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Development of 3D Spheroids from Human Primary Hepatocytes as an in vitro Culture Model

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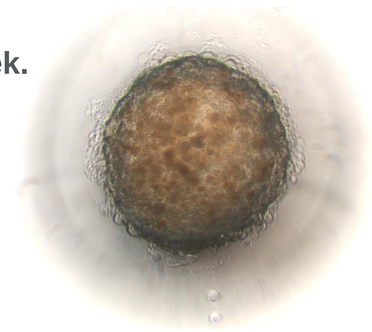


ABSTRACT

Recently our group has developed an easy-to-assemble user-friendly in vitro Primary Human Hepatocyte (PHH) 3D-spheroid model. Our initial work shows that hepatic spheroids can consistently and reproducibly assemble using super low attachment 96-wellplates and standard centrifugation method in 3-5 days following seeding. They can live up to 21 days in culture and remain phenotypically stable, retaining the hepatocyte-specific functions. We have shown that seeding 1,500 PHH/well resulted in spheroid formation with homogenous morphology and consistent size (~200 µm diameter). To assess whether hepatocyte-specific functions were maintained in the PHH spheroids during prolonged culture, albumin secretion, CYP3A4 activity and levels of ATP synthesized were analyzed. These parameters were found to remain stable during prolonged culture period. Also, gene expression profiles at 5, 7, 14 and 21 days showed a relatively higher expression of hepatocyte specific genes compared to that of the 2D-culture.

These results indicate that the PHH 3D-spheroid system developed by us constitutes a promising in vitro tool to evaluate hepatic function. As part of our future work, we are investigating the possibility of introducing nonparenchymal liver cells like Kupffer and Stellate cells to the spheroid system to assess feasibility of creating various liver disease models.

INTRODUCTION

Primary Human Hepatocytes (PHH) are the gold standard in vitro models for studying hepatic biology, liver function, and drug induced hepatotoxicity. The conventional way of culturing PHH in 2-dimension (2D) has major pitfalls. The PHH rapidly de-differentiate and lose the hepatic specific functions in a week. Therefore, there is a need for more robust in vitro models that reflects in vivo liver biology more accurately and maintains the liver functions for a longer time. 3-dimensional (3D) hepatic in vitro models are gaining a lot of attention for their ability to recapitulate the hepatic function and greater longevity.

Here we describe the development of a primary hepatocyte 3D-culture system that can be used as an easy to use hepatocyte culture for high throughput screening and longer viability.

MATERIALS AND METHODS

Media and Reagents:
Hepatocyte Plating medium – 500mL Williams Media E, WEM (Gibco™ A1217601), Plating Supplements (Gibco™ CM3000)
Hepatocyte Maintenance Medium - WEM (Gibco™ A1217601), Maintenance Supplements (Gibco™ CM4000)
Hepatocyte Thaw Medium - HTM (Gibco™ CM7500)
3D Plates - Nunclon™ Sphera™ super low attachment U-bottom 96-well Microplates (174925)
2D Collagen Plates (controls) – Gibco™ Collagen Coated 24-well plates (A1142802)

Hepatic Spheroid Protocol

1. Cryopreserved Primary Hepatocyte (Gibco™) vial was thawed quickly in a 37°C water bath. Upon thawing the cells were promptly transferred into a 50 mL centrifuge tube containing HTM.
2. The tube was centrifuged at 100X g for 10 minutes. After centrifugation the supernatant was discarded.
3. The cell pellet was gently re-suspended in 3 mL hepatocyte plating media. Cell counting was performed with Trypan blue using hemocytometer.
4. Appropriate volume of cell suspension was prepared to contain 7,500 cells/mL. Using a multichannel pipette 200uL of the cell suspension was added into individual well of Nunclon™ Sphera™ low attachment U-bottom 96-well Microplates.
5. The Sphera™ microplates were centrifuged at 200g for 2 minutes to allow cells to group at the bottom of the wells.
6. The Sphera™ Microplates were transferred to an incubator (37° C, 5% CO_2, humidified) and allow to sit undisturbed in Plating Media for at least 3-5 days before changing media.
7. In 3-5 days the PHH formed spheroid with compact spherical mass of cells (Figure 1 and 2). On day 5, upon confirmation of spheroid formation, 50% the plating media was exchanged with hepatocyte maintenance media.
8. Spheroids were maintained in the maintenance media up to 21 days with media change every 48-72 hours.

