Development of semi-solid cell culture medium for providing 3D matrix support and enhancing the survival of liver organoids

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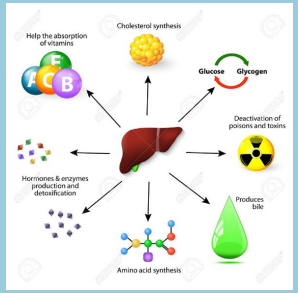
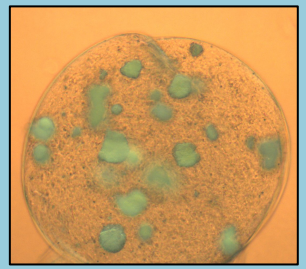
Medical Applications of Liver Organoids

The liver is the largest organ in our body responsible for regulating chemicals in our blood stream, excreting bile, and detoxifying food. Liver organoids are smaller 3D models of the liver. They us help understand regeneration of the liver, model common liver diseases, and metabolism studies.

- **Similar composition and architecture to organ:** A liver organoid is a smaller version of an actual human organ. Individual Hepatocyte cells (2D) cannot fully reveal the mechanism and characteristics of liver diseases
- **Relevant model to study drug discovery and disease conditions:** Liver organoids help us identify disease mechanisms in the body. They help understand the function different drugs have on our body because they replicate cellular microenvironments
- **Stable system for extended storage:** Liver organoids can be cryopreserved as biobanks. They can then be cultured at anytime to study disease conditions.
- It is used in drug toxicity testing, regenerative medicine, personalized medicine, disease pathology- in both basic and translational research

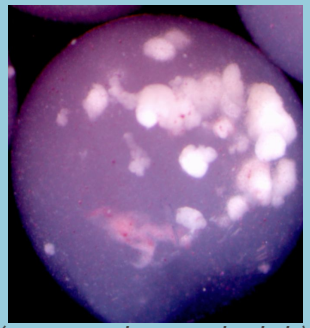




(Organoid Image taken at the lab. Other image reference from hangzhou)

Drug Toxicity Testing in Liver Organoids

Hepatoprotective activity of the *Phyllanthus* species on *tert*-butyl hydroperoxide (*t*-BH)-induced cytotoxicity in liver organoids



(Image taken at the lab)

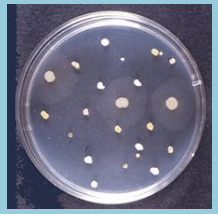
- Liver disorders, like liver cirrhosis, benefit from therapeutic strategies employing compounds extracted from plants and herbs
- *Phyllanthus niruri* (PN) is a herbal plants widely used against viral hepatitis and liver cirrhosis.

- It contains several chemical agents including phyllanthin, hypophyllanthin, phyltetralin, niranthin, nirtetralin, hinokinin and isolintetralin
- In this study, the previously implicated hepatoprotective function of the PN extract was further evaluated and its efficacy as a therapeutic agent was experimentally tested on rat liver organoids **against t-BH (tert-butyl hydroxide) -induced toxicity.**

We were tested the Effect of the Phyllanthus niruri extract (commercially available) on cell viability against t-BH-induced toxicity (upto 100 µg/ml) in liver organoids

(Image taken from the Morpheme)

How are Liver Organoids Generated?



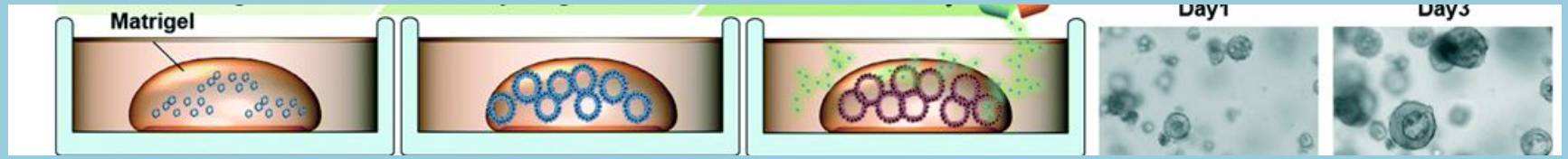
(Taken at the lab)

Liver organoids are generated either from adult tissues, fetal liver or pluripotent stem cells.
If we provide appropriate physical and biochemical stimulus they will form an organoid.

Physical cues: Provide support for cell attachment and survival. For example a 3D matrix home environment.

Biochemical cues: Growth factors and cell culture supplements. Thereby modulating signaling pathways as well as influencing proliferation, differentiation and self-renewal.

