

The Impact of a 3D Human Liver Microtissue Model on Long-term Hepatotoxicity Studies

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most highly prescribed drug families around the world, and consequently, along with antimicrobial agents, are the most frequent causes of drug-induced liver injury (DILI).¹ Diclofenac, in particular, frequently used to treat chronic pain and inflammatory disorders, elicited an FDA warning in 2009 concerning potential hepatic effects.²

Multiple *in vitro* cell-based models help to determine the varied mechanisms of action (MOA) of NSAID-related hepatotoxicity. Studies using rat liver mitochondria and freshly isolated rat hepatocytes demonstrate that the common NSAID structure, diphenylamine, uncouples oxidative phosphorylation and decreases hepatic ATP content.³ Mitochondrial permeability transition (MPT) is also shown to be important in diclofenac-induced liver injury, as well as the role that oxidative stress plays in MPT induction.⁴ Finally, cytochrome P450 (CYP)-related metabolic activation of diclofenac and reactive metabolite formation are also related to diclofenac hepatotoxicity.⁵ These mechanisms do not necessarily manifest independently from each other, yet their combined hepatotoxic effects may not appear until weeks after therapy commencement.

When performing hepatotoxicity studies, *in vivo* models remain the gold standard, however, they are costly and time-consuming. Consequently, *in vitro* screens using primary hepatocytes are less costly, reduce animal exposure, and are more amenable to higher-throughput platforms. A typical toxicity study involves repeatedly dosing cultured hepatocytes with a potential drug over multiple days to assess any cumulative effects. A challenge to this method is that primary hepatocytes cultured on a hard, flat surface tend to undergo rapid dedifferentiation and decreased metabolic capacity.^{6,7,8} In addition, these 2D cultured hepatocytes are less inclined to form the complex cell-cell and cell-matrix communication networks found *in vivo*.⁹ These functional and communication shortcomings limit the scope of current hepatotoxicity studies, and may impede true understanding of the drug's cumulative and long-term effects in the body.

Newer 3D cell culture models exist that allow cells to aggregate and retain the functionality and communication networks found *in vivo*. The favorable environment created by the 3D culture model allows for longer term dosing experiments that analyze a potential drug's cumulative effects. This application note evaluates the suitability of 3D human liver microtissues for use in long-term toxicity studies. The microtissues are formed by proprietary hanging drop technology that allows primary human hepatocytes to reaggregate into functional microtissues. The microtissues demonstrate *in vivo*-like cell-cell and cell-matrix interactions and retain viability over weeks.

The experiments incorporated fluorescent probes to facilitate 2D and 3D total- and dead-cell counting, while mitochondrial superoxide concentration was used to monitor cellular oxidative stress. Apoptosis was assessed by measuring MPT pore opening. Additionally, live-cell imaging was performed to visually monitor the toxin's effects on the 2D and 3D cultured cells, and confirm assay findings.

Materials and methods

Materials

Cells

3D liver microtissues created from human hepatocytes (lot IZT, BioreclamationIVT (Baltimore, MD) were purchased from InSphero, Inc. (Cambridge, MA) and supplied in ready-to-use 96-well GravityTRAP plates. Cryopreserved human hepatocytes from the same lot were also obtained directly from BioreclamationIVT.

Reagents, kits, and consumables

CellTox Green Cytotoxicity Assay (part number G8731) was donated by Promega Corporation (Madison, WI). MitoSOX Red Mitochondrial Superoxide Indicator (part number M36008), and Image-iT Live Assay (containing Calcein AM, MitoTracker Red CMXRos, Hoechst 33342, and Cobalt II chloride hexahydrate) (part number I35103) were purchased from Life Technologies (Carlsbad, CA). BioCoat Collagen I 384-well black, clear bottom plates (part number 354667) were donated by Corning Life Sciences (Corning, NY).

Instrumentation

Agilent BioTek Cytation 3 cell imaging multimode reader

Agilent BioTek Cytation 3 cell imaging multimode reader was used to perform all fluorescence cellular imaging.

Methods

Cell culture and propagation

3D liver microtissues:

Medium was exchanged in 96-well GravityTRAP plates containing 3D liver microtissues, and the plate was incubated overnight at 37 °C/5% CO₂.

