Corning® HepatoCells Spheroids: A New High Throughput 3D Model for Hepatotoxicity Studies

Application Note

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Feng Li, Ph.D. and Rongjun Zuo, Ph.D.
Corning Incorporated, Life Sciences
Bedford, MA USA

Introduction

In vitro hepatic 3D cultures based on single cell line or mixed cell types have been shown to better reflect the *in vivo* behaviors of liver cells than 2D cultures. 3D cultures have been shown to have proper polarization, cell-cell contacts, and hepatic cellular functions[1]. These advanced systems are currently being evaluated by both industrial and academic scientists for drug discovery and drug development research[2].

In this study, we combine two novel innovations: Corning HepatoCells and Corning spheroid microplates to create a high throughput 3D model suitable for cytotoxicity screening. Corning HepatoCells are a renewable hepatic cell source derived from primary human hepatocytes. They have consistent phenotypes for cytochrome P450 enzymatic and drug transporter activities, which are necessary for ADME applications such as CYP induction, metabolism, and cytotoxicity studies.

Corning spheroid microplates feature an Ultra-Low Attachment surface and round well bottom geometry that facilitate the generation of single spheroids within each well. Single spheroids made from Corning HepatoCells are uniform in size and shape. At lower seeding densities, ATP levels measured for these single spheroids closely correlate with the numbers of initial seeding cells.

To characterize this novel 3D hepatic culture system, the activity of CYP3A4, a major drug metabolizing cytochrome P450 enzyme, was measured using Corning HepatoCells spheroids. Our data indicate that CYP3A4 activity is significantly increased in spheroids in comparison to 2D cultures. Furthermore, CYP3A4 activity in spheroid culture is also inducible (3-fold) in response to the treatment of rifampicin, which is a prototypic CYP3A4 inducer.

For hepatotoxicity tests, Corning HepatoCells spheroids were treated with known liver toxins such as aflatoxin B1 and acetaminophen. Bioluminescent ATP assays were performed for endpoint viability measurement. Consistent with increased CYP3A4 activity in 3D spheroid culture, HepatoCells spheroids are much more susceptible than 2D culture to aflatoxin B1-induced cell death, which is CYP-mediated drug metabolism-dependent. Importantly, the dose response EC_{50} values of known liver toxins from HepatoCells spheroids were consistent with other *in vitro* models. Together, our results demonstrate a promising 3D spheroid culture-based platform using Corning HepatoCells and Corning spheroid microplates for high throughput screening of liver toxins.

Materials and Methods

- Corning 96-well spheroid microplate, Ultra-Low Attachment surface (Corning Cat. No. 4515)
- Corning 384-well spheroid microplate, Ultra-Low Attachment surface (Corning Cat. No. 4516)
- Cryopreserved Corning HepatoCells (Corning Cat. No. 354881)
- Corning culture medium for HepatoCells (Corning Cat. No. 354882)
- Fetal Bovine Serum (FBS) (Corning Cat. No. 35016-CV)
- Pen/Strep (100x) (GIBCO Cat. No. 15140)
- 0.4% Trypan blue (Sigma Cat. No. T8154)

Medium Preparation

Spheroid Plating Medium

Corning culture medium for HepatoCells	100 mL
FBS	10 mL
Pen/Strep	1 mL

Spheroid Culture Medium

Corning culture medium for HepatoCells	100 mL
Pen/Strep	1 mL

Procedures

Thawing Corning HepatoCells

- Prepare a 50 mL conical tube with 15 mL pre-warmed spheroid plating medium.
- Thaw cryovial quickly in a 37°C water bath.
- Spray cryovial with 70% ETOH and bring to tissue culture hood.
- Transfer cells from cryovial to the 50 mL conical tube with plating medium.
- Rinse cryovial once with 1 to 2 mL spheroid plating medium.
- Gently pipet the cell suspension to break any cell clumps.
- Add spheroid plating medium to 50 mL.
- Centrifuge at 150 g for 10 minutes.
- Aspirate media.

