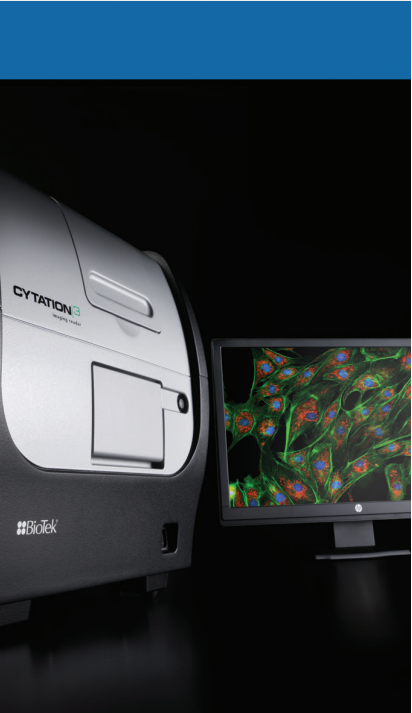


Incorporation of a Novel 3D Cell Culture System to Perform *in vitro* Cytotoxicity Analyses using Human Primary Hepatocytes



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Introduction

Once a lead molecule is identified in the drug discovery process, cytotoxicity studies identify whether the molecule may induce harm to unintended cells. Hepatocytes, being the primary cell type of the liver, provide the major detoxification function. This then increases the potential for cellular dysfunction and death, and has led to the issuance of an FDA guidance on drug-induced liver injury (DILI). While *in vivo* studies remain the gold standard for hepatotoxicity assessment, *in vitro* screens are now being adapted at a higher rate as they reduce animal exposure, are more amenable to higher-throughput platforms, and allow for the determination of cellular mechanisms of action.

Typically in this type of toxicity study, hepatocytes are exposed to a potential drug for multiple days to assess cumulative effects. However, primary hepatocytes cultured in traditional two-dimensional (2D) formats, where the cells adhere to a flat, solid surface, tend to undergo rapid de-differentiation and loss of key functions^{1,2,3}.

In addition, cells grown in a monolayer format are unable to properly form cell-cell and cell-matrix communication networks seen *in vivo*⁴. These two key drawbacks limit the length and scope of hepatotoxicity studies, and may prevent true comprehension of the drug's effects.

Here, we demonstrate differences in cytotoxicity response between human primary hepatocytes cultured using 2D and 3D methods. Cells were challenged with the DNA topoisomerase I inhibitor, camptothecin, as it is known to cause short-term oxidative stress and induction of apoptosis, and eventual cytotoxicity.

We incorporated commonly used tests such as kinetic and endpoint reactive oxygen species (ROS) measurements, kinetic external lipid membrane bilayer exposure of phosphatidylserine (an early apoptosis indicator) and endpoint caspase 3/7 activation. Additionally, kinetic live cell imaging was performed to monitor the toxin's effects on the cells in real time for a thorough assessment of the toxin profile. Image overlay allowed for discrete cellular analysis.

RAFT 3D Cell Culture System Principle

The novel RAFT system (Figure 1) uses a collagen matrix at physiologically relevant concentrations to create a realistic environment, where a wide variety of cells can grow into biomimetic, dimensionally stable 3D structures. In process, cells and neutralized collagen are mixed and dispensed into wells of a 96-well culture plate, and subsequently incubated to allow formation of a cell-seeded hydrogel. A specialized RAFT plate is placed on top of the hydrogels, where sterile absorbers gently remove medium, thus concentrating the cell/collagen hydrogel to a layer approximately 120 μm thick, mimicking physiological conditions. The cultures are then ready for use, or additional layers or epithelial or endothelial cell overlays may be added to study co-cultures or more complex stratified cultures.

