**Methylcellulose as a scaffold in the culture of liver-organoids for the potential of treating acute live failure**

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**Key words:** Organoids, matrigel, methylcellulose, transforming growth factor-β, epidermal growth factor, acute liver failure, hepatocytes, mesenchymal stem cells, albumin, urea, drug screening,

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**Abstract**

Much progress has been made in understanding the development of human organs through advanced cellular and molecular techniques. Acute liver failure (ALF) in children is a life-threatening condition that relies on liver or hepatocyte transplantation. The translation of novel regenerative medicine strategies for the treatment of ALF is however somewhat limited. Here, we show that *in vitro* liver-organoids derived from human umbilical cord derived mesenchymal stem cells and human cadaveric donor derived hepatocytes, cultured in a clinically appropriate manner, exhibit liver function. We obtained organoids that varied in size and morphology which produced albumin, and detoxified ammonium chloride into urea. Immunohistochemistry of these organoids revealed hepatocyte specific, non-parenchymal markers and histological organisation. Our *in vitro* findings indicate that these organoids may be a useful bridge in ALF while awaiting liver recovery or transplant. The organoid culture system we have established is also well suited to understanding organ development and drug screening.

**Introduction**

There have been significant conceptual and practical advances with regards to regenerative medicine for the treatment of disease. This has ranged from the classic use of embryonic stem cells, their culture and ability to differentiate into practically any cells of the body, to reprogramming of fully differentiated cells into an embryonic state followed by differentiation to a chosen cell type, which is now mainstream research ((1)). These now significantly impact not only on treating disease but the potential for modelling disease in culture dishes that was previously not possible. The development of liver has been of prominent interest, as liver disease is the fifth biggest cause of deaths in the UK.

Acute liver failure (ALF) in children is a life-threatening condition. The prognosis of ALF is generally poor and medical intervention relies on a liver(2, 3) or hepatocyte(4, 5) transplantation if the native liver is unable to recover. The major limitation of the technique today is the availability of donor organs. Many patients who might benefit from a transplant die before a suitable organ can be found. Whilst much progress has been made in the study and understanding of the biology of liver(6), there have been limited clinical advancements in the treatment of ALF utilising novel regenerative techniques, such as the use of embryonic stem cell techniques to induced pluripotent stem cell derived hepatocytes. Human bio-compatible scaffolds to grow liver type/functional cells is ever increasing. The main challenges in translating advances in basic science of cell therapy into the clinic has been determining the best route of delivery, the rapid elimination of transplanted cells by the recipient, poor engraftment and proliferation of transplanted cells within the liver(7).

Stem cell-based technologies have been extensively researched in vitro and in vivo. Mesenchymal Stem/Stromal cells (MSC) are a population of immature cells that can give rise to more mature cell types such as adipogenic, osteogenic and chondrogenic cells. These cells can be easily cultured from adult bone marrow and full term umbilical cord (blood or the Wharton’s jelly). MSC have also been isolated from muscle connective tissue, adipose tissue and in some circumstances peripheral blood. Culture protocols usually include direct plastic adherence or enrichment by CD marker selection. The well-established characteristics of MSC, has been basis of the extensive use in clinical applications. MSC have been used in the treatment of liver disease with varying results(8).

Tissue engineering strategies for regenerative medicine are yet another avenue of great advancement. Novel scaffolds-engineered(9), naturally occurring(10) or synthetic material(11) are under extensive scrutiny. There is an unmet need to use novel organoid-based strategies that is suitable for transplantation in ALF until the patient receives a liver transplant or the native liver recovers. As such, we proposed to exploit novel organoid-strategies as a means for clinical application. Here we hypothesized that using methyl cellulose as a scaffold to obtain liver-organoids maybe a useful approach towards clinical use.