Cell Suspension Preparation

- Resuspend cells in 10 mL spheroid plating medium.
- Break any cell clumps by pipetting with a 10 mL pipet to prepare single cell suspension.
- Take 0.5 mL from cell suspension for Trypan blue cell counting with a hemocytometer.

Cell Counting with Trypan Blue

- Mix 50 µL cells with 50 µL 0.4% Trypan blue (1:1 dilution).
- Count 4 squares of diluted cells on hemocytometer.
- Record live and dead cells.
- Calculate cell density and viability.
- Cell density (10^4/mL) = live cell count/2
- Cell viability (%) = live cell count/(live cell count + dead cell count) x 100

Setting Up Corning® HepatoCells Spheroid Culture

For 96-well Corning spheroid microplate:

- Dilute cell suspension at 15 x10^3 cells/mL in plating medium.
- Plate 100 µL/well (1.5K cells/well) diluted cell suspension.
- Transfer the plate to incubator at 5% CO_2, 37°C.
- No media change for the next 2 days.

For 384-well Corning spheroid microplate:

- Dilute cell suspension at 30 x10^3 cells/mL in spheroid plating medium.
- Plate 50 µL/well (<5K/well) diluted cell suspension.
- Transfer the plate to incubator at 5% CO_2, 37°C.
- No media change for the next 2 days.

Note: At low seeding densities (<5k/well), cells first appear as loose clumps followed by restructuring to form a single spheroid with a smooth outline in 48 to 72 hours.

Spheroid Culture Maintenance

Day 3 feeding with spheroid culture medium

For 96-well Corning spheroid microplate:

- Add 100 µL/well spheroid culture medium.

For 384-well Corning spheroid microplate:

- Add 50 µL/well spheroid culture medium.

Perform half-medium change every other day with spheroid culture medium.

For 96-well Corning spheroid microplate:

- Remove 100 µL/well old medium.
- Add 100 µL/well spheroid culture medium.

For 384-well Corning spheroid microplate:

- Remove 50 µL/well old medium.
- Add 50 µL/well spheroid culture medium.

Note: If minimal FBS is desired, half-medium change steps can be repeated 5 to 6 times on day 3 to minimize the residual amount of FBS.

Results

Characterization of Corning HepatoCells Spheroids

As shown in Figure 1, a serial dilution of Corning HepatoCells was performed to determine the optimal cell density for spheroid culture using 384-well Corning spheroid microplates. At lower