Key Words:

- 3D
- RAFT
- Collagen Hydrogel
- Hepatocytes
- Hepatotoxicity
- ROS
- Apoptosis
- Cytotoxicity
- Cytation 3
- Cytochrome P450
- CYP3A4
- Fluorescence Imaging

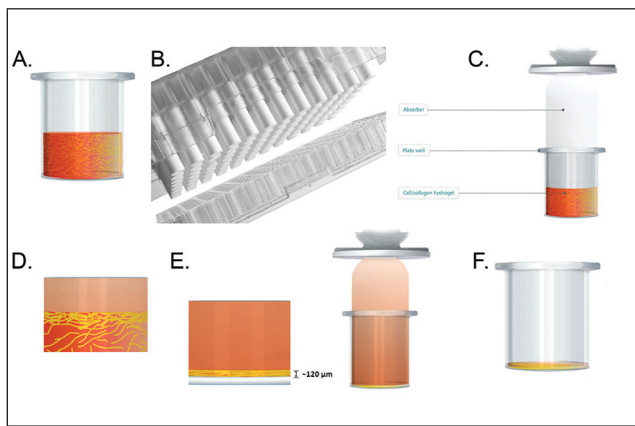


Figure 1. Creation of 3D Hepatocyte/Collagen Hydrogel using the RAFT System. (A) Cells and the prepared collagen solution are mixed, plated, and incubated. After incubation, a 96-well RAFT plate containing individual sterile absorbers (B) is inserted into the cell plate's wells. (C) An additional incubation allows medium to be absorbed, thus concentrating collagen and cells to *in vivo* strength (D) and creating a 120 μm thick hydrogel (E). Finally, the absorbers are removed (F), and new media is added to the concentrated cell/collagen hydrogel.

Materials and Methods

Materials

Cells

Cryopreserved plateable human hepatocytes (Lot IZT) were provided by BioreclamationIVT (Baltimore, MD).

Reagents, Kits, Consumables

CellTox Green Cytotoxicity Assay (Catalog No. G8731) was procured from Promega Corporation (Madison, WI). The DCFDA Cellular ROS Detection Assay (Catalog No. ab113851) and Kinetic Apoptosis Kit (Catalog No. ab129817) were purchased from Abcam (Cambridge, MA), while the CellROX® Deep Red Oxidative Stress Reagent (Catalog No. C10422), CellEvent™ Caspase-3/7 Green Detection Reagent (Catalog No. C10423) and MitoTracker Red CMXRos (Catalog No. M7512) assay kits were purchased from Life Technologies (Carlsbad, CA). 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 Reader

Cytation 3 Cell Imaging Multi-Mode Reader was used to perform all kinetic and end point fluorescence cellular imaging.

Methods

Cell Culture and Propagation

Collagen Hydrogel Cultured 3D 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. 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.

2D Hepatocytes

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

Kinetic Oxidative Stress

Medium was removed from the wells, and the cells were washed with 100 μL of 1x buffer, then stained with DCFDA, Hoechst 33342, and MitoTracker Red at final 1x concentrations of 25 and 5 μM and 200 nM, respectively, for 60 minutes at 37 °C/5% CO_2 . After staining, the cells were washed with 1x buffer, followed by a 100 μL test compound addition, and the plate was immediately placed into Cytation 3 for kinetic imaging every 20 minutes for four to nine hours.

Apoptotic Activity Monitoring

Compound dilutions were prepared in medium containing 10 $\mu\text{L}/\text{mL}$ of pSIVA™ (Polarity Sensitive Indicator of Viability and Apoptosis) reagent contained in the Kinetic Apoptosis Kit. Medium was removed from the wells and replaced directly with the various test compound concentrations along with Hoechst 33342 nuclear stain at a final 1x concentration of 5 μM . The plate was then placed immediately into the Cytation 3 for kinetic imaging once per hour for twenty-four hours.

Long-term Cytotoxicity Detection

CellROX, CellEvent, CellTox Green reagents, and Hoechst 33342 nuclear stain, were added to medium at 6x concentrations of 30 μM , 42 μM , 6x, and 5 μM , 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 , 1x, and 5 μM , and incubated at 37 $^{\circ}\text{C}/5\% \text{CO}_2$ for 60 minutes. All wells were then washed 3x with phosphate buffered saline and imaged with Cytation 3. The percentage of untreated wells was calculated using the following formula:

$$\text{RFU}_{(\text{Compound Treated Well})} / \text{RFU}_{(\text{Untreated Well})} * 100.$$

This process was repeated every day for seven days after initial compound treatment.

