Bioscience Solutions

Primary Human Hepatocyte Spheroid Generation and Performance in Different Culture Systems

S. Buesch[1], M. Bunger[2], J. Schroeder[1], M. Stosik[1]

[1]Lonza Cologne GmbH, Cologne, North Rhine-Westphalia, Germany; [2]Lonza RTP Inc., Research Triangle Park, North Carolina, United States of America

1. Introduction

In the field of toxicology, there is a strong need for *in vitro* human liver model systems that support the long-term culture of primary human hepatocytes (PHH) and closely mimic *in vivo* conditions of liver tissue. Unfortunately, standard 2 dimensional (2D) hepatocyte cultures rapidly lose typical hepatocyte functionality, e.g. cytochrome P450 (CYP) activity (1; 2). To bridge this gap, complex culture systems are studied and optimized, including fluidic culture, 3 dimensional (3D) hydrogels and so called liver spheroids or micro-tissues (3). Compared with standard 2D culture, spheroids generated from PHH allow for more in-vivo-like cell organization, including extensive contact of adjacent cells. Agitation on a shaker can imitate the movement of fluids within the body at least to some extent. Thus, spheroid culture may support hepatocyte functionality for extended periods of time. In this study, we analyzed and optimized the formation, culture and performance of PHH in different spheroid culture systems and under various culture conditions.

2. Material and Methods

Cells and Media: Cryopreserved Primary Human Hepatocytes (PHH; Plateable, Induction Qualified; Lonza) were cultured according to the supplier's instructions (4) in sandwich culture. Briefly, 50.000 cells per well were plated in collagen coated 96-well plates. 4-6 hours post-seeding, Matrigel™ (Corning) overlay was added in Hepatocyte Culture Medium (HCM™; Lonza). Media was renewed daily.

Spheroid formation: 1.500 or 3.000 PHH were seeded in different culture plates including ultra-low attachment (ULA) V-bottom and U-bottom plates as well as hanging drop culture, micro-well- and other dedicated spheroid culture plates. HCM™ (Lonza) supplemented with 25 mM HEPES and 20% FBS (PAN-Biotech) was used as spheroid formation medium. Cells were left undisturbed for 5 days at 37°C 5% CO_2 with or without gentle agitation on an orbital shaker (KL-2, Edmund Bühler) set to 90 rpm. On day 6 after seeding and then every 2 to 3 days, 50% of the medium was renewed with serum-free HCM™. Remaining FBS content was thus below 1% at 15 days of culture and virtually serum-free in week 3 and 4. On day 6, spheroids generated in the hanging drop system were transferred into standard ULA U-bottom plates to allow for identical culture volumes and thus comparable assay readouts.

Analysis: Spheroid formation was observed and documented by bright field microscopy (Zeiss AxioObserver Z.1 microscope equipped with a Zeiss N-Achroplan 10x Objective and an AxioCam MRm camera). Viability / metabolic capacity of the culture was assessed via CellTiter-Blue® Cell Viability Assay (Promega) according to manufacturer's instructions. Incubation period with CellTiter-Blue® Reagent was 24h for spheroid culture and 1,5h for adherent culture. Albumin content in the supernatant was quantified with the Human Albumin ELISA Kit (Bethyl) according to manufacturer's instructions. CYP3A4 activity was assessed via the P450-Glo™ CYP3A4 Assay (Promega) using Luciferin-IPA as a substrate according to manufacturer's instructions. For measuring the inducibility of CYP3A4, spheroids were incubated with 10 µM rifampicin or dimethyl sulfoxide (DMSO) control for 72 hours before measuring the CYP3A4 activity. Cholyl-lysyl-fluorescein (CLF) staining (Corning) was used for visualization of bile canaliculi formation. Cells were incubated for 2 h in medium containing 4 µM CLF followed by three washing steps consisting of 50% medium replacement and 1h incubation each prior to examination by fluorescence microscopy. To reflect the specific culture conditions, spheroids cultured under agitation on an orbital shaker were stained under agitation. Spheroids cultured without agitation were stained and washed without agitation.

Statistics: Outlier were identified by Dixon's Q test (95% confidence) and rejected. Error bars represent standard deviation.

3. Results

In order to set a bench mark for our liver spheroid cultures, we first analyzed the functionality of PHH in standard sandwich culture in 96-well plates. Exemplary of one functional read-out, it was observed that basal CYP3A4 activity clearly decreased within 7 days (Figure 1). This is consistent with previous observations of PHH sandwich cultures in 96-well plates and also 24-well plates (1). Interestingly, long-term PHH viability and functionality is better supported in 24-well culture (data not shown). The reason for this is not clear. One can speculate that oxygen and nutrient supply is better in 24-well plates.