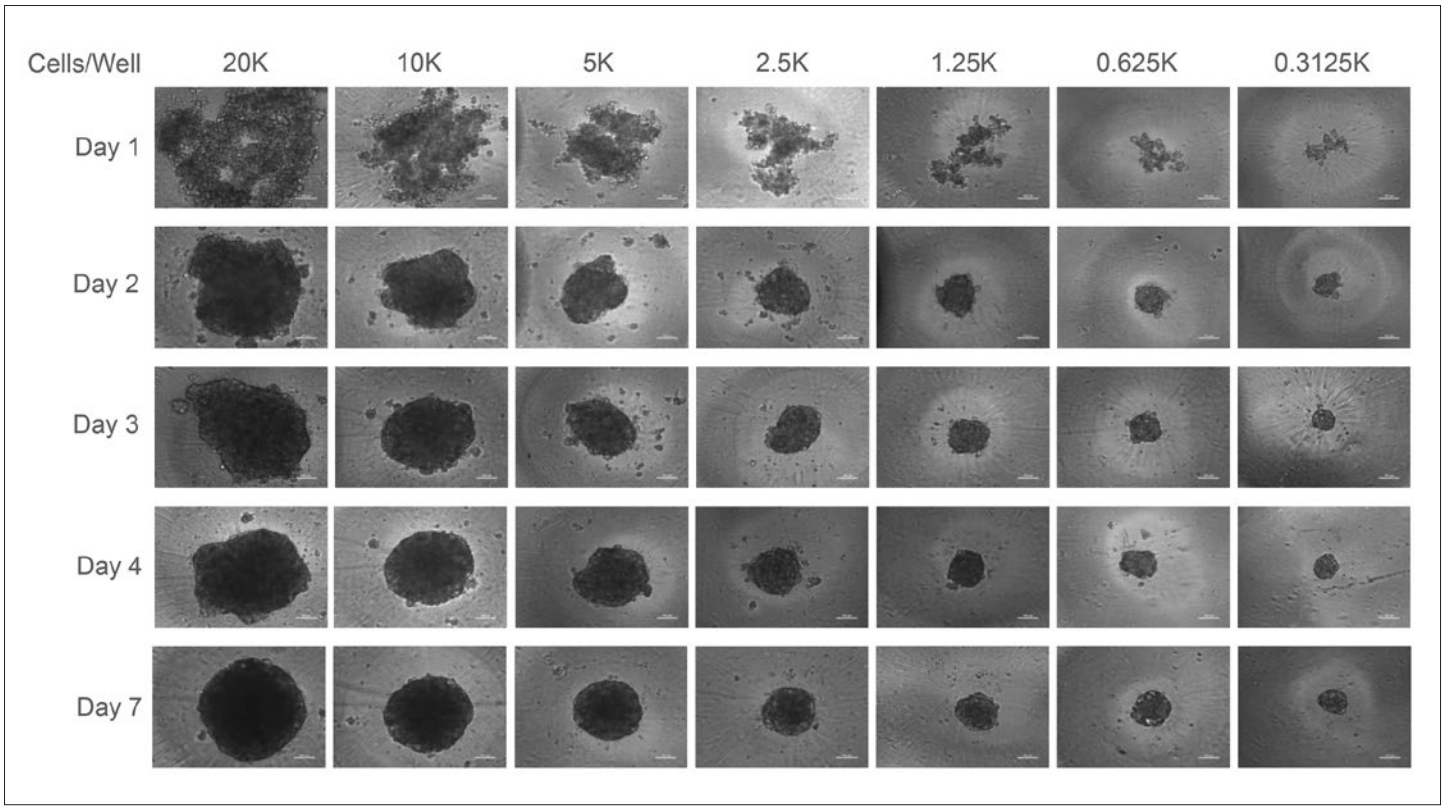

Figure 1. Correlation between sizes of spheroids and initial seeding density. Corning HepatoCells were seeded at indicated cell densities in 384-well Corning spheroid microplates (Corning Cat. No. 4516) and cultured over 7 days. No medium changes were made for the first 2 days and spheroid cultures were then maintained with half-medium changes every other day. Bar = 100 µm.

seeding densities (<5K/well), it took 2 to 3 days for single spheroids to form under the test conditions. It takes extra days for single spheroids to form at higher seeding densities. Spheroids were uniformly round with a smooth outline, and the sizes of spheroids correlated with the initial seeding density (Figures 1 and 2).

To evaluate the structure and cell morphology, Corning® HepatoCells spheroids (1.5K/well) were fixed in 10% formalin and were paraffin-embedded prior to sectioning on day 7. An image of Hematoxylin and Eosin (H&E) staining shows the intact core structure and no signs of necrotic cell death in a spheroid (Figure 2). At a given seeding density, the size and shape of HepatoCells spheroids are very uniform and consistent between individual wells. Measurement of the diameter of HepatoCells spheroids during the 7 day culture showed excellent consistency and clear correlation between initial seeding density and the sizes of the spheroids. This correlation was further confirmed by ATP bioluminescent assay for day 7 spheroids. Quantitative analysis indicated that at lower seeding densities (<5K/well), the ATP levels closely correlated to the cell numbers with very small variations. Based on these results, 1.5K cells/well seeding density is recommended for routine spheroid culture using Corning HepatoCells.