Results and Discussion

Kinetic Oxidative Stress

A mechanism of action study was conducted to determine whether oxidative stress plays a role in camptothecin's hepatotoxic effects. Oxidative stress is an early cytotoxicity marker, and ROS induction has been shown to occur rapidly following camptothecin addition⁴, so it was necessary to perform kinetic imaging immediately following compound addition. Cells manifesting fluorescence values above 5000 RFU from the DCFDA probe were considered to exhibit evidence of the respective marker, and the Hoechst 33342 nuclear stain allowed for determination of total cell number and percentage of affected cells per image.

Per the graph in Figure 2A, camptothecin is confirmed to cause oxidative stress with 2D cultured cells where μM concentrations of camptothecin typically induce ROS production in about 10-20% of the cells in the field of view, peaking after about an hour post treatment. However, the 3D cultured hepatocytes showed a lack of response to the camptothecin within the treatment time range (Figure 2B). This difference was confirmed via the kinetic images captured during the incubation period (Figures 2C-H). Fluorescence from the green DCFDA probe peaks at approximately 30 minutes, and then begins to decrease after a 60-minute incubation for 2D cultured hepatocytes. This contrasts to the 3D cultured hepatocytes, where no appreciable change in fluorescence is seen.

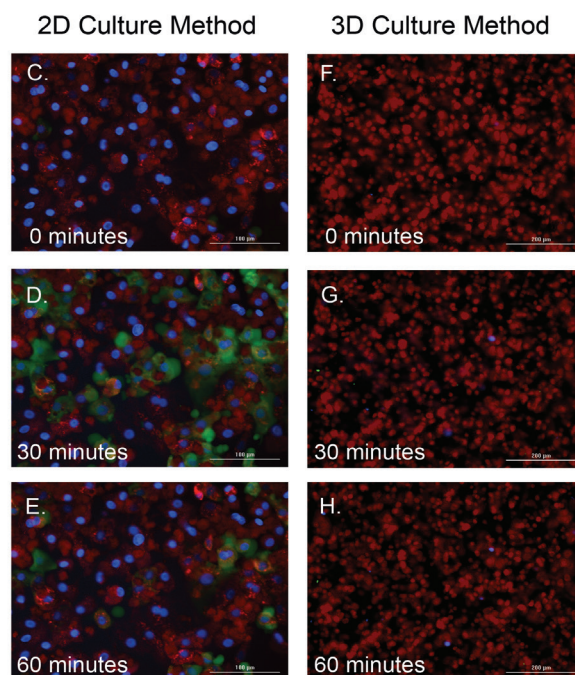
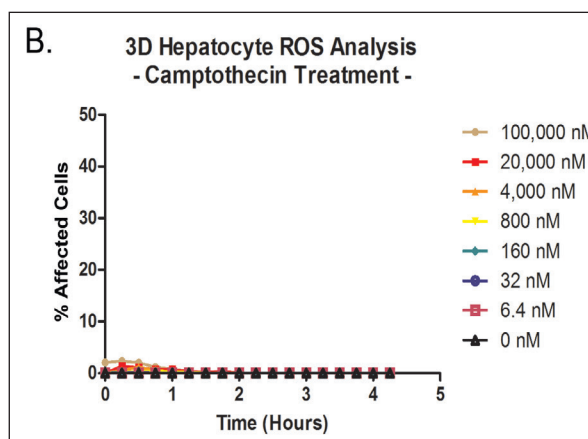
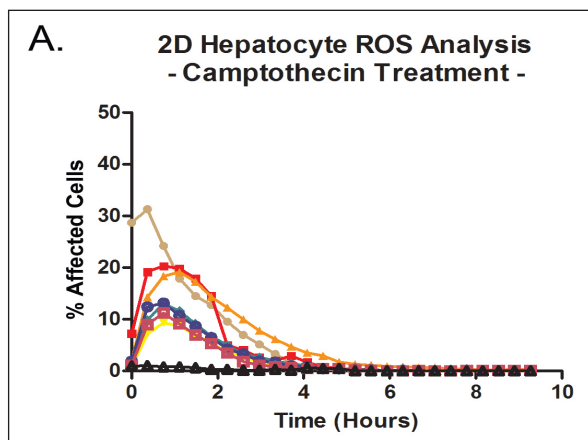