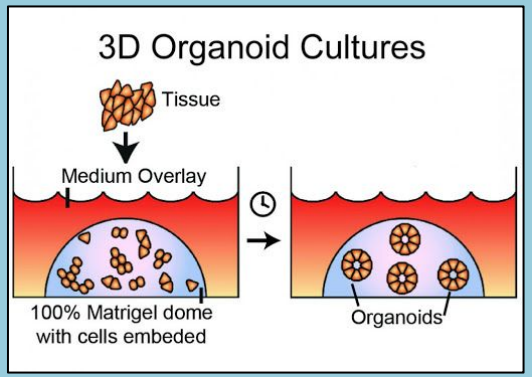
In order to grow liver organoids we need to culture in a matrigel which is a liquid in cold temperatures. It solidifies in room temperature forming thick coating around the organoid.



(Curr Gene Ther. 2013 Apr;13(2):120-32)

Current Problem in Liver Organoid Culture

- Current method for culturing liver organoid: today scientists use matrigel. This is a liquid at cold that solidifies arounds the organoid in room temperature.
- The biggest problem with liver organoids is retrieving the cells without killing the cells
- When you are trying to remove the cells an enzyme solution is used, this not only very bad for the cells in many cases it kills many of the cells
- Matrigel is derived from cancer cells, so it is not ideal for clinical applications
- Due to solidification process, it does not allow organoids to grow large.
- In many cases the matrigel matrix loses it integrity



(Reference from "scientific reports")

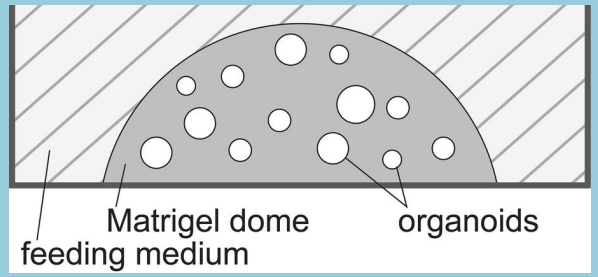
(Taken at the lab)

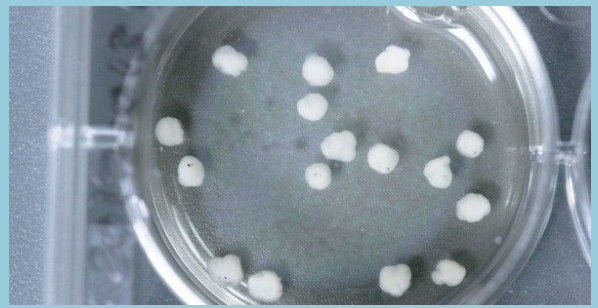
My Research Question and Hypothesis

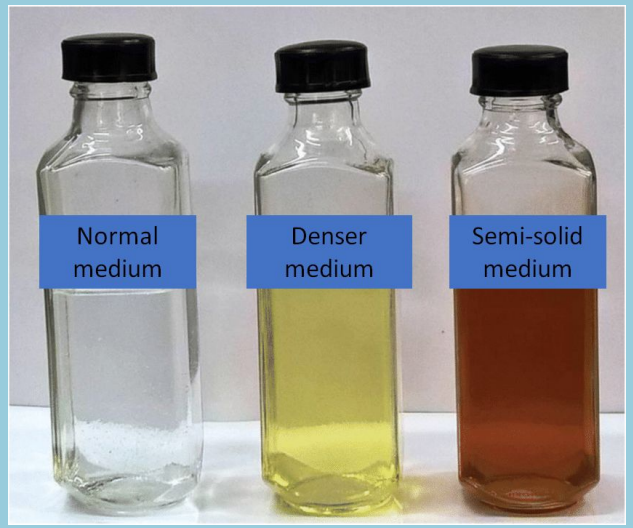
Research Question: Is it possible to culture liver organoid in a new culture medium for easy retrieval?

Hypothesis: If a high viscosity culture medium (semi-solid) is used to culture a liver organoid then this will provide a 3D environment for the organoid, similar to what matrigel provides. As well as easy retrieval of organoid from the semi-solid medium and it will allow the formation of large organoids

(Taken by my mentor)







(Organoid and cell culture bottles from the lab)

Experimental Design

- Extensive Literature review to identify new culture medium compound; I learned that culturing organoids in a semi-solid medium was not done before.
- Creating the semi-solid medium in RPMI medium (n=10)
- Creating the liver organoid from mouse liver (n=10 mice).
- Culturing liver organoids in the new semi-solid, matrigel, and liquid medium (n=20)
- Functional assessment (structural integrity, cell viability, cellular functional assays, and gene expressions) of liver organoids in semi-solid, matrigel, and liquid medium (n=20)
- Testing the effect of the Phyllanthus niruri extract (commercially available) on cell viability against t-BH-induced toxicity (upto 100 µg/ml) in liver organoids (n=10)
- Data collection and data analysis
- Statistical analysis by student T- test and ANOVA