RESULTS

Figure 1. Work Flow of assembly and characterization of primary hepatocyte into 3D-spheroid

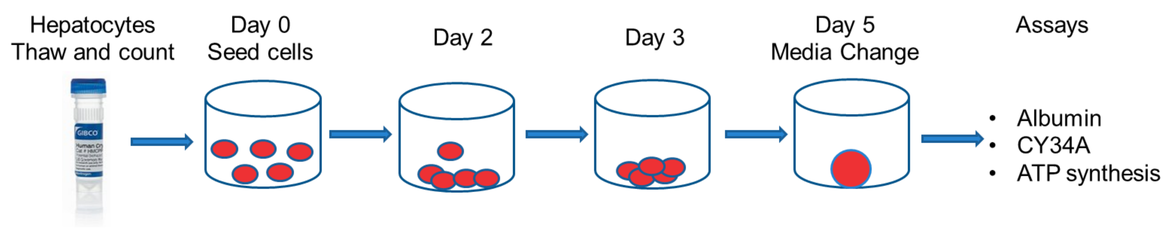
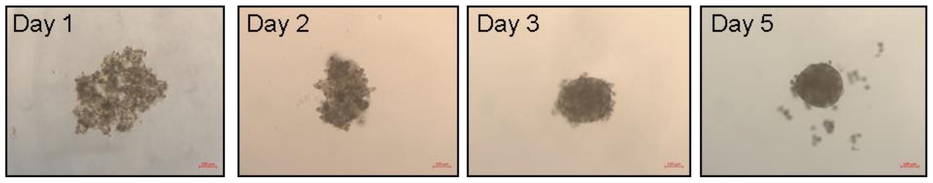
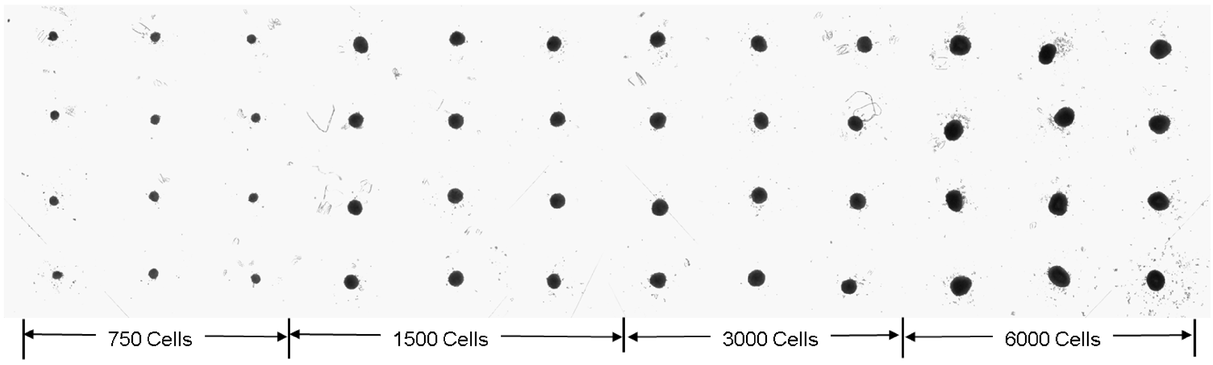


Figure 2. Self assembly of primary hepatocytes into 3D-spheroid



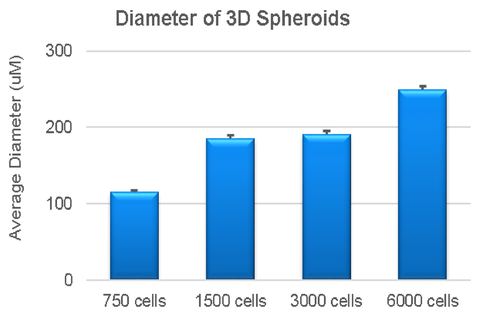
Primary Hepatocytes are seeded in Nunclon™ Sphera™ super low attachment U-bottom 96-well Microplates, where they self assemble into 3D-spheroids by day 5. By day 7 they are ready for further biochemical assays and characterization.

Figure 3. Optimization of Hepatic Spheroids: Variable Cell Numbers



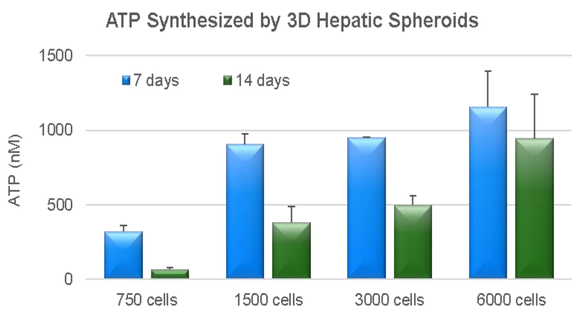
Primary Hepatocytes were seeded in Nunclon™ Sphera™ super low attachment U-bottom 96-well Microplates in variable cell numbers. .