Since viability and functionality of PHH is still limited in 24-well plates and assay throughput is limited, we evaluated different PHH spheroid culture options in a 96-well format.

In presence of serum, formation of compact, transferable spheroids occurred in the majority of tested culture systems within five days without addition of any supporting cell types (Figure 2). Both 1.500 and 3.000 PHH per well resulted in stable, compact spheroids independent of agitation. In absence of serum, no spheroid formation could be observed. After an initial formation period of 6 days, serum was not required for further spheroid culture. Due to the following medium replacements, remaining FBS content was thus below 1% at day 15 of culture and the culture was basically serum-free during week 3 and 4. Not only spheroid formation itself, but also albumin production and viability (data not shown) of spheroid culture at days 7-9 was comparable between U-bottom and V-bottom ULA plates and hanging drop culture. The tested micro-well and other specialized plates were not suitable for our purpose (data not shown). V-bottom ULA plates were not ideal for imaging (data not shown). In conclusion, U-bottom plates and hanging drop systems were chosen for all subsequent experiments.

To get an initial understanding of PHH spheroid performance, basal and induced CYP3A4 activity was determined. Both basal and induced CYP3A4 activity were slightly higher but still within similar range in hanging drop culture compared to U-bottom ULA plate culture (Figure 3). The resulting CYP induction was 5-fold in both culture systems. To examine spheroid stability over time, spheroids were cultured for up to 4 weeks with and without agitation in different culture systems. The generated spheroids remained compact and stable over the complete examination period (Figure 4), independent of culture vessel and agitation.

Bile canaliculi formation was observed in all tested culture systems. Long term persistence of bile canaliculi was donor dependent. For example, spheroids generated with PHH from donor B maintained a 3D branched bile canaliculi network for 4 weeks in all tested culture systems when cultured without agitation (Figure 5). Bile canaliculi visibility was negatively affected by orbital agitation. This was observed repeatedly for different hepatocyte donors (data not shown) and could represent altered bile canaliculi formation or stability due to fluidic shear stress or more intensive movement of the spheroids. It could also be a result of forced "washing off" of the CLF staining on a shaker.

Metabolic activity and albumin secretion was stable for at least 2 weeks of spheroid culture. After that a donor specific decline was observed (Figure 6). Spheroids generated with PHH from donor B showed reduced, but still clearly detectable metabolic activity after 4 weeks of culture as well as sustained albumin secretion. Accordingly, basal CYP3A4 activity was clearly detectable during the full examination period of 4 weeks in spheroids generated with PHH from donor B (Figure 7).To confirm the long term metabolic activity of CYP3A4 and to also address CYP2B6 and CYP1A2 function, testosterone, phenacetin and bupropione metabolism will be tested by mass spectroscopy (data pending).

The gradual decline in PHH spheroid function and metabolic activity is in accordance with currently published observations (5). In recent literature, only minor variability in sensitivity to different compounds was found between three donors – however, PHH from these donors were pre-characterized for long-term culture before (2). Our results confirm the importance of pre-selection of cryopreserved PHH lots suitable for long-term culture.

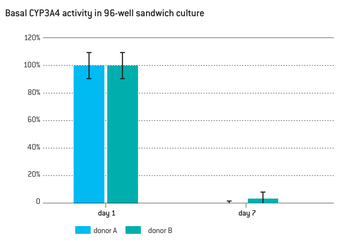

Figure 1: Basal CYP3A4 activity decreases rapidly in 96-well sandwich culture. PHH from two different donors were cultured in collagen coated 96-well plates with Matrigel™ overlay. CYP3A4 activity was assessed with the P450-Glo™ CYP3A4 Assay (Promega). Day 1 values were set to 100% for both donors, n = 4. Error bars indicate standard deviation.