CYP3A4 Activity in Corning HepatoCells Spheroids

A specific bioluminescent based Luciferin-IPA probe (Promega) was used to assess the CYP3A4 activity in Corning HepatoCells spheroids in comparison to 2D culture. As shown in Figure 3, CYP3A4 activities were measured for both 3D spheroid and 2D cultures. ATP levels were measured at the same time for both cultures and used for normalizing the measured CYP3A4 activities. After normalization, CYP3A4 activity in 3D spheroids from Corning HepatoCells is 538% greater than that of 2D culture (set at 100%, Figure 3C). Similar observations of increased CYP activities have been made for 3D cultures of other hepatic cells lines[3]. CYP3A4 activity is also inducible in HepatoCells spheroids. Table 1 shows that following a 3-day induction with Rifampicin at 10 µM, there was ~3-fold induction in HepatoCells spheroids. This lower CYP3A4 induction fold change in spheroids compared to 2D culture (19.6-fold and data not shown) is likely the result of higher basal activities as shown above.

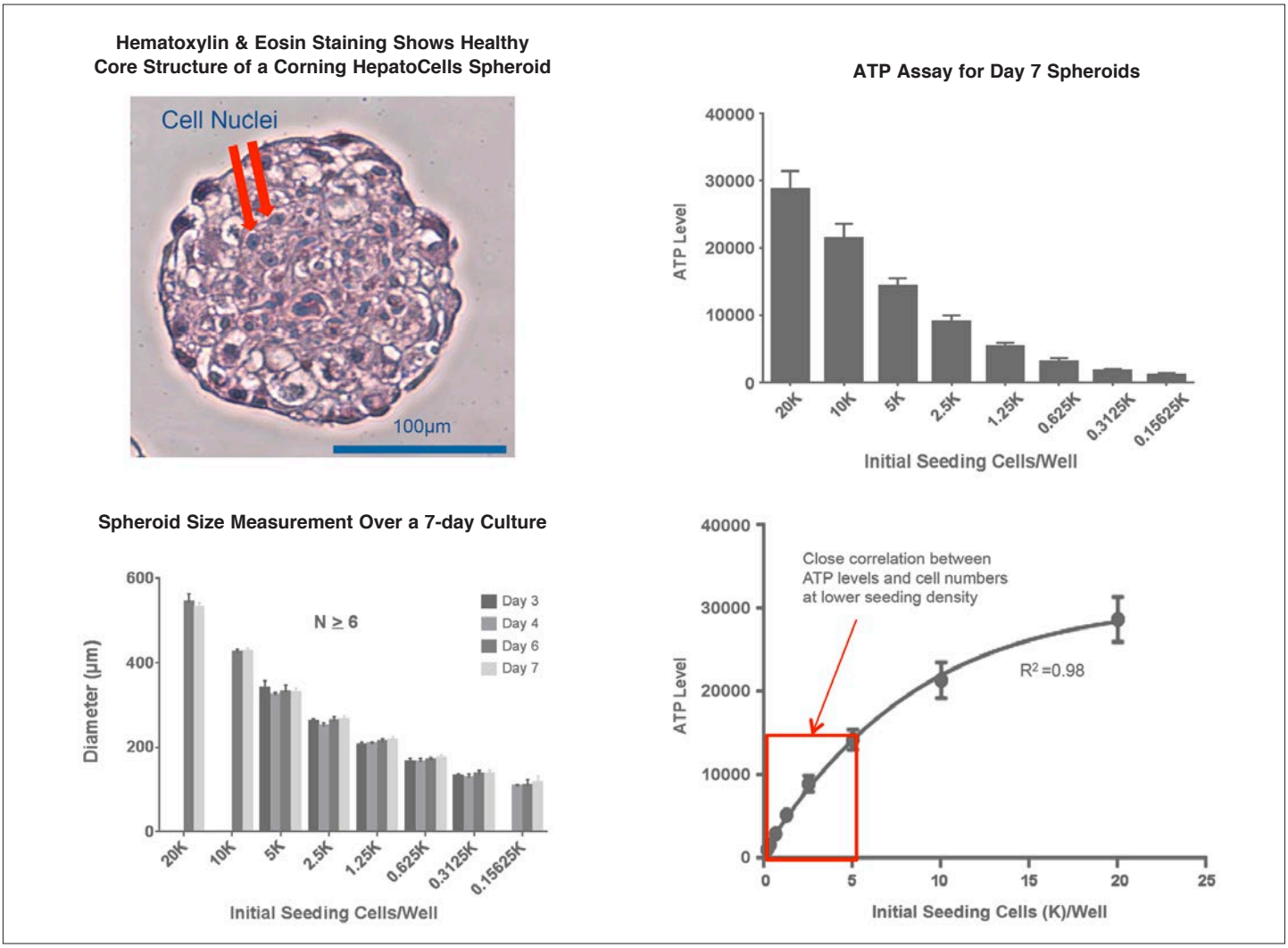