**Results**

**Establishment of organoids in matrigel**

Our initial strategy to develop liver organoids, stems from the seminal research of Takebe and colleagues(12). Whilst Takebe et al used Human Induced Pluripotent Stem Cell (hiPSC) derived hepatocytes, MSC and Human Umbilical Vein Endothelial Cells (HUVECS) cultured in matrigel with a host of factors to obtain organoids, we used adult hepatocytes from cadaveric donors and MSC cultured in serum free matrigel. Organoids were analysed after 14 days in culture (Figure S1A). Confocal imaging of these organoids revealed strong expression of hepatocyte markers (Figure S1B). Our rationale for the ratio of cells to be cultured was based on our previous experience of a 2-D co-culture system of Hepatocytes and MSC, which resulted in improved hepatocyte function(13). We tested four different ratios of hepatocytes cultured with MSC. Albumin production and ability to metabolise ammonium chloride to urea were used as functional readouts of the resulting organoids after two weeks in culture. Hepatocytes and MSC cultured in a two to one ratio respectively, gave rise to the highest albumin production (Figure S2A). Interestingly, the urea production was the highest when using a one to one ratio (Figure S2B). When using a two to one ratio of cultured cells, these organoids produced albumin over a period of two weeks, peaking from day 10 onwards (Figure S3A). These organoids were also able to metabolise ammonium chloride to produce urea from day 4 peaking at day 14 as well (Figure S3B).

**Establishing organoids using methylcellulose as a scaffold**

Matrigel whilst proving to be an excellent scaffold for studying and understanding basic biology of organ development, it’s clinical application is somewhat restricted as it is a gelatinous substance derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. Therefore, we hypothesised that substituting matrigel with methylcellulose as a scaffold for the growth of organoids might be a useful approach to using organoids for future clinical application (Figure S4). Having studied the composition of growth factors present in matrigel (Corning), we tested the effects of Transforming Growth Factor-β (TGF-β), Hepatocyte Growth Factor (HGF) and Epidermal Growth Factor (EGF); individually and in combination, in methylcellulose for the ability to produce organoids. A two-part hepatocyte to one-part MSC ratio were maintained and cultured in the presence of methyl cellulose and growth factors in non-tissue culture 6-well plates. After 14 days in culture, we observed organoids of varying shapes and sizes (50-600µm) (Figure 1A). Interestingly no organoids were obtained when these growth factors were added individually in cultures (Figure 1B). Surprisingly, the addition of all three growth factors in culture yielded organoids that weren’t as big in comparison to organoids from TGF-β and EGF growth factor combination (Figure 1C, D).

**Liver function activities of organoids cultured in methylcellulose**

We next tested the ability of organoids to produce albumin and detoxify ammonium chloride, in optimally cultured in the conditions. We compared these conditions, to that of organoids obtained using matrigel as a reference point. Albumin production was highest from organoids derived from cultures containing TGF-β and EGF (Figure 2A). Again, surprisingly, organoids derived from the culture in all three growth factors, yielded the least amount albumin production. This pattern of albumin production co-related with the morphology of organoids obtained as above. When organoids were assayed for the ability to detoxify ammonium chloride to urea, a similar pattern was observed. Urea production was highest from organoids derived from the culture containing TGF-β and EGF in comparison to the organoids derived from all three growth factors (Figure 2B). A similar co-relation of function to morphology was observed.

**Histological and biochemical properties of organoids derived from methylcellulose**

Having established the functionality of these organoids, we then analysed the histology of these optimally cultured organoids. These organoids expressed hepatocyte specific marker, OCH1E5 and; biliary marker, cytokeratin 7 (Figure 3B) and Control H&E section (Figure 3A) indicating that cells of both hepatocyte and biliary lineages were present in the organoids. These organoids also expressed Cytokeratin 19 (Figure 3B, right and middle panels). Furthermore, confocal, microscopy also revealed CD31 expression in these organoids suggesting endothelial cell formation (Figure 3C).

Finally, we assessed the ability of organoids to conjugate bilirubin. Albeit relatively lower levels compared to the HepG-2 cell line, these organoids could conjugate bilirubin (Figure 4A). Strikingly, we found the expression of MRP-2 in these organoids, suggesting an attempt at organisation of the hepatocytes into a lobule (Figure 4B, middle panel) and compellingly, bile staining as shown in a Fouchet stain (Figure 4C).

**Discussion**

In this study, we show that methylcellulose, an inert semi-solid media, could be utilised as a scaffold to establish liver-organoids that resemble liver structure and function. The results we obtained here are comparable to that of using matrigel as a scaffold. There was also a good correlation of the morphology of organoids obtained to functional activity. Albeit the lowered functional activities (albumin and urea production) using methylcellulose as opposed to matrigel, the suitability of using methyl cellulose towards clinical grade expansion of organoids is highly compelling. Further optimization and scaling up of the process is underway. Methylcellulose is a synthetic chemical product derived from cellulose. Cellulose is heated with sodium hydroxide and the resulting substitution of hydroxyl residues on cellulose are replaced with methoxide(14). Methylcellulose is used in a wide array of applications in industry and in the clinic. It is a well-known agent for the treatment of constipation(15) and its derivatives can be used as artificial tears(16, 17) or saliva. In the laboratory, methylcellulose has long been used as a scaffold for the development of colony forming cells from haematopoietic stem/progenitor cells and is a good support for studying stem cell proliferation and differentiation(18-20). As such, it should be relatively easy to implement a clinical grade strategy using methylcellulose as a scaffold to obtain liver-organoids.