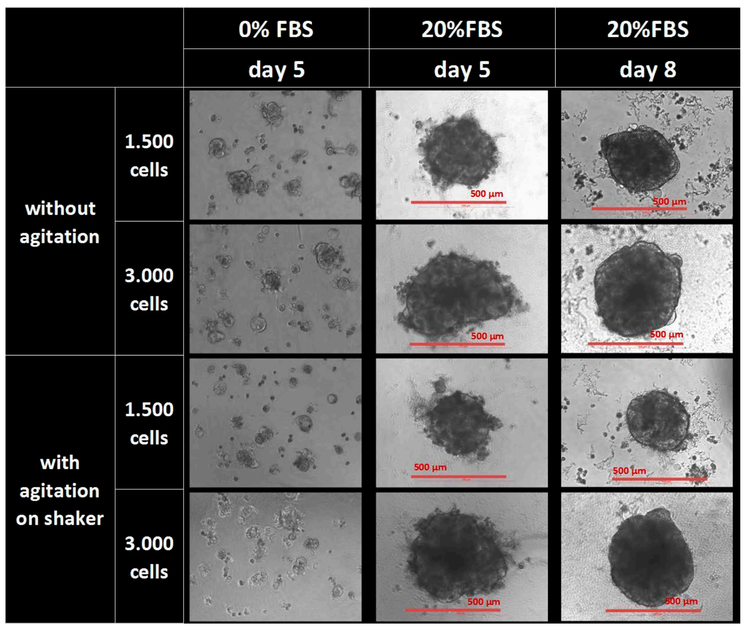

Figure 2: Succesfull spheroid formation in presence of serum. 1.500 or 3.000 PHH were seeded in ULA U-bottom plates with or without 20% FBS. Cells were left undisturbed for 5 days with or without gentle agitation. Starting on day 6, media changes were performed as described in Materials and Methods.

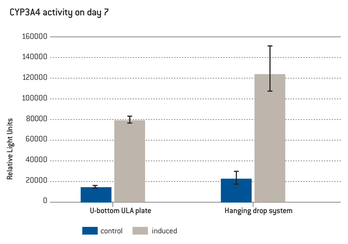

Figure 3: 5-fold CYP3A4 induction in U-bottom ULA plates and hanging drop system. 3.000 PHH were seeded in ULA U-bottom plates or into a hanging drop system in serum-containing spheroid formation medium. Cells were left undisturbed without agitation for 5 days. Starting on day 6, media changes were performed as described in Materials and Methods. CYP3A4 activity was assessed via the P450-Glo™ CYP3A4 Assay (Promega). For measuring the inducibility of CYP3A4, spheroids were incubated with 10 µM rifampicin for 72 hours. Results of one typical experiment are shown. Comparable basic and induced CYP3A4 activity as well as fold-induction rate (data not shown) was observed in both culture systems. (n = 3; error bars indicate standard deviation)

Figure 4: Spheroids remain stable over 4 weeks of culture. 3.000 PHH were seeded into the indicated spheroid culture formats in serum-containing spheroid formation medium. Cells were left undisturbed for 5 days with or without gentle orbital agitation. Starting on day 6, media changes were performed as described in Materials and Methods. Typical results are shown.

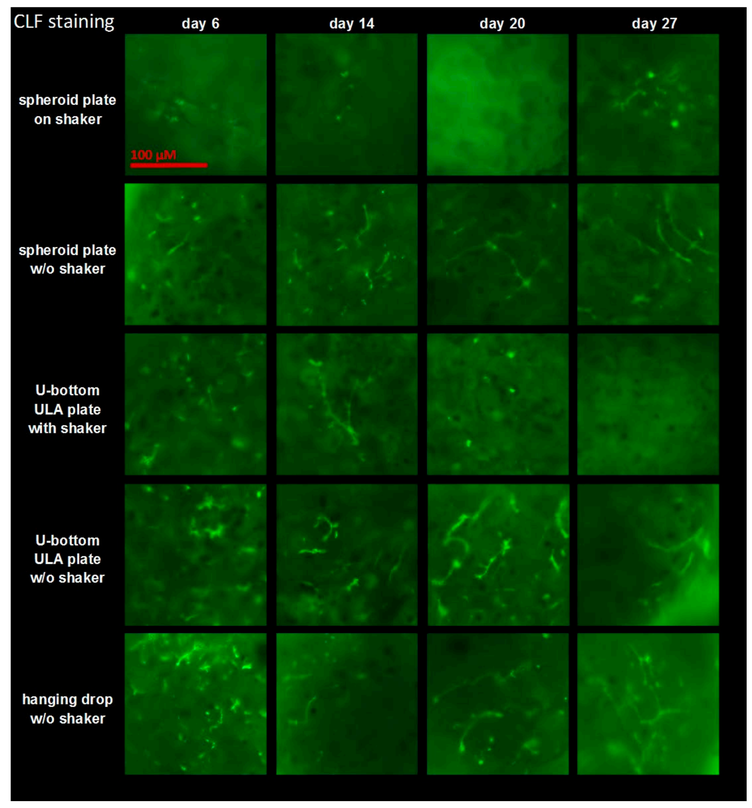

Figure 5: Bile canaliculi formation within spheroids over time. 3.000 PHH were seeded into the indicated spheroid culture formats in serum-containing spheroid formation medium. Cells were left undisturbed for 5 days with or without gentle orbital agitation. Starting on day 6, media changes were performed as described in Materials and Methods. CLF staining was used for visualization of bile canaliculi formation.