Figure 2. Kinetic ROS Activation Analysis. (A-B) Total cell percentage exhibiting oxidative stress per captured 4x (2D) or 10x (3D) image. (C-E) 20x images of 2D cultured hepatocytes captured after 0, 30 and 60 minute treatments with 800 nM camptothecin. (F-H) 10x images of 3D cultured hepatocytes captured after 0, 30 and 60 minute treatments with 800 nM camptothecin. Blue: Hoechst 33342; Green: DCFDA reagent; Red: MitoTracker Red CMXRos Mitochondrial probe.

Kinetic Apoptotic Activity Monitoring

Apoptosis, another early cytotoxicity marker, was also studied to determine if it is involved in camptothecin's hepatotoxic effects. Cells with fluorescence values above 5000 RFU from the pSIVA probe were considered to exhibit evidence of the respective marker, and the Hoechst 33342 nuclear stain was again used to determine total cell number and compute the percentage of affected cells per image.

The percentage of affected cells per Figure 3A confirms previously published reports that camptothecin also causes apoptosis⁵. The pSIVA probe measures phosphatidyl serine exposure on the cell's plasma membrane and thus is a probe of early stage apoptosis. The time profiles evident in Figure 3A are consistent with early stage apoptosis. However, as seen in the previous kinetic oxidative stress experiment, there is a significant difference in percentage of affected cells between the 2D and 3D cultured hepatocytes (Figure 3B). The 3D cultured hepatocytes exhibit no significant affect from camptothecin when compared to the 2D cultured hepatocytes. This difference was once again confirmed via imaging (Figures 3C-H).

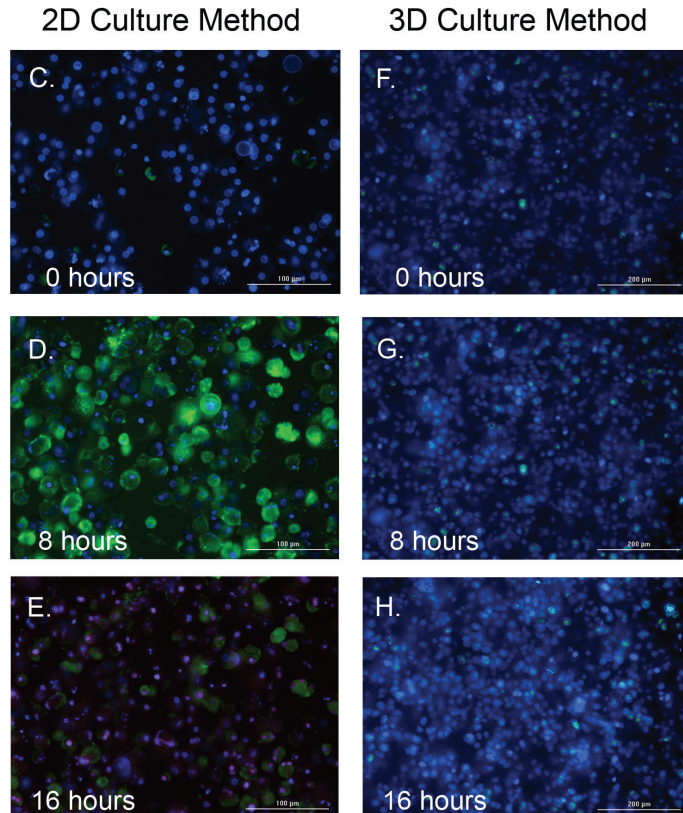
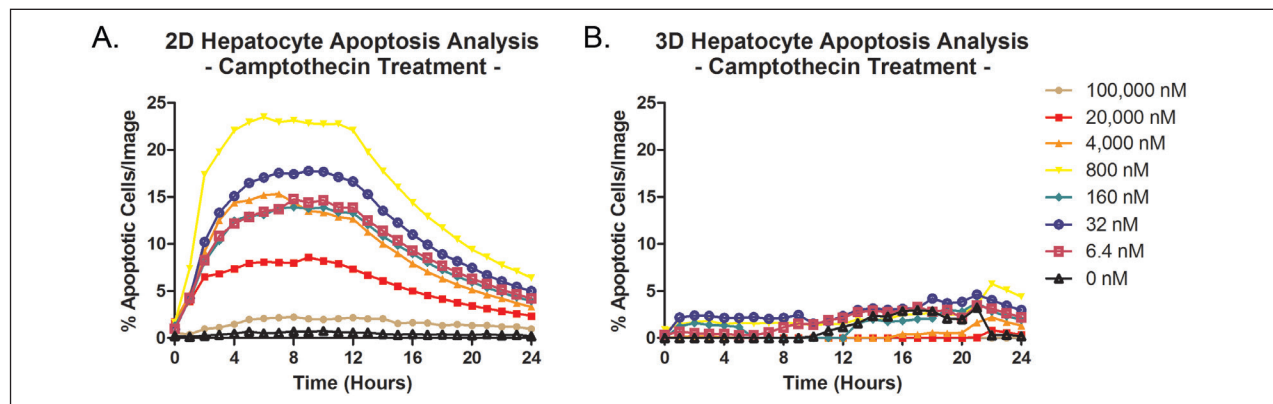


