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INNOVATIONS

Three-dimensional perfused cell culture enhances the infectability of primary human hepatocytes with Hepatitis B virus

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Introduction

Research into the biology and treatment of hepatitis B virus (HBV) requires an *in vitro* infection model that fully supports all the steps of the HBV life cycle and accurately recapitulates virus–host interactions. However, the molecular mechanisms underlying HBV infection remain poorly understood. The sodium-taurocholate cotransporting polypeptide (NTCP) has recently been described as the HBV receptor expressed on hepatocyte baso-lateral membranes (1), but current models relying on NTCP overexpression in hepatoma cell lines and non-polarised culture of hepatocytes, do not fully capture the complexity of HBV infection (2). As the principle host cell infected by HBV *in vivo*, primary human hepatocytes (PHH) represent the gold-standard for studying HBV interactions with the host. Prior studies have shown that PHH support HBV infection, although infection is usually not robust and PHH rapidly lose their hepatic phenotype shortly after isolation from the *in vivo* microenvironment (3, 4).

Here we utilised the perfused 3D cell culture system, LiverChip® (Fig. 1) and compared the infectivity of PHH cultured in 2D plates to those cultured in 3D. The same cell lots were infected with patient-derived HBV positive serum and maintained for up to 21 days.

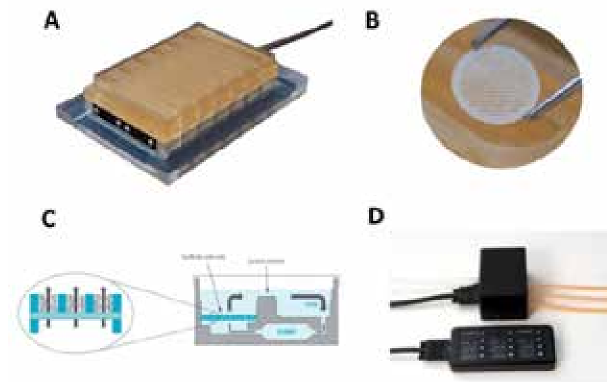


Figure 1: LiverChip® Hardware

A. LiverChip® is a 12 well perfused cell culture system.
B. Hepatocytes form three dimensional tissue structures in an array of channels through a collagen I-coated scaffold.
C. Media flows through the channels due to the action of a pneumatically operated pumping mechanism.
D. The speed and direction of flow can be adjusted using an electronic controller.

Methods

Cryopreserved human hepatocytes were obtained from Life Technologies (USA) or QPS Hepatic Biosciences (USA). 0.6 x 10^6 hepatocytes were seeded into each LiverChip® well (3D) in Williams' E medium containing supplements. Equivalent cells were cultured in parallel in collagen-coated 24-well plates (2D), with 0.35 x 10^6 hepatocytes seeded into each well. The same culture medium was used to maintain cells in 2D, as in LiverChip®. Cultures were infected between Day 0 and 3 in culture with commercially available HBV positive serum or plasma (Quest Biomedical Ltd and SeraLab). Cultures were infected in the presence or absence of 4% polyethylene glycol 8000 (PEG) at various multiplicity of infection (MOI) expressed as genome equivalents per cell. Complete media changes were performed on all cultures every 2 – 3 days.

Assays

Albumin was measured by ELISA (AssayPro). P450-Glo (Promega) was used to monitor CYP3A according to the manufacturers' protocol. Total RNA was extracted using the RNeasy mini kit (Qiagen). RNA was converted to cDNA using the high capacity cDNA synthesis kit (Life Technologies). Gene expression profiles were quantified in equivalent cDNA samples using TaqMan assays on an ABI QuantStudio 6 real time PCR system. Cells were stained with 50 µg/ml Phalloidin-TRITC, counterstained with Hoechst and imaged on an Upright Ziess LSM 510 confocal microscope.

HBV surface antigen (HBsAg) was measured using a CLIA according to the manufacturer's instructions (AutoBio Diagnostics, China). For HBV DNA, total DNA was extracted from cell culture medium using QIAamp MinElute Virus Spin Kit along with a standard curve generated with Acrometrix HBV panel. qPCR was run with TaqMan reagent and specific probes and primers.