2D hepatocytes:

Cryopreserved hepatocytes were thawed, diluted with media provided by BioreclamationIVT, and plated in 384-well black, clear bottom plates at a concentration of 10,000 cells/well and incubated overnight 37 °C/5% CO₂.

Diclofenac cytotoxicity study

3D liver microtissues:

Diclofenac was added to designated wells in final 1x concentrations of 300, 30, 3 and 0 μM. The microtissues were then redosed daily with diclofenac over two weeks by removing medium with compound and replacing with an equal volume of new medium and compound. Diclofenac induced cytotoxicity levels were analyzed 1, 3, 7, 10, and 15 days following initiation of treatment. On each appointed day, medium containing compound was removed and 30 microliters of medium containing 5 μM Hoechst 33342 and either 1x CellTox Green and 5 μM MitoSOX probes, or 1x concentrations of the Image-iT components (1 μM Calcein AM, 200 nM MitoTracker Red CMXRos, 1 μM Hoechst 33342, and 1 mM CoCl₂) were added to designated wells and incubated at 37 °C/5% CO₂ for four hours. Following incubation, the wells were washed three times with phosphate-buffered saline (PBS) and imaged at 4x and 10x magnification.

2D hepatocytes:

Diclofenac was added to designated wells in final 1x concentrations of 300, 30, 3 and 0 μM . The cells were then redosed daily with diclofenac over approximately two weeks by removing medium with compound and replacing with an equal volume of new medium and compound. Diclofenac induced cytotoxicity levels for the 2D cultured hepatocytes were analyzed 3 and 10 days following initiation of treatment. On each appointed day medium containing compound was removed and 25 microliters of medium containing Hoechst 33342 and either CellTox Green and MitoSOX probes, or the Image-iT components, at equal concentrations as what was used for the 3D cytotoxicity studies, were added to designated wells and incubated at 37 °C/5% CO₂ for one hour. Following incubation, the wells were washed three times with PBS and imaged at 4x and 20x magnification.

Results and discussion

Diclofenac cytotoxicity study

3D liver microtissues

3D hepatocyte microtissue cytotoxicity was assessed using the aforementioned diclofenac concentrations spanning 3 logs. Total cells were quantified using Hoechst stain, and measured using an RFU threshold of 5,000, with a 5 to 30 μm object size range (minimum and maximum, respectively). Dead cells were quantified using CellTox Green stain, and measured using an RFU threshold of 9,000, with a 5 to 30 μm object size range (minimum and maximum, respectively). Dead cell percentages were calculated using the following formula:

$$\left(\frac{\text{Dead Cell Number}}{\text{Total Cell Number}}\right) \times 100$$

Results from the diclofenac cytotoxicity study (Figure 1) demonstrate that total cell counts from the 3D liver microtissues remain relatively constant over the diclofenac dosing range, while the number of dead cells increases with increased diclofenac concentration during the 16-day dosing period.