Figure 3. Kinetic Apoptosis Analysis. (A-B) Total cell percentage exhibiting apoptotic activity per captured 4x (2D) or 10x (3D) image. (C-E) 20x images of 2D cultured hepatocytes captured after 0, 8 and 16 hour treatments with 800 nM camptothecin. (F-H) 10x images of 3D cultured hepatocytes captured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

Long-term Compound Cytotoxicity Analysis

Due to the lack of response within the treatment times incorporated with the initial kinetic analyses for hepatocytes cultured in 3D, seven-day dosing experiments measuring oxidative stress and apoptotic activity were then performed to ascertain whether the 3D cells were impervious to camptothecin, or if they experienced delayed ROS induction and apoptotic activity compared to 2D cultured hepatocytes. 2D and 3D cultured cells were dosed with a range of camptothecin concentrations on a daily basis, with end point assessments of oxidative stress and apoptotic activity performed at 1, 3 and 7 days after initial compound treatment.

Results from the multi-day oxidative stress and apoptosis analyses (Figures 4A and 4B, respectively) demonstrate that camptothecin does, in fact, cause oxidative stress and apoptosis in 3D cultured hepatocytes. The 3-day incubation was found to elicit the greatest response, which was also confirmed via imaging. For the 2D cultures, ROS production and phosphatidyl serine exposure has long since run their course by the end of day 1 and thus, as expected, Figures 4A and B demonstrate no response over this time frame. Per Figure 4C-E, an increase in signal was seen from the CellROX and CellEvent fluorescent ROS, and caspase 3/7 probes, respectively.