A further advantage to using methylcellulose as a scaffold is the flexibility to assess the effects of different growth factors,tailor-made chemicals or synthetic combinations on organoid formation. We found that the use of EGF and TGF-β together were crucial in establishing organoids. EGF is a member of the Tyrosine Kinase receptor family(21) and is important in the upregulation of MAP kinase signalling pathway leading to DNA synthesis and cell proliferation(22). TGF-β on the other hand is a growth factor known to induce apoptosis in lymphocytes and hepatocytes in mice(23). It is also thought to stop proliferation and induce differentiation in stem cells(24). It is intriguing that the synergistic effect of EGF and TGF-β in our experiments was necessary for the formation of organoids, to which the exact mechanism of organoid development remains to be determined. The simplicity of this system means that it can also be scaled up for drug screening and cell signalling experiments as well.

We and others have previously shown that hepatocytes isolated from a donor liver, can be used to treat children with ALF effectively(25-27). Further improvements to hepatocyte transplantation has been the method of encapsulating hepatocytes with alginate beads(28, 29). This encapsulating method eliminates the need for immunosuppressing drugs that are routinely used in liver or non-encapsulated hepatocyte transplantation. Such alginate encapsulated human hepatocyte transplants in humans although not necessarily curative, have shown to be useful as a bridge to native liver recovery or until a suitable donor liver is found (Personal communication/unpublished data, AD).

Recently, bio-fabrication techniques have been developed using 3D plotting with methylcellulose and alginate. MSC cultured in these 3D scaffolds retained viability and differentiation properties(30, 31). Taken together, it is highly conceivable to be able to establish organoids in methylcellulose, as done here and then encapsulating the resulting organoids in alginate-methylcellulose 3D scaffolds(32). Such a strategy should be highly applicable in the treatment of ALF.

**Methods**

**Hepatocyte and MSC isolation**

Primary hepatocyte isolation and research activities were ethically approved via the National Research Ethics Service (King’s College Hospital local research ethics committee; LREC 01-016). Organs were donated through the National Health Service Blood and Transplant (NHSBT) and offered for hepatocyte transplantation and/or research following decline for solid organ transplantation. Hepatocytes were derived from donor as previously described(33). All hepatocyte isolation procedures were performed to Good Manufacturing Practises (GMP) standards (Cell Therapy Unit, King’s College Hospital) and governed by the Human Tissue Authority for the storage, clinical and research use of these cells. Briefly, surgically obtained split liver lobes were weighed and cannulated(26). This was followed a by an in-house modified collagenase perfusion step, followed by a perfusion to inactivate the collagenase. Perfused liver lobes were then macerated using scissors and forceps and filtered through gauze until clumps had reduced. Hepatocytes were obtained by centrifugation at 50g, where the hepatocyte fraction was collected from the cell pellet. Cell number and viability was determined by trypan blue exclusion. Cells were frozen in cryovials (research use) and cryobags (clinical use), using a controlled rate freezer and stored at -180°C vapour phase nitrogen freezers.

MSC were isolated from Wharton’s jelly (WJ) of umbilical cords obtained following caesarean section deliveries at King’s College Hospital. These umbilical cords were procured via the Anthony Nolan Trust, according to King’s College Hospital Institutional guidelines. MSC were isolated at research laboratory grade standards. Briefly, cords were collected in PBS containing 40µg/ml of gentamicin (33). Cords were cut into sections to expose the WJ, which was minced into fine pieces (1-3 mm2). The explants were placed in sterile petri dishes and immersed with MSC culture medium, consisting of MEM α (Life Technologies), 5% Stemulate (Cook Regentec), 40µg/ml of gentamicin and allowed to adhere for up to 5 minutes. Cord samples were then incubated at 37°C, 5% CO2 in atmosphere and half medium replenished every 3-6 days. Gentamicin was used in the MSC medium for the first 1-2 weeks of culture and then replaced by penicillin/streptomycin (100 units/mL and 100 µg/mL, respectively). Once cell cultures were established, they were expanded, quality controlled and cryopreserved. Cells were positive for MSC markers (i.e. CD73, CD90 and CD105; expression higher than 75%) and negative for hematopoietic markers (i.e. CD14, CD34 and CD45; expression lower than 2%) by flow cytometry.