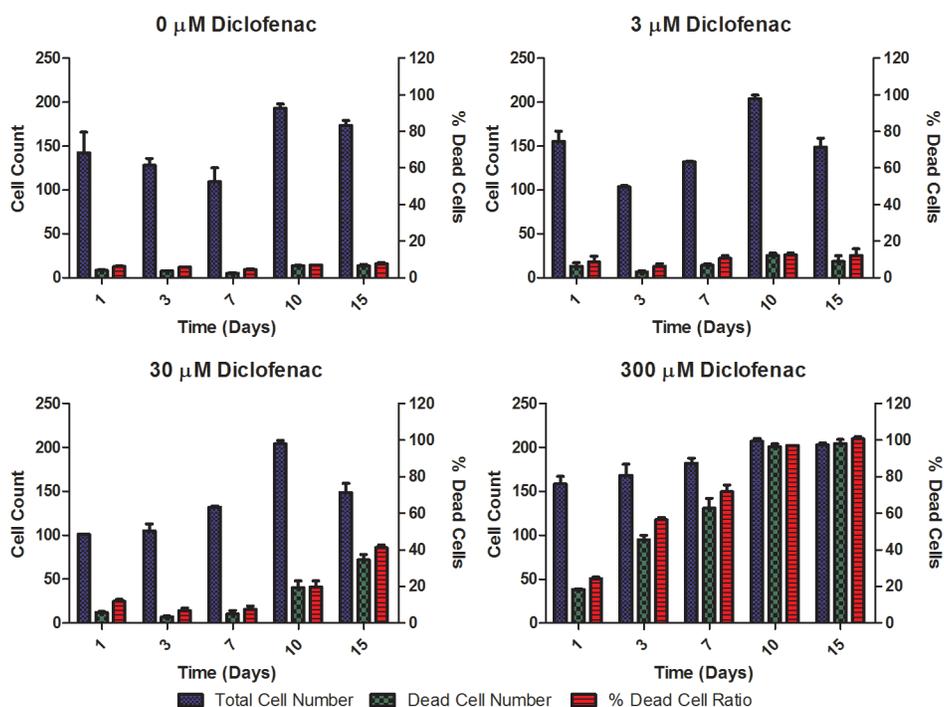


Figure 1. Diclofenac hepatotoxicity results using liver microtissues. Total cell and dead cell counts (left y-axes) were calculated for liver microtissues treated with diclofenac for dosing times listed (x-axes). Ratio of dead:total cell counts represented using right y-axes.

This is confirmed via imaging of the 3D liver microtissues at each of the dosing concentrations following a 10-day diclofenac treatment (Figure 2). This demonstrates that the 3D microtissues are a viable option when monitoring a test compound's potential cytotoxicity, particularly during chronic dosing evaluations.

Additionally, it can be seen that significant cytotoxicity does not occur until day 3 of diclofenac dosing, using a 300 μM concentration. This contrasts with previously published data demonstrating that cytotoxicity may be seen in as few as 24 hours using 200 to 450 μM diclofenac concentrations on 2D cultured hepatocytes, and in as few as two hours when using suspension hepatocytes.^{4,10} This contrast underlies the need to use the most relevant cell model for cytotoxicity studies.

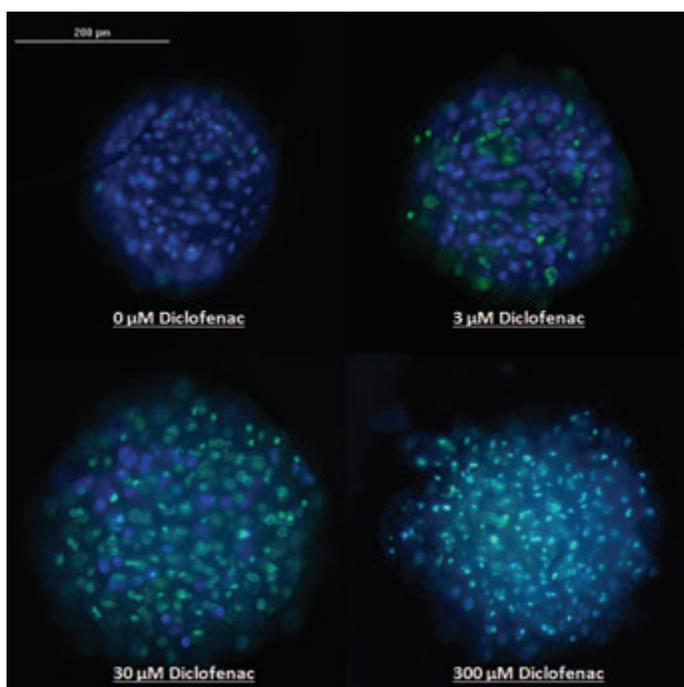


Figure 2. 3D Liver microtissue cytotoxicity imaging. Representative overlaid images of liver microtissues after a 10-day diclofenac treatment, stained with Hoechst 33342 (blue) and CellTox Green (green), and captured using the DAPI or GFP Agilent BioTek Cytation 3 imaging channels, respectively. Autofocus performed on CellTox Green stained cells.

2D hepatocytes

The same cytotoxicity assessments were performed using 2D cultured hepatocytes at a concentration of 10,000 cells/well and diclofenac concentrations spanning 3 logs to assess whether there were differences in induced toxicity between the 2D and 3D cell models. Assessments were made at day 3 and day 10 of the diclofenac treatment. Hoechst 33342 and CellTox Green stains were used to detect total and dead cells respectively, with an RFU threshold of 10,000 and a 5 to 35 μm object size range (minimum and maximum, respectively).

