stress per captured 4x (2D) or 10x(3D) image. (E-G) 20x images of 2D cultured hepatocytes, and (H-J) 10x images of 3D cultured hepatocytes captured after 0, 30, and 60 minute treatments with 200 µM pycocyanin. Blue: Hoechst 33342; Green: DCFDA reagent; Red: MitoTracker Red Mitochondrial probe.

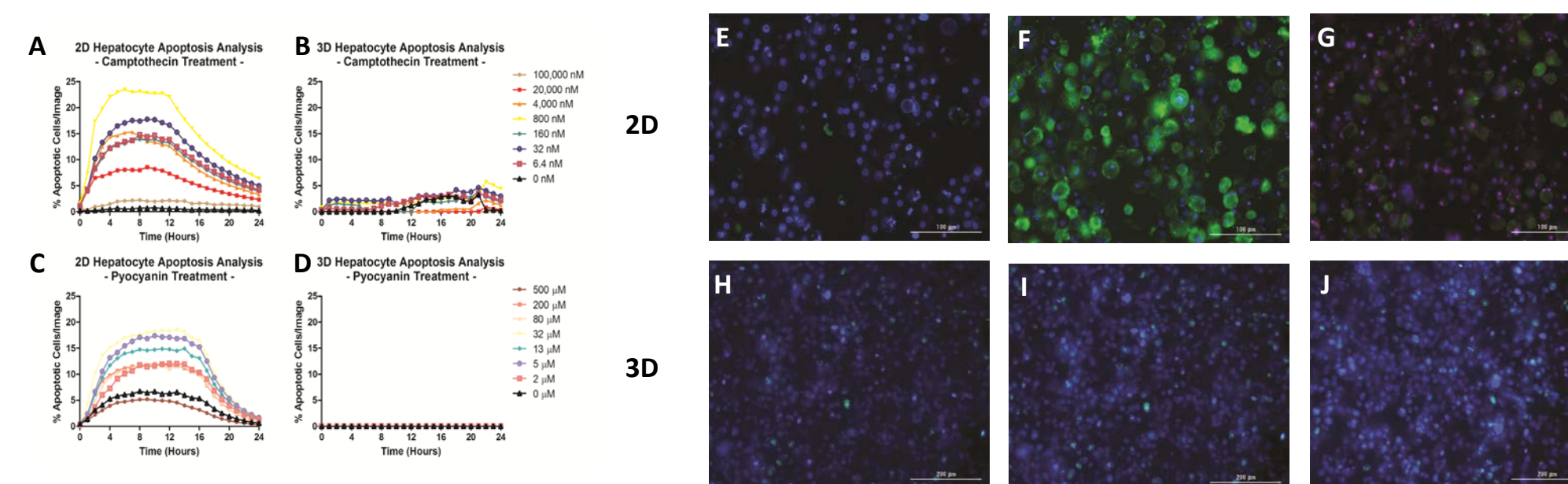


Figure 3. Kinetic Apoptosis Analysis. (A-D) Total cell percentage exhibiting apoptotic activity per captured 4x (2D) or 10x (3D) image. (E-G) 20x images of 2D cultured hepatocytes, and (H-J) 10x images of 3D cultured hepatocytes captured after 0, 8, and 16 hour treatments with 800 nM camptothecin. Blue: Hoechst 33342; Green: pSIVA reagent.

Robustness of 2D and 3D Culture Systems

Hepatocytes were cultured in 2D and 3D as previously described. Following the initial 3 day incubation period cell viability and CYP3A4 activity were then assessed for an additional 14 days. Medium exchanges were performed on a daily basis throughout the entire culturing period. Assays were performed regularly to track any potential changes in CYP3A4 activity or in cell health.

From the results in Figure 4A it can be seen that CYP3A4 activity for 2D cultured wells containing 50,000 cells/well decreases beginning by the third day of monitoring and continues to decrease until approximately day 10. This is in contrast to 3D cultured hepatocytes, which maintain consistent CYP activity across the entire incubation period. The loss of CYP activity results in eventual cell death after day 7, for the 2D cultured hepatocytes, as seen in Figure 4B. Again no loss of cell viability is seen during the 14 day monitoring period in the 3D RAFT systems. These findings demonstrate that hepatocytes cultured in a 3D collagen matrix exhibit a greater level of viability and normal activity over extended culturing periods.