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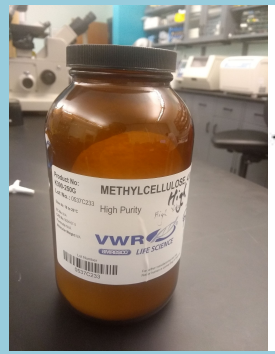
(Taken by my mentor)

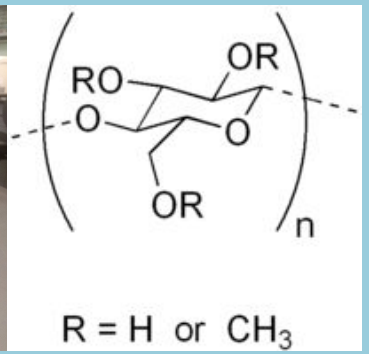
Preparation of semi-solid culture medium

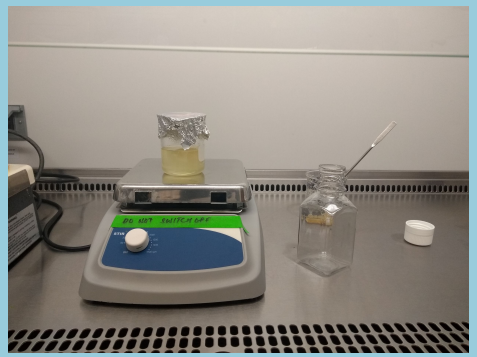
To create a strong semi-solid culture I need the best compound. Through literature review I identified “methylcellulose”. This mixture is ideal allowing you to control the the viscosity. It is common enough and even found in food products. It is not tested in cell culture.

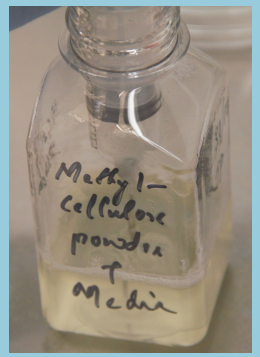
- Methylcellulose powder, 4000 centipoises (high purity, VWR, AMRESCO, LLC)
- Is weighed (3mg) and collected in 100 ml RPMI culture medium
- After overnight mixing SSCM with viscosity (i.e. similar to honey, centipoises 1500) is obtained.
- 20 ml of SSCM is aliquoted to 5 sterilize 50cc conical and stored
- Viscosity (1500 centipoises)and pH (7.4) were adjusted.
- Stored at cold and bring it to room temperature on the day of experiment.

(Images below taken at the lab and reference from the polymer properties database)





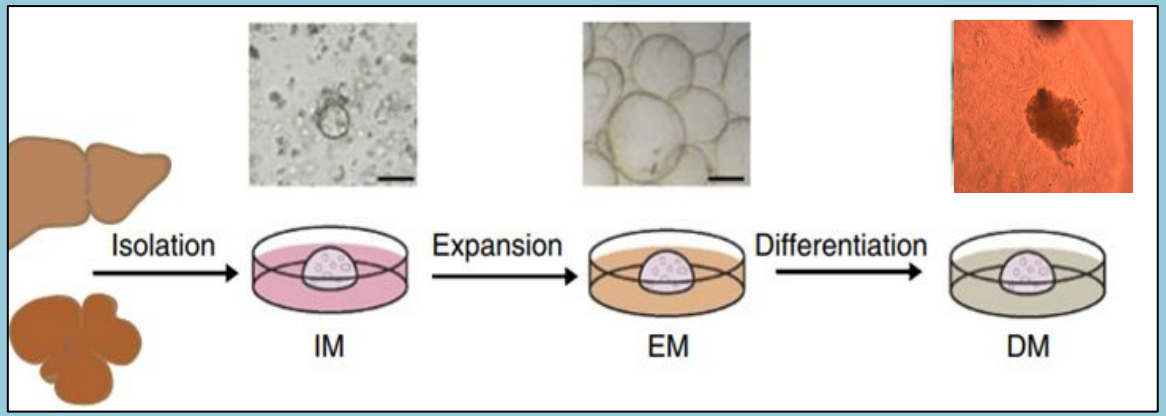




Creation of the Liver Organoid

- Mouse liver tissues were digested in digestion solution (Collagenase) for 2 h.
- When digestion was complete, bile ducts were pelleted by mild centrifugation and washed with PBS
- Isolated ducts were then resuspended in Matrigel (BD Bioscience) and cast in 30 µl droplets in the centre of the 48-well plate
- After the gels were formed, 250 µl of isolation medium (RPMI) was added to each well.
- Isolation medium was composed of Invitrogen supplemented with B-27 and N-2 (both GIBCO), 1.25 µM N-acetylcysteine , 10 nM gastrin and the following growth factors: 50 ng ml−1 EGF, 1 µg ml−1