Figure 4. Diameter of Hepatic Spheroid shows remarkable consistency.



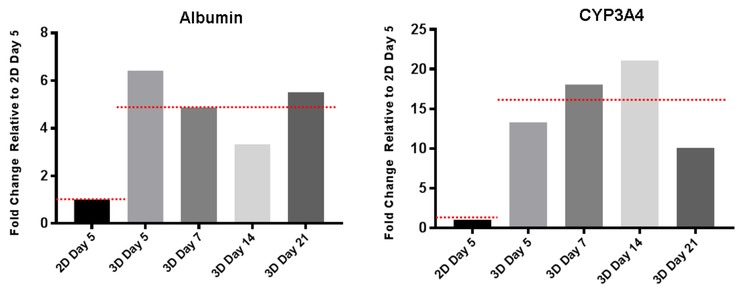
Diameters of Primary Hepatocyte Spheroids were measured at day of culture using Zeiss microscope . Results are mean +/- SD, n = 6.

Figure 5. ATP synthesis by 3D spheroids is proportionate to the number of cells.



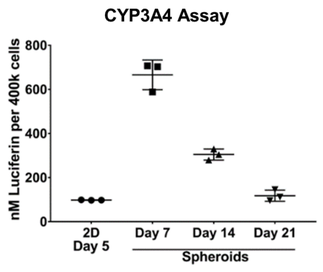
ATP synthesis by individual spheroids was measured using CellTiter-Glo® 3D cell viability assay (Promega) at day 7 and day 14 of culture. Results are mean +/- SD, n = 3.

Figure 6. Comparison of Gene Expressions between 2D and 3D cultures.



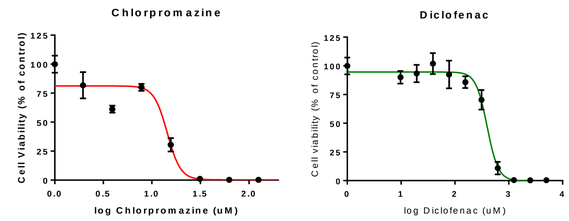
Albumin and Cyp3A4 mRNA levels were measured by qRT-PCR to compare the levels of gene expression between day 5 of 2D culture and various days of 3d culture. Gene expression levels of Albumin and CYP3A4 were consistently higher in the 3D-spheroids than that of the 2D-culture

Figure 7. Comparison of CYP3A4 activity between 2D and 3D cultures.



CYP3A4 activity was measured using P450 ProGlo assay (Promega). Cyp3A4 activity of day 7 was found to be much higher than that of 2D culture, indicating higher metabolic function of 3D culture.. Results are mean +/- SD, n = 3.

Figure 8. Drug induced Cytotoxicity assay using 3D primary human hepatocytes.



3D spheroids from Primary Hepatocytes containing 1500 cells/well were treated with variable levels of chlorpromazine and diclofenac at day 14 of culture. Cell viability was assayed 24 hours post treatment using CellTiter-Glo® 3D cell viability assay (Promega). Results are mean +/- SE, n = 4. Non-linear regression was performed for variable slope of log(inhibitor) vs response using GraphPad Prism7.

Table 1. Drug induced cytotoxicity assay.

Drugs	IC_{50} - 2D culture	IC_{50} - 3D culture
Chlorpromazine	34.22 µM[1]	14.39 µM
Diclofenac	331 µM[2]	396.2 µM

Drug induced cytotoxicity assay shows comparable IC_{50} levels between 2D and 3D cultures using primary human hepatocytes at 24 hours with Chlorpromazine and Diclofenac.

CONCLUSIONS

- Gibco™ Primary Human Hepatocytes can easily be assembled into a 3D culture in 5 days using Nunclon™ Sphera™ super low attachment U-bottom 96-well Microplates, Gibco™ plating media and plating supplements.
- The PHH are functionally viable for at least 21 days, which is a significant progress in primary hepatocyte culture considering the conventional 2D-culture methods.
- The 3D hepatocyte culture requires a significantly lower number of cells than that of the 2D counterpart, which opens new possibilities for high throughput assays using PHH.
- 3D hepatocyte culture has comparable cytotoxic effects as that of the 2D cultures using compounds causing drug induced liver injuries (DILI), such as chlorpromazine and diclofenac.

FUTURE DIRECTIONS

- Coculture of PHH with non-parenchymal cells to establish in vitro liver model.
- 3D culture of PHH isolated from diseased livers (such as NAFLD, NASH and hepatic fibrosis).

REFERENCES

1. de Joannon AC. Pharmacologyonline, 3, 77-87-2002.
2. Bort R. et al. Journal of Pharmacology. and Experimental Therapeutics, 288(1), 65-72, 1999.

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