Results – Hepatocyte culture

PHH were cultured in both 2D and in 3D (in the LiverChip® platform) for 21 days and their functionality was compared (Fig. 2). Cells cultured in 3D had higher production of albumin and urea, had greater CYP3A4 activity and had increased expression of key hepatic genes (Fig. 2). PHH cultured in 3D formed microtissues, forming polarised cells and bile canaliculi (Fig. 3). For effective HBV infection PHH must be polarised and express the NTCP receptor and PHH cultured in 3D were found to express higher levels of NTCP than those cultured in 2D (Fig. 4).

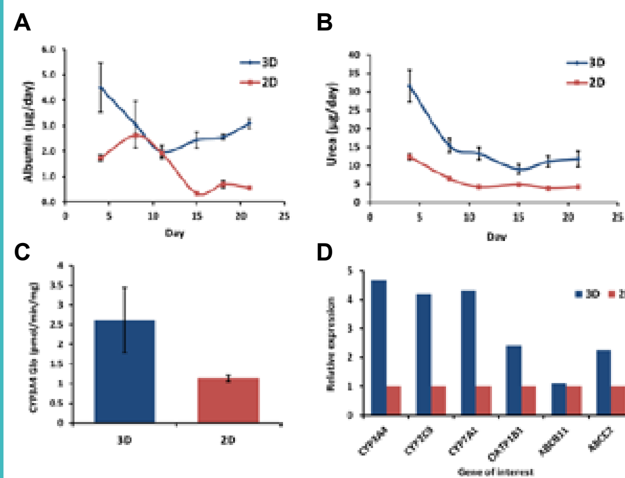


Figure 2: Hepatocytes cultured in 3D outperform those cultured in 2D

PHH were cultured in 3D or on collagen-coated 24-well plates (2D) for 21 days and compared for (A) albumin production; (B) urea production; (C) CYP3A4 activity; (D) total RNA was extracted from cultured cells, converted to cDNA and gene expression was determined qPCR. Gene expression shown as a fold change of 3D vs 2D.

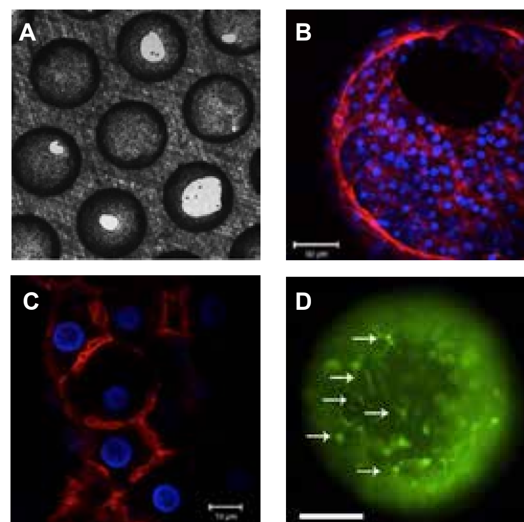


Figure 3: Hepatocytes cultured in 3D form liver microarchitecture

PHH were cultured in 3D and imaged by microscopy; (A) phase contrast; (B, C) stained for actin (red) and nuclei (blue) to show polarised cells; (D) CFDA staining of bile canaliculi.

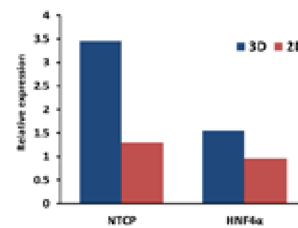


Figure 4: The expression of genes required for HBV infection are increased in 3D cultured hepatocytes

Total RNA was extracted from PHH cultured in 3D and in 2D. Gene expression analysis was performed using TaqMan assays and expressed as fold increase over 2D.

Results – HBV infection

PHH were infected with HBV in both 2D and in 3D and compared for the level of infection by measuring HBV DNA and HBsAg production in the culture medium (Fig. 5). PHH cultured in 3D demonstrated a more productive infection, when compared to PHH cultured in 2D (Fig. 5). The addition of PEG promoted an increase in progeny virus production from PHH in both conditions (Fig. 5). Using an alternative PHH lot and serum donor varying MOI's of HBV were observed to infect PHH when cultured in 3D, with an MOI of < 2 able to launch an infection (Fig. 6).