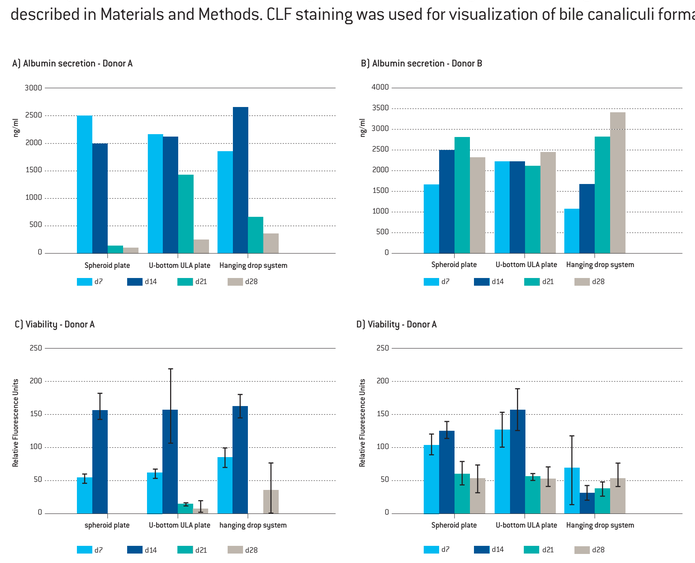

Figure 6: Long term functionality in spheroid culture is donor-dependent. 3.000 PHH were seeded into the indicated spheroid culture formats in serum-containing spheroid formation medium. Cells were left undisturbed and without orbital agitation for 5 days. Starting on day 6, media changes were performed as described in Materials and Methods. Albumin content in the supernatants was quantified with the Human Albumin ELISA Kit (Bethyl) (A, B). Results of two experiments with 2 independent donors are shown. Bars represent pooled supernatants of ≥ 3 samples. Viability / metabolic capacity of the spheroid cultures was assessed via CellTiter-Blue® Cell Viability Assay (Promega) (C, D). Results of two experiments with two independent donors are shown (n ≥ 3; error bars indicate standard deviation)

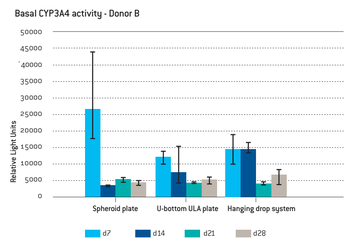

Figure 7: Long term basal CYP3A4 activity in PHH spheroids. 3.000 PHH were seeded into the indicated spheroid culture formats in serum-containing spheroid formation medium. Cells were left undisturbed and without orbital agitation for 5 days. Starting on day 6, media changes were performed as described in Materials and Methods. CYP3A4 activity was assessed via the P450-Glo™ CYP3A4 Assay (Promega). Results of one experiment are shown, n ≥ 3. Error bars indicate standard deviation.

4. Conclusion

We developed simple protocols for the successful *in vitro* generation of primary human hepatocyte spheroids in a variety of culture systems. We demonstrated that standard analysis methods like cell viability or cytochrome P450 enzyme activity assays can be applied to spheroid culture. While spheroid formation in serum containing medium was successful in a variety of systems tested, the long term performance of spheroids in serum-free medium was donor-dependent, emphasizing the need for careful donor selection and thorough examination of available cryopreserved hepatocyte lots in advance. The resulting *in vitro* spheroid cultures offer more *in vivo* like conditions for long-term exposure and repeated dosing toxicology studies.

Literature

1. Buesch S, Schroeder J, Bunger M, D'Souza T, Stosik M. A Novel *In Vitro* Liver Cell Culture Flow System Allowing Long-Term Metabolism and Hepatotoxicity Studies. Applied *in Vitro* Toxicology. 2018. Published Online: 14 Jun 2018.
2. Bell CC, Dankers ACA, Lauschke VM *et al*. Comparison of Hepatic 2D Sandwich Cultures and 3D. Toxicological Sciences. 2018, Vol. 162, (2).
3. Underhill GH, Khetani SR. Bioengineered Liver Models for Drug Testing and Cell Differentiation Studies. Cell Mol Gastroenterol Hepatol. 2017, Vol. 6;5, (3).
4. Lonza. Suspension and Plateable Cryopreserved Hepatocytes - Technical Information & Instructions. Available: URL bio.lonza.com/go/literature/5334. 2017. Document # BR-CryoHep 05/17.
5. Vorrink SU, Ullah S, Schmidt S, Nandania J, Velagapudi V, Beck O, Ingelman-Sundberg M, Lauschke VM. Endogenous and xenobiotic metabolic stability of primary human hepatocytes in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. FASEB J. 2017, Vol. 31, (6).