- After the first 4 days, isolation medium was changed with expansion medium (EM), which consists of isolation medium without Noggin, Wnt and Y-27632.
- One week after seeding, organoids were formed inside a differentiation medium
- After creating liver organoids I started using semi-solid culture medium



(Gut. 2019 Dec;68(12):2228-2237). Other images taken at the lab

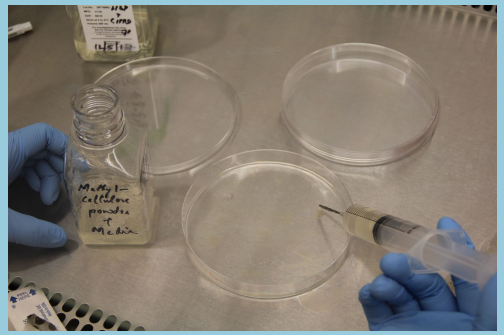
Culturing liver organoids in semi-solid medium

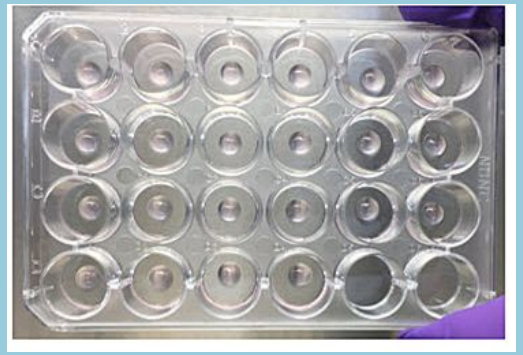
After liver organoids were prepared, they were then cultured in 24 well plate. About 20 organoids were mixed with 50µL of Matrigel and dropped in the middle of the culture plate (n=20). Fresh culture medium (RPMI) was added surrounding the matrigel (250µL).

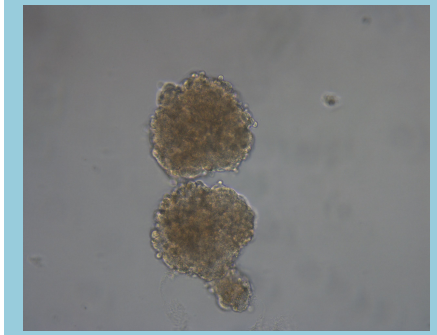
Similar number of 20 organoids were cultured in semi-solid culture medium (250µL) as well as in normal liquid culture medium (n=20). All cultured were incubated in 5%CO2 incubator.

Cultures were monitored for 10 days and subjected to functional assessments for comparing culture conditions and survival of the organoids.

(Images taken at the lab)

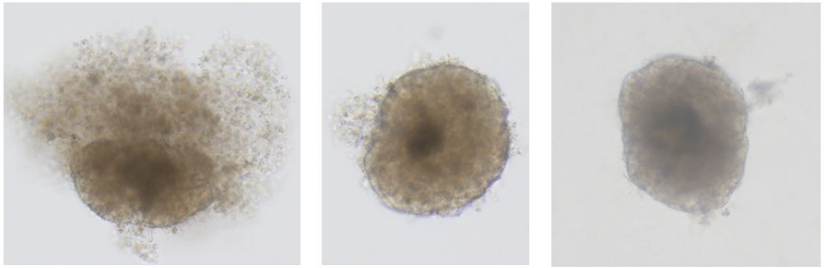






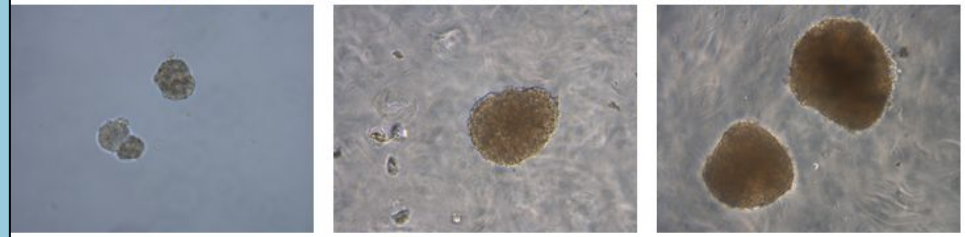
Results-Organoid Integrity, Size and Viability