Figure 2. Uniform formation of Corning HepatoCells spheroids in a Corning spheroid microplate. Corning HepatoCells were seeded at indicated cell densities per well in a 384-well Corning spheroid microplate. For H&E staining, day 7 spheroids from HepatoCells (1.5K/spheroid) were collected and fixed in 10% PBS-buffered formalin for >24 hours prior to paraffin embedding and preparation and staining of 10 µm sections. Spheroid images were taken and sizes (diameters) were measured during the culture period. ATP levels (viability) were measured with a Promega CellTiter-Glo® 3D kit following the manufacturer's instructions.

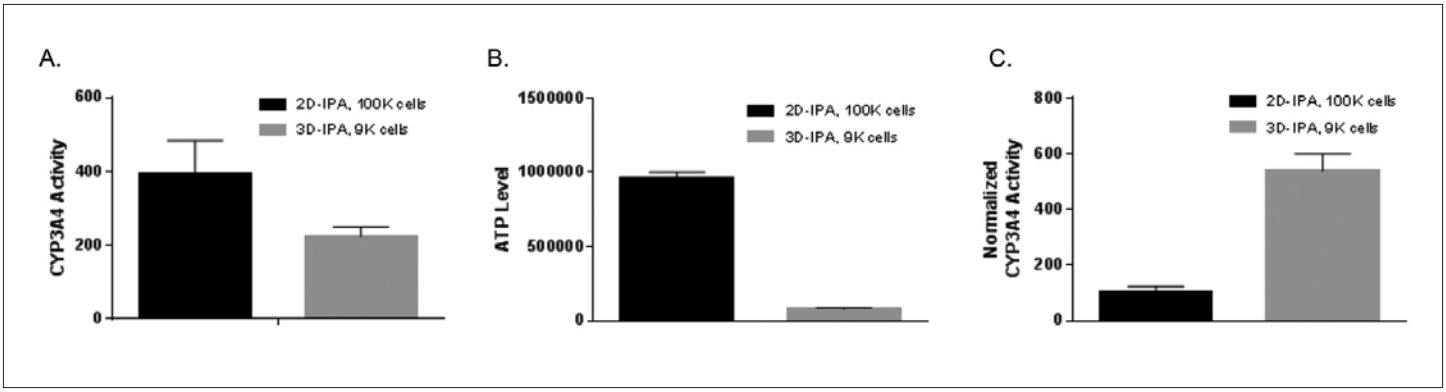

Figure 3. Higher CYP3A4 activities in Corning HepatoCells spheroid microplate vs. 2D culture (normalized to ATP levels). Corning HepatoCells were seeded at 1.5K/well for spheroid culture in 96-well spheroid microplates. At the same time, 100K/well HepatoCells were used to set up 2D culture in a 96-well Corning® BioCoat™ Collagen I microplate. CYP3A4 activity was measured with a Promega Luciferin-IPA kit for day 7 spheroid culture (pool of 6 spheroids = 9K cells; 2D = 100K cells/well) (A). ATP levels were measured for both spheroids and 2D cultures (B), and the levels were used to calculate the normalized CYP3A4 activities (C). Error bars = standard deviation, n = 3.