As illustrated in Figure 3, total hepatocyte and dead cell counts decrease at the highest diclofenac concentration causing percentage dead cell ratios to remain unchanged or lower than untreated cell ratios, contrary to expectations. This may falsely suggest that diclofenac toxicity is not concentration dependent, and also shows little change over time.

However, imaging at the various diclofenac concentrations (Figure 4) confirms that an increasing number of cells are completely cytotoxic and have detached from the well bottom. Detachment artificially lowers total and dead cell counts, with the end effect being that the percentage dead cell ratio does not change, as was seen in Figure 1. This phenomenon complicates analysis using 2D cell cultures and further supports incorporation of 3D cell culture models into long-term cytotoxicity assessments.

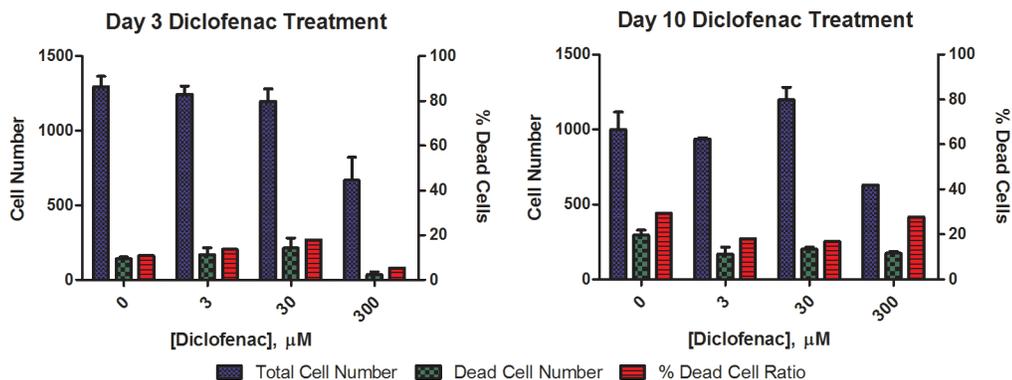


Figure 3. Diclofenac hepatotoxicity results using 2D cell culture. Total cell and dead cell counts (left y-axes), in addition to percent dead cells (right y-axes), were calculated for 2D cultured hepatocytes treated with diclofenac concentrations over three and ten days.

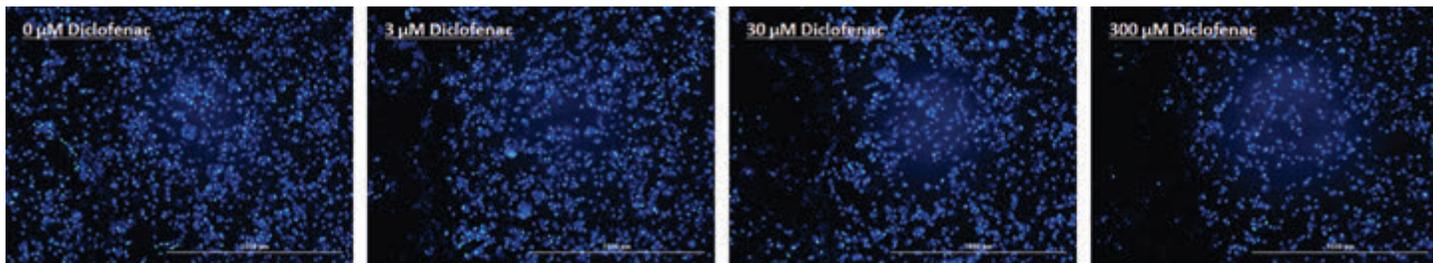


Figure 4. 2D Hepatocyte cytotoxicity imaging. Representative overlaid images of 2D cultured hepatocytes after a 10 day diclofenac treatment, stained with Hoechst 33342 (blue) and CellTox Green (green), and captured using the DAPI or GFP Agilent BioTek Cytation 3 imaging channels, respectively. Autofocus performed on Hoechst 33342 stained cells.