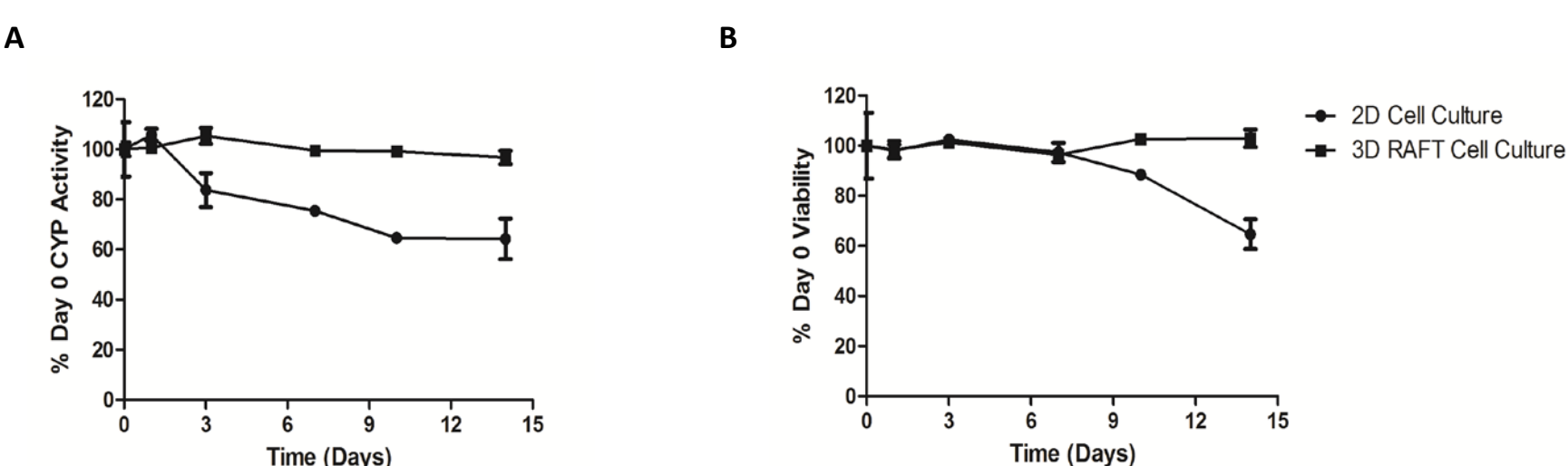


Figure 4. Cell Viability and CYP450 Enzyme Activity Assessment for Extended Culturing of Hepatocytes in 2D and 3D. (A) CYP3A4, and (B) Cell viability findings for hepatocytes cultured in 2D or in 3D using the RAFT system. Left side graphs display raw luminescent values for each cell culture method. Right side graphs represent normalized percentages compared to the first day of analysis. % Viability and CYP Activity calculated by the following formula: RLU(Day X)/RLU(Day 0)*100.

Long-Term Cytotoxicity Markers

A seven day dosing experiment was then performed to ascertain whether the improvement in cell health observed from the results of the viability and CYP3A4 activity study caused hepatocytes cultured in 3D to be impervious to the effects of camptothecin and pycocyanin, or delayed the ROS induction and apoptotic activity seen previously with 2D cultured hepatocytes. Cells were re-dosed with a range of compound concentrations on a daily basis. Endpoint assessments of ROS and apoptotic activity were then performed 1, 3, and 7 days after initiation of treatment.

The results from the graphs in Figure 5A and B demonstrate that camptothecin and pycocyanin do cause oxidative stress and apoptosis in 3D cultured hepatocytes. The incubation time eliciting the greatest response was found to be after a 3 day dosing, which can be seen by the increase in signal from the CellROX and CellEvent fluorescent ROS, and caspase 3/7 probes, respectively (Figure 5C-G). These results contrast with the previous findings, as well as the 2D culture results in Figure 5, indicating that induction of ROS and apoptotic activity occurs in less than 24 hours.