Corning® HepatoCells Spheroid Treatment with Classic Liver Toxins

Drug-induced liver injury (DILI) is the leading cause of acute liver failure and post-market drug withdrawal[4,5]. 3D human hepatic culture systems have been documented to better mimic the *in vivo* behaviors of liver cells and could be used to improve the *in vitro* model systems for liver toxicity tests. Aflatoxin B1-induced hepatotoxicity in Corning HepatoCells 2D culture is dependent on CYP activity (e.g., CYP3A4) and is reversible by pretreatment with a CYP inhibitor, Ketoconazole, at 20 µM (Figure 4). In comparison, HepatoCells day 4 spheroids are more sensitive to aflatoxin B1-induced cell death than 4 day 2D culture as shown by calculated EC_{50} values, 20.7 µM versus 78.4 µM, respectively. This is consistent with higher CYP metabolic activity in 3D spheroids than 2D culture of HepatoCells.

Figure 5 shows the viability of day 7 Corning HepatoCells spheroids (1.5K/well) after 24-hour treatment with serial dilutions of liver toxins (aflatoxin B1 and acetaminophen) or non-toxic menthol. After normalized to untreated samples, the EC_{50} values were calculated for HepatoCells spheroids. These values were comparable, or better, than some reported values using other *in vitro* models with either primary human hepatocytes or HepG2 cell line in 2D cultures (Figure 5, Table 2). It is noteworthy that large variations have been documented for primary hepatocyte-based *in vitro* models. On the other hand, CYP-mediated hepatotoxicity could be missed in screening for potential liver toxins due to the low drug metabolism activities in HepG2 cells.

Conclusion

Corning HepatoCells were successfully adapted to a high throughput 3D culture system using Corning spheroid microplates. CYP3A4 activity in spheroids was significantly increased compared to 2D culture. In addition, CYP3A4 activity remained inducible in spheroid culture, albeit at lower fold changes. Tests with classic liver toxins aflatoxin B1 and acetaminophen demonstrated that HepatoCells spheroids are a promising 3D high throughput model system for liver cytotoxicity studies.

Table 1. Inducible CYP3A4 activity in Corning HepatoCells spheroids. CYP3A4 3-day induction was performed with 10 µM Rifampicin (RIF) on days 4, 5, and 6 for either single spheroids made from Corning HepatoCells or 5 spheroids pooled together in the same well. 0.1% DMSO was included as negative control. CYP3A4 activity was measured on day 7 with a Luciferin-IPA probe.

	Single Spheroid		5 Spheroids	
	DMSO	RIF	DMSO	RIF
CYP3A4 Activity	167.1 (n = 5)	535.7 (n = 4)	983.9	3,483.8
Fold Change		3.2		3.5