Diclofenac cytotoxicity mechanism of action confirmation

A final experiment was conducted to determine if diclofenac's purported cytotoxicity MOA in rat hepatocytes could also be observed in 3D cultured human hepatocyte microtissues. Mitochondrial oxidative stress and MPT pore opening were monitored on the same days in which cytotoxicity was assessed. The percentage of MitoSOX or Image-iT calcein signal for diclofenac-treated wells was calculated using the following formula:

$$\left(\frac{\text{Diclofenac Treated Well Signal}}{\text{Untreated Well Signal}} \right) \times 100$$

Diclofenac induction of mitochondrial oxidative stress was confirmed using the MitoSOX assay (Figure 5). Following a 300 μM diclofenac dosing over three days, a significantly increased signal from the red fluorescent probe was seen, indicating elevated superoxide levels. This is consistent with

previously published data showing that diclofenac cytotoxicity is a result of ROS formation.¹⁰

Increases in mitochondrial oxidative stress also led to MPT pore opening, as witnessed by the decreased calcium signal at increased diclofenac concentrations (Figure 6). Cobalt chloride, a component of the Image-iT assay, quenches cytosolic calcein, but is prevented from quenching mitochondrial calcein as it cannot freely pass through the mitochondrial membrane. Once the MPT pore is opened, cobalt chloride enters the mitochondria to quench the calcein signal, which was observed after a 7-day compound dosing, and agrees with previously published literature.⁴ This assessment confirms that these important MOA are observed using 3D cultured human hepatocyte microtissues.

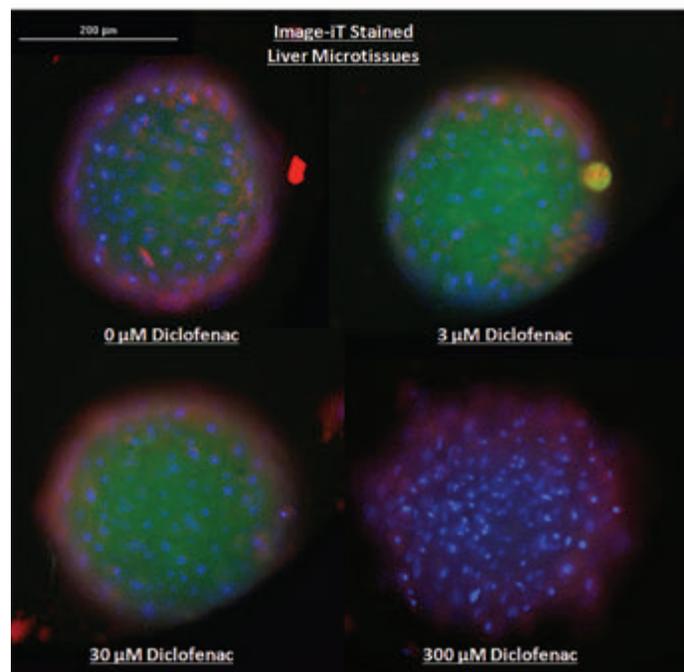
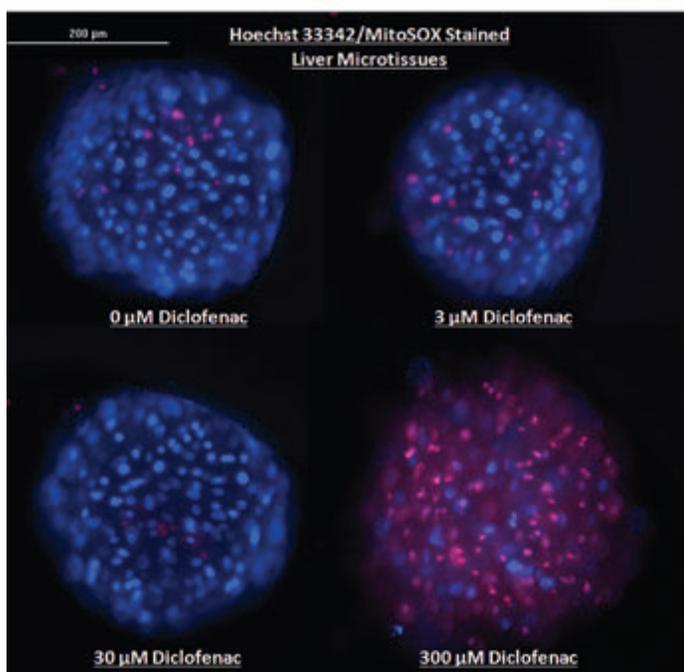
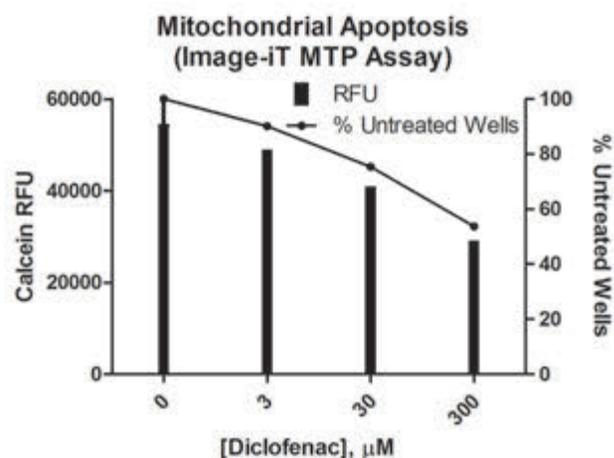
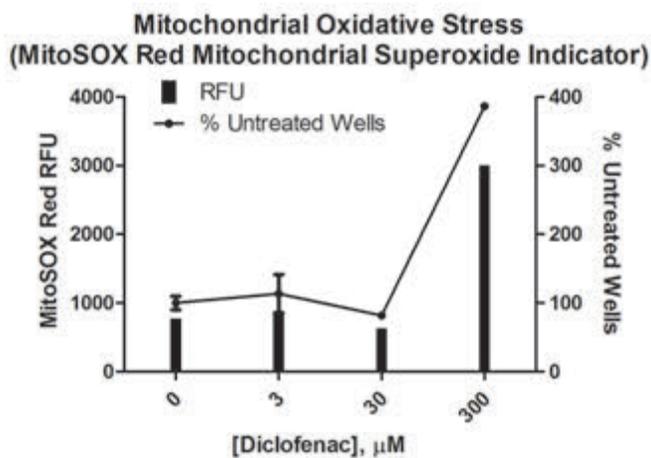