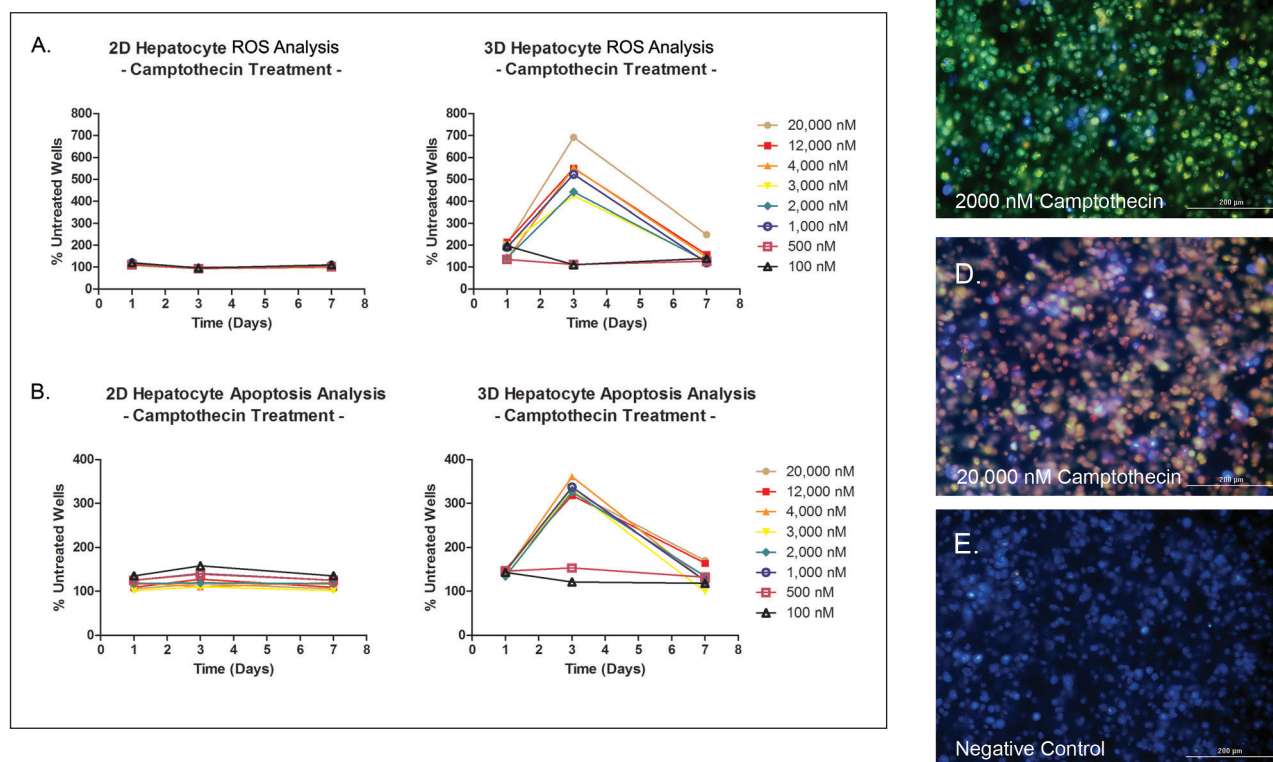


Figure 4. Long-term (A) oxidative stress and (B) apoptosis analysis of 2D and 3D cultured hepatocytes treated with camptothecin. (C-D) 10x images of 3D cultured hepatocytes after 3-day camptothecin treatment, as well as no treatment (E). Blue: Hoechst 33342; Green: CellEvent Caspase-3/7 Reagent; Red: CellROX Deep Red Oxidative Stress Reagent.

A final long-term incubation experiment was performed to determine whether the variations observed in early cytotoxicity marker induction also led to differences in final cytotoxic effects from camptothecin. 2D and 3D cultured cells were again dosed with a range of camptothecin concentrations on a daily basis, and the number of total and dead cells was calculated at 1, 3 and 7 days after initial compound treatment.

Camptothecin was found to cause significant hepatotoxicity, again agreeing with previously published findings⁶. Significant differences were seen in the timing and toxicity levels between the 2D and 3D cultures (Figure 5A-B). 20 μ M camptothecin caused 50% cytotoxicity in the 2D culture on Day 3 (Figure 5D), and complete cell death by Day 7 as confirmed by Figure 5E showing cell detachment. 3D cells dosed with the same 20 μ M camptothecin concentration exhibit only 8% cytotoxicity on Day 3 (Figure 5G), and 51% cytotoxicity on Day 7 (Figure 5H). These results agree with the aforementioned differences in ROS induction and apoptotic activity seen between the two culture models.