Organoid cell integrity after culture for 10 days in different culture medium



Liquid culture | Matrigel | Semi-solid culture

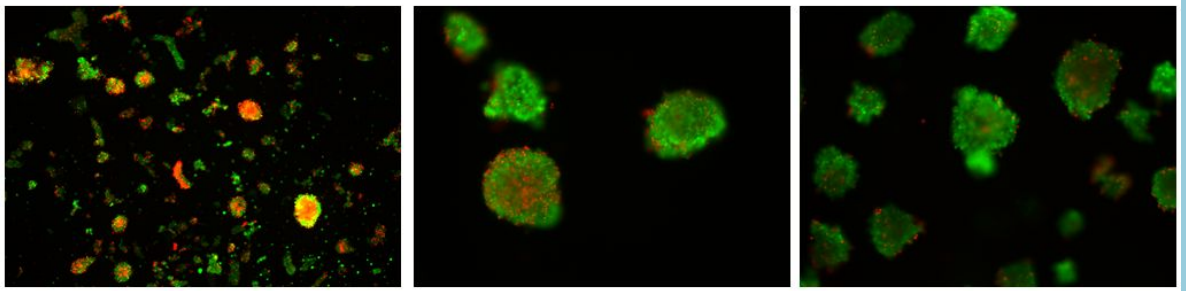
Organoid size variations in different culture (40X)



Liquid medium | Matrigel | Semi-solid

- Image to the left shows the organoids cell integrity after 10 days in a liquid culture, matrigel, and my semi-solid
- In the liquid culture you can see the cell does not hold it's integrity while in the matrigel and the semi-solid hold the shape very well.
- Cell viability shows dead cells and viable cells or cells that have survived
- The red cells are the dead cells and green show viable cells
- In the liquid medium there were more dead cells compared to the others

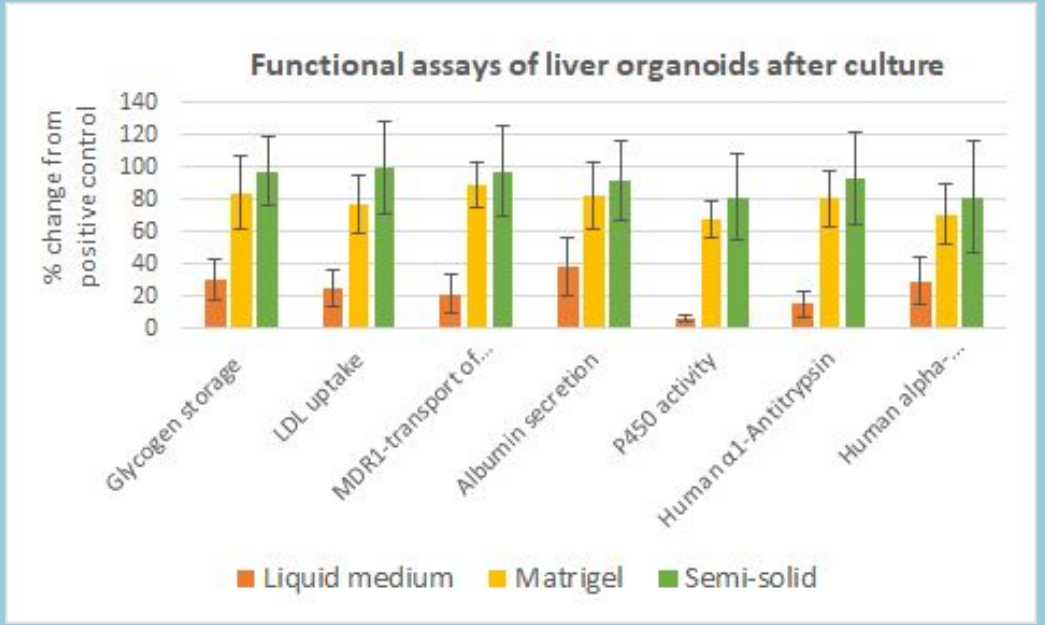
Organoid cell viability by Acridine Orange/Propidium Iodide double fluorescence stain (Red=dead cell; Green=viable cells) after culture for 10 days in different culture medium



Liquid culture | Matrigel | Semi-solid culture

Functional Assessment of Organoids after culture

Assay	Technique
Glycogen storage assay	Periodic acid-Schiff (PAS, Sigma) staining
LDL uptake	DiI-Ac-LDL (Biomedical Technologies)
MDR1-mediated transport of rhodamine 123	Detected over a 10–15 minutes incubation.
Albumin secretion	Bethyl Elisa