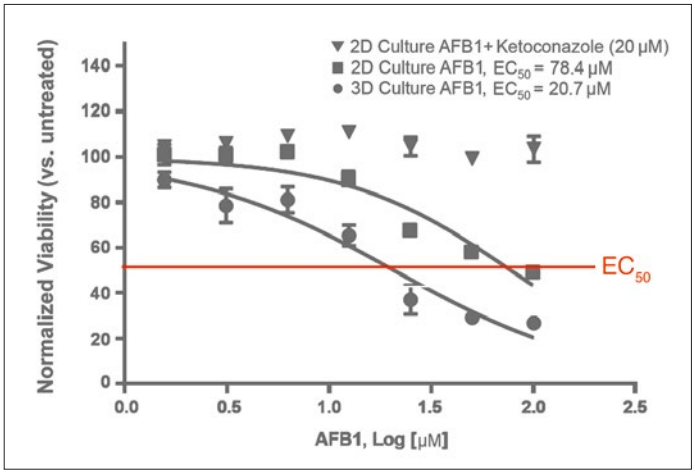

Figure 4. Day 4 spheroid culture vs. 2D culture to aflatoxin B1 (AFB1) treatment. Corning HepatoCells were seeded at 1.5K/well for spheroid culture in 96-well Corning spheroid microplates. For 2D cultures, HepatoCells (100K/well) were used in a 96-well Corning BioCoat Collagen I microplate. Serial dilutions of aflatoxin B1 (AFB1) were prepared and used to treat either 2D culture or spheroid cultures for 24 hours. Two-hour pretreatment with Ketoconazole (20 µM) were included in some 2D samples to inhibit CYP activities. ATP levels or cell viability were measured using a Promega CellTiter-Glo® 3D kit following the manufacturer's instructions. After normalization with untreated samples, non-linear regression and EC_{50} values were calculated using Graphpad software. Error bars = standard deviation, n = 3.

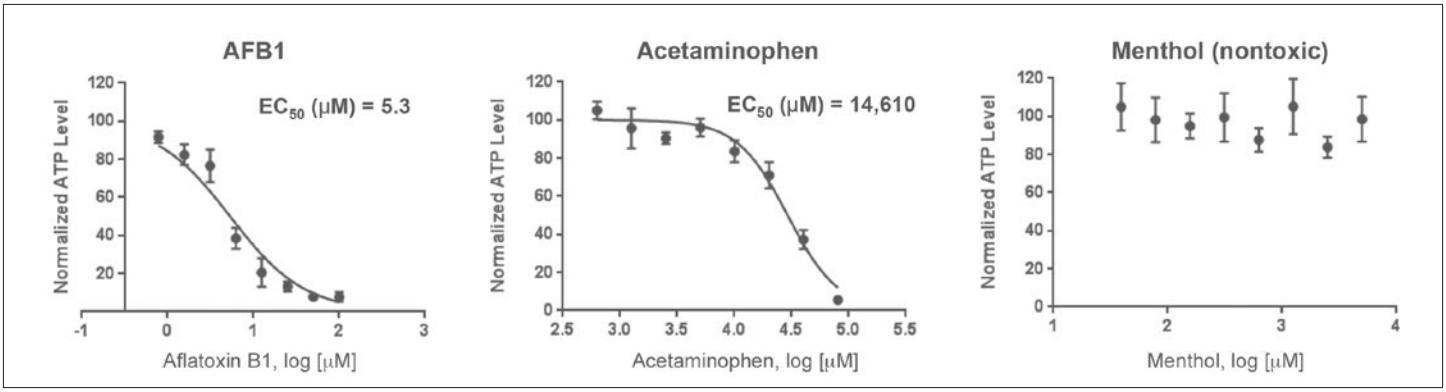

Figure 5. EC_{50} values of day 7 Corning® HepatoCells spheroid exposed to classic liver toxins. Day 7 spheroid cultures (1.5K/spheroid) in 96-well format were treated for 24 hours with classic liver toxins and nontoxic control for 24 hours. ATP levels were measured at the end of incubation for cell viability. Non-linear regression and EC_{50} values were calculated. Error bars = standard deviation, n = 3.

Table 2. Corning HepatoCells EC_{50} Values Comparisons to Literature

Toxin	EC_{50} (µM) Corning HepatoCells 3D culture	Reported EC_{50} (µM) (2D Culture)	References
Aflatoxin B1	5.34	10 (HH) >100 (HepG2) 78.4 (Corning HepatoCells)	Avior Y, et al. (2015)[6] In-house data
Acetaminophen	14,610	28,200 (HH) 29,755 (HepG2)	Jemnitz K, et al. (2008)[7] Wang K, et al. (2002)[8]
Menthol	Nontoxic	Nontoxic	Avior Y, et al. (2015)[6]

HH = human hepatocytes.

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