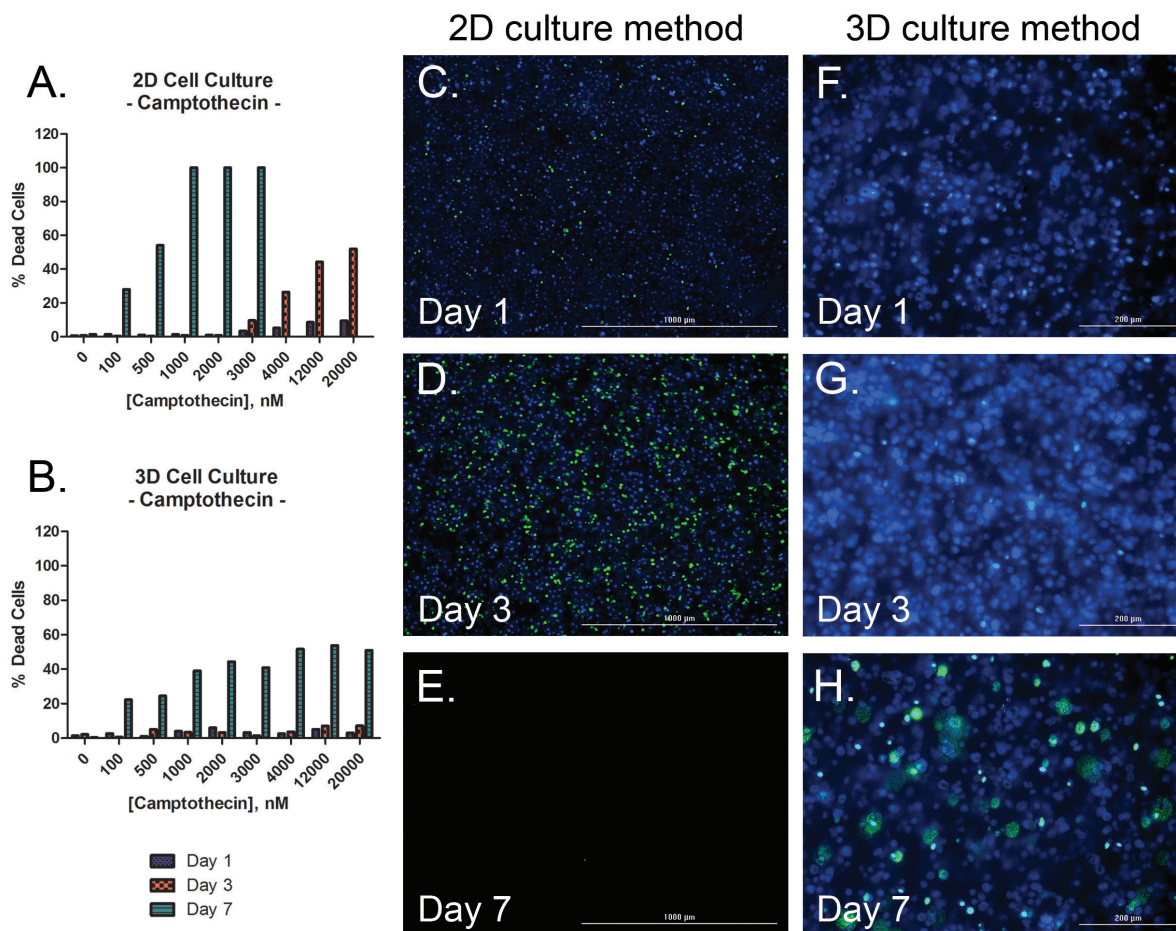


Figure 5. Hepatotoxicity Results. (A-B) Dead cell percentage per captured 4x (2D) or 10x (3D) image. (C-E) 20x images of 2D cultured hepatocytes and (F-H) 10x images of 3D cultured hepatocytes showing live (blue) and dead (green) cells. Blue: Hoechst 33342; Green: CelTox Green Cytotoxicity Reagent.

Conclusions

We have shown that the incorporated cytotoxicity assays are capable of being used with 2D and 3D cultured cells, yielding accurate, repeatable results during detection and imaging. Results show that 3D cultured hepatocytes demonstrated cytotoxicity effects from camptothecin, however they were less sensitive to the toxin than traditional 2D cultures. This was exhibited by the 3D cell's lack of immediate reactive oxygen species induction, and variations in observed cytotoxicity levels over time with multiple camptothecin treatments. This may indicate that the cell-cell and cell-matrix communications exhibited with 3D cell cultures create a more robust, biologically relevant cell culture system. Finally, variations in the degree and timing of cytotoxic effects from camptothecin, when comparing 2D and 3D cultured hepatocytes, highlights the necessity to incorporate relevant 3D cell models when performing experiments to determine potential hepatotoxic effects from repeated and extended dosing of lead compounds.

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