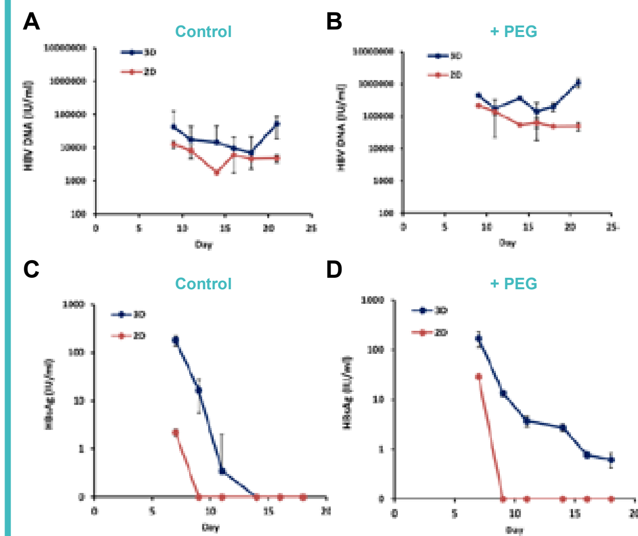
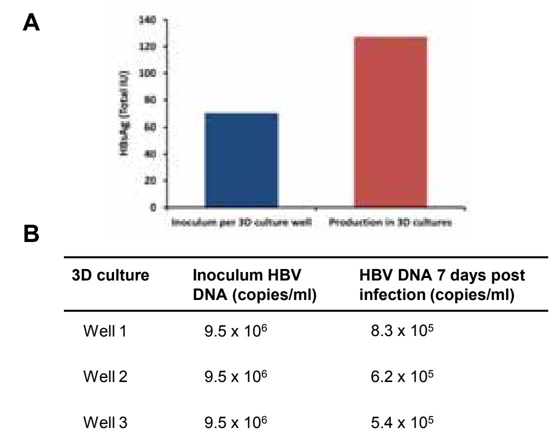


Figure 5: Infectivity of hepatocytes by HBV is more efficient in 3D

PHH were cultured in 2D or 3D for 21 days and were infected with HBV (MOI: 303 genome equivalents per cell) in the presence (B, D) or absence (A, C) of PEG. (A, B) HBV DNA production in culture medium was measured by qPCR; (C, D) HBsAg production was measure in culture medium by ELISA.



3D culture	Inoculum HBV DNA (copies/ml)	HBV DNA 7 days post infection (copies/ml)
Well 1	9.5 x 10^6	8.3 x 10^5
Well 2	9.5 x 10^6	6.2 x 10^5
Well 3	9.5 x 10^6	5.4 x 10^5

Figure 6: Hepatocytes cultured in 3D can be infected by low quantities of HBV

PHH were cultured in 3D and infected with HBV serum (MOI - 4). (A) Cumulative production of HBsAg over 8 days and (B) HBV DNA at day 7, were measured in cell culture medium at varying time points to determine HBV infection.

Conclusions

- Hepatocytes cultured in the LiverChip® platform (3D) maintained an enhanced hepatic phenotype, compared to those cultured in 2D.
- The expression of NTCP, a key receptor required for HBV infection, is expressed at higher levels in 3D cultured hepatocytes.
- PHH infected with patient-derived HBV positive serum produced a more productive infection when cultured in 3D rather than 2D.
- Hepatocytes cultured in 3D can be infected with HBV using low MOI's. The amount of virus particles produced in these cultures is greater than the number of virus particles used to launch infection, confirming *de novo* progeny virus production.

References

1. Yan, H, et al. (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife*, 1: e00049.
2. Iwamoto, M, et al. (2014). Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP. *Biochem. Biophys. Res. Commun.* 443: 808–813
3. Gripon P, et al. (1993). Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: Effect of polyethylene glycol on adsorption and penetration. Virology 192(2):534–540.
4. Guillouzo A (1998). Liver cell models in in vitro toxicology. Environ Health Perspect 106 (Suppl 2):511–532.

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