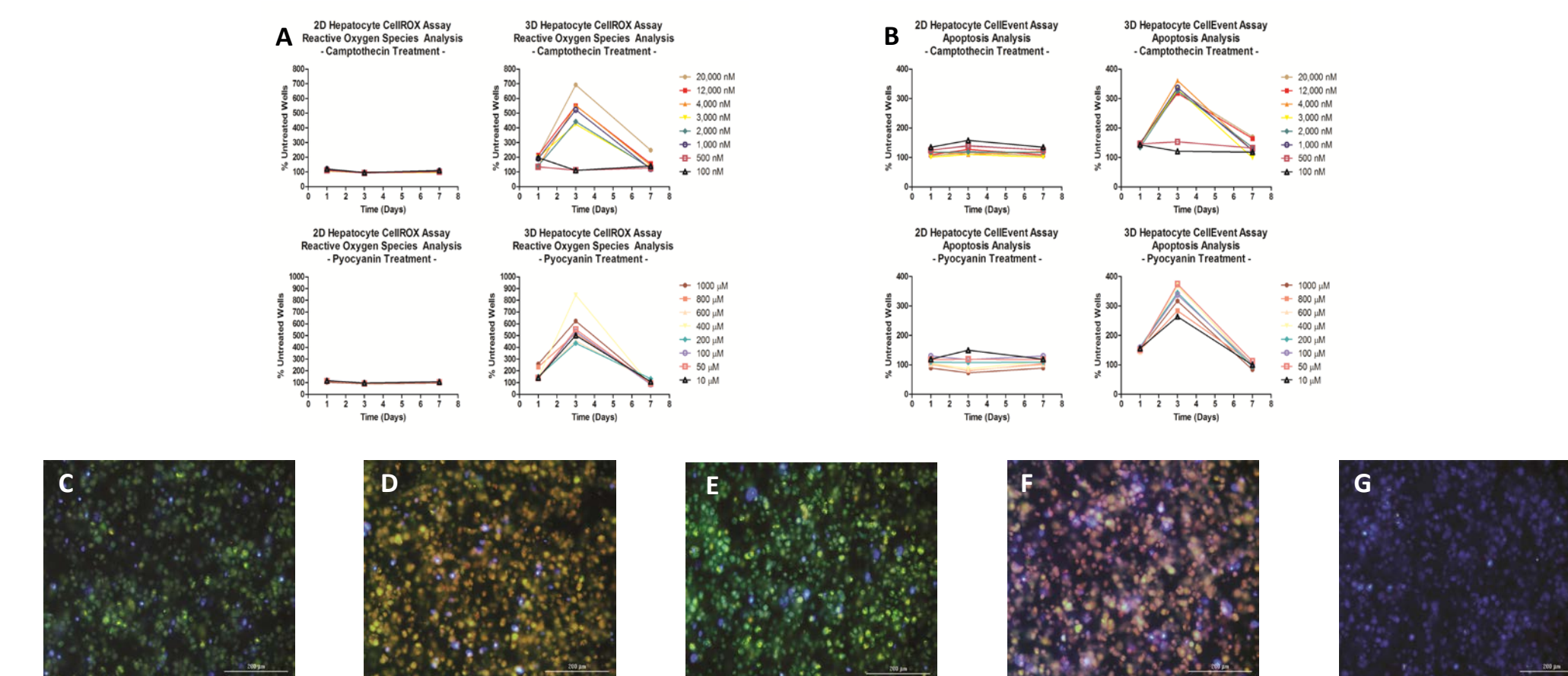


Figure 5. Long-term Oxidative Stress and Apoptosis Analysis. Results of multi-day camptothecin and pycocyanin incubations on (A) ROS induction, and (B) apoptotic activity for cells cultured in 2D and 3D. % Untreated Wells calculated using the following formula: RFU(Compound Treated Well)/RFU(Untreated Well)*100. 10x images also shown for 3D cultured hepatocytes after 3-day treatment with (C-D) pycocyanin, (E-F) camptothecin, as well as no treatment (G). Blue: Hoechst 33342; Green: CellEvent Caspase-3/7 Reagent; Red: CellROX Deep Red Oxidative Stress Reagent.