**Organoid formation**

The initial establishment of liver organoids was adapted from Takebe et al. Briefly, hepatocytes and MSC were thawed and cultured in round bottom non-tissue culture treated 96 well plates. The culture media consisted of DMEM/F12 high glucose (Gibco) as the basal media supplemented with N2 (Gibco) and B27 (Gibco) supplements, Penicillin and Streptomycin and L-glutamine. Matrigel (Corning) was added to a final concentration of 1%. The initial ratio of hepatocytes to MSC was 1:1 and 105 cells in 200 µl total volume used. Plates were incubated at 37°C, 5% CO2 in atmosphere. Different cell ratios were investigated. Plates were cultured for two weeks and monitored daily for the growth of organoids.

Having established the system in Matrigel, methyl cellulose (BioTechne) was substituted as a scaffold. We also scaled up the process into a non-tissue culture treated 6 well plates, omitting the use of Penicillin and Streptomycin. Methylcellulose was added to a similar culture media as above to a final concentration of 1.2%. 10ng/ml final concentration of Transforming Growth Factor-β (TGF-β), Hepatocyte Growth Factor (HGF) and Epidermal Growth Factor (EGF) (all from BioTechne) was used in cultures, either individually and/or in combination. Plates were incubated at 37°C in 5% CO2 in atmosphere. Plates were cultured for two weeks and monitored daily for the growth of organoids.

**Albumin Assay**

Albumin secretion from organoids were measured by a standard ELISA protocol as previously described(13). Briefly, organoids were transferred to fresh plates and incubated with media (less the scaffold) for 8 to 18 hours, and media was then removed and stored at -20°C until the assay.

**Urea assay**

Urea assay was performed according to previously established methods(13). Again, organoids were transferred to new plates and incubated with media (less scaffold) in the presence of 4mM Ammonium Chloride. Urea production in the media was measured 6 hours later using QuantiChrome Urea Assay Kit (BioAssay Systems, Cambridge, UK)

**Bile conjugation assay**

Total and conjugated bile was determined by using a bilirubin assay kit from Sigma. One µM unconjugated bilirubin was used to spike organoids. This assay is based on the Jendrassik-Grof method which utilizes the reaction of bilirubin with diazotized sulfonic acid resulting in a colorimetric product measured at 530 nm, proportionate to the bilirubin present in the sample.

**Histology**

Developing and established organoids were fixed in 4% paraformaldehyde. Fixed organoids were embedded in 2.5% agarose. The set agarose-organoid material was then routinely processed. Five-micron thin paraffin embedded sections were placed on poly-lysine coated slides. Standard immunohistochemistry protocols were employed. Details of antibodies used in Table S1. All immune-stained sections were counter stained with Haematoxylin and permanently mounted.

Confocal microscopy was performed on live organoids mildly permeabilised with 0.01% triton-x in PBS. Organoids were then stained with respective antibodies (Table S1), washed twice in PBS and mounted onto glass slides. Confocal microscopy was performed on a Leica SP5 microscope.

**Figure legends**

**Figure 1 Morphology of organoids grown in methylcellulose**

A. Left hand panel: Hepatocytes and MSC cultured in a 2:1 ratio respectively in EGF only, Right hand panel: As in left hand panel but at higher magnification. No organoid formation was observed after 14 days in culture. Scale bar=100 micron

B. Left hand panel: Organoids obtained from hepatocytes and MSC cultured in a 2:1 ratio respectively in EGF and TGF-β, Right hand panel: As in left hand panel but at higher magnification. Organoids formation was observed after 14 days from culture initiation Scale bar=100 micron

C. Left hand panel: Organoids obtained from hepatocytes and MSC cultured in a 2:1 ratio respectively in EGF, HGF and TGF-β, Right hand panel: As in left hand panel but at higher magnification. Organoids formation was observed after 14 days from culture initiation, however morphologically not the same as in B Scale bar=100 micron

D. Organoids grown in non-tissue culture treated 6-well plates. After two weeks in culture many were macroscopic and heterogenous in shape. Scale bar=200 micron.