Figure 5. Mitochondrial oxidative stress assessment following a 3-day diclofenac dosing. (A) Mean pixel RFU values from the field of view (left y-axis) and percent signal for diclofenac treated wells (right y-axis) measured from MitoSOX assay signal. (B) Representative overlaid images of 3D liver microtissues, stained with Hoechst 33342 (blue) and MitoSOX Red (red); captured using the DAPI or RFP Agilent BioTek Cytation 3 imaging channels, respectively. Autofocus performed on DAPI stained cells.

Figure 6. MPT induction assessment following a 7-day diclofenac dosing. (A) Mean pixel RFU values from the field of view (left y-axis) and percent signal for diclofenac treated wells (right y-axis) measured from Image-iT calcein signal. (B) Representative overlaid images of 3D liver microtissues, stained with Hoechst 33342 (blue) and MitoTracker Red (red) and calcein (green); captured using the DAPI, Texas Red or GFP Agilent BioTek Cytation 3 imaging channels, respectively. Autofocus performed on DAPI stained cells.

Conclusion

This application note has shown that primary human hepatocytes, cultured into 3D liver microtissues demonstrated long-term mitochondrial injury and cytotoxicity effects from diclofenac over multiple concentrations. The microtissues also appeared to be less reactive to the toxin than traditional 2D cultures. However, direct comparison to 2D cultures was complicated by detachment of hepatocytes from the tissue culture-treated surface of the microplate well such that cell counting was skewed. This may indicate that the 3D culture's cell-cell and cell-matrix communications create a more robust and biologically relevant cell culture system. Additionally, the incorporated assays delivered accurate and reliable results when used with the 3D cell culture model and imaged with the Agilent BioTek Cytation 3 cell imaging multimode reader. Finally, differences seen in diclofenac-induced cellular events using the 3D liver microtissues, when compared to published results using other hepatocyte models, highlights the necessity to incorporate relevant 3D cell models when performing repeated and extended dosing hepatotoxicity experiments.

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