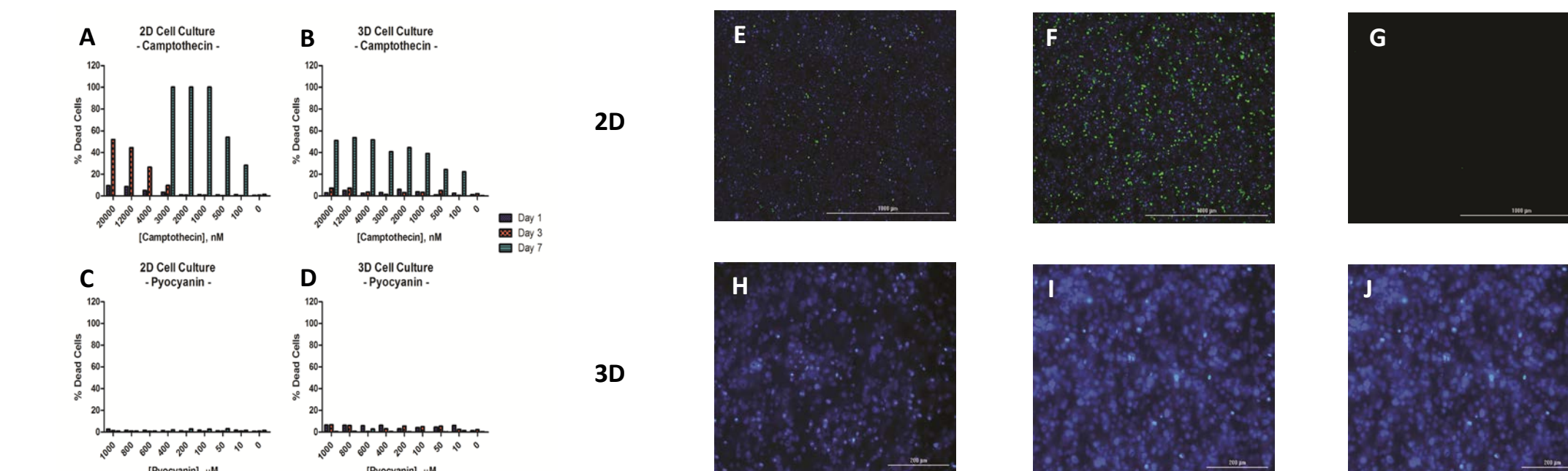


Figure 6. Hepatotoxicity Results. (A-D) Dead cell percentage per captured 4x (2D) or 10x (3D) image following 1, 3, or 7 day treatment with camptothecin or pycocyanin. (E-G) 20x images of 2D cultured hepatocytes, and (H-J) 10x images of 3D cultured hepatocytes showing live (blue) and dead (green) cells. Blue: Hoechst 33342; Green: CellTox Green Cytotoxicity Reagent.

A final long-term incubation experiment was then performed using a similar process, to determine whether the variations observed in early cytotoxicity marker induction also led to differences in final cytotoxic effects from the compounds. The number of total and dead cells was calculated following 1, 3, and 7 day dosings with equivalent compound concentrations. The findings of the cytotoxicity experiment illustrate that pycocyanin causes no appreciable increase in hepatotoxicity over that seen with untreated control wells for 2D and 3D cultured cells (Figure 6C-D). This agrees with previously published data by Cheluvappa *et al.*, 2008 showing that pycocyanin causes ultrastructural changes in liver endothelial cells in the absence of hepatocyte injury. Camptothecin, in contrast, causes significant hepatotoxicity; again agreeing with literature references (Fulco *et al.*, 2000). However dramatic differences are seen in the timing and levels of toxicity between the two cell culture methods (Figure 6A-B). 20 µM camptothecin causes 50% cytotoxicity in 2D culture on day 3 (Figure 6F), and complete cell death by day 7, as witnessed by the cell detachment seen in Figure 6G. The same camptothecin concentration, in comparison, exhibits only 8% cytotoxicity on day 3 (Figure 6I) and 51% cytotoxicity on day 7 (Figure 6J). These results are in agreement with the differences seen between the two culture models for induction of ROS and apoptotic activity.

Conclusions

- Hepatocytes cultured in 3D using the RAFT system demonstrate improved cell health over extended culturing periods, when compared to cells cultured in 2D, allowing for use in long-term cytotoxicity analyses with retained hepatocyte function
- The incorporated cell health and cytotoxicity assays are capable of being used with cells cultured in 3D using the RAFT system, and yield accurate, repeatable results when detected and imaged using the Cytation 3 and analyzed with the Gen5 data analysis software
- Hepatotoxic effects from camptothecin, as observed by others, were also detected in 3D cultured hepatocytes
- The variation in degree and timing of cytotoxic effects from camptothecin, when compared to that seen from 2D cultured hepatocytes highlights the necessity to incorporate relevant 3D cell models when performing experiments to determine potential hepatotoxic effects from repeated dosing of lead compounds