**Figure 2 Albumin secretion and urea production from organoids**

Albumin secretion from organoids was measured by ELISA method. A. Albumin production from organoids cultured in Matrigel (as a base line) was compared to that which was cultured in methylcellulose. The combination of TGF-β and EGF growth factors in methylcelluose yielded the highest amounts of albumin production compared to other cytokine combinations in methycellulose (n=3 independent experiments, each performed in triplicate)

B. Urea production of similar organoids were measured by colorimetric means. Here too the combination of TGF-β and EGF growth factors yielded the highest amounts of urea production compared to other cytokine combinations in methylcellulose (n=3 independent experiments, each performed in triplicate)

**Figure 3 Immunohistochemical profile of organoids**

Immunohistochemistry was performed to characterize organoids. A. Organoids stained by H&E (left hand panel), and organoids expressing hepatocyte (Orange arrow) and biliary marker (blue arrow)(right hand panel).

B. Organoids expressing CK-19 (left and middle panel) and control H&E stained sections of a liver biopsy (right hand panel)

C. Organoids expressing CD31 (green) and nuclear stained DAPI (Blue)

**Figure 4 Organoids exhibiting biliary function**

A. Organoids were able to conjugate bile as in control cells, as measured biochemically

B. Immunohistochemistry confirmed this as organoids stained positively for MRP-2 antibody (middle panel), H&E left panel and control tissue (right panel) thus suggesting structure formation

C. Fouchet staining of organoids showing bile stain, indicating the presence of conjugated bile

**Supplementary figure legends**

**Figure S1 Organoids cultured in matrigel**

1. Morphology of organoids from a 96-well plate
2. Confocal imaging of hepatocyte (top right panel, in green) and CD105 marker (bottom left panel-in red and merged image (bottom right panel). Dapi stain of nuclei (Top left panel-in blue)

**Figure S2 Cell ratio analysis for organoid development**

1. Albumin ELISA demonstrating that a 2:1 hepatocyte: MSC ratio gave rise to the highest expression
2. Urea production was highest using a 1:1 hepatocyte: MSC ratio

**Figure S3 Time function of organoids**

1. Albumin production was detected from day two in culture and peaking at day 10
2. Urea production was like albumin production except that a slightly higher level was noted on day 2. Urea production peaked at day 12

**Figure S4 Schematic production for developing organoids suitable for transplantation**

**Table S1 Antibodies used in this study**

| **Antibody** | **Supplier** | **Catalogue #** | **Pretreatment** | **Dilution** | **Bond**  **Protocol** | **Control** | **Result** |
| --- | --- | --- | --- | --- | --- | --- | --- |
| cMOAT **(F)** | Abcam | Ab3373-250 | ER2 | 1:40 | F | Normal liver | Canaliculi +ve |
| CK 7 | Dako | M7018 | ER1 | 1:200 | F | Normal liver | Bile ducts +ve |
| OCH1E5 | Dako | M7158 | ER1 | 1:100 | F | Normal liver | Hepatocytes +ve |
| Cytokeratin 19 | Abcam | ab52625 | - | 1:200 | - | Normal liver | Bile ducts +ve |
| CD31 | Abcam | ab28364 | - | 1:50 | - | - | Endothelial cells +ve |
| CD105-PE | Biolegend | 323205 | - | 1:50 | - | MSC-P1 | MSC +ve |

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**Acknowledgements**

We would like to thank Ms Shirin E Khorsandi for reading the manuscript. The authors would also like to thank Mr Simon Walker (MSC and hepatocyte isolation), Dr Charlotte Lee and Dr Raquel Fernandez-Dacosta for help with hepatocyte isolations. Our gratitude also goes to all the families of the liver donors and the liver transplant coordinators for their tireless work. This work was funded by the Medical Research Council, UK under the UK Regenerative Medicine Programme-II; Stem Cells and the Niche. Ms Simona Asan, a research student involved in the project passed away shortly before drafting the paper and this work is dedicated in her memory.

**Author contribution**

AC, AD conceived and designed experiments; AC, TP, CS performed experiments; AC, MD, AD analysed and interpreted data; SL, VI, AC for GMP hepatocyte isolation; VI responsible for MSC isolation; SB, EF obtained and provided clinical samples; RM, CF, DH provided useful discussion, insight and support. AC, AD wrote the paper.

**Conflict of interest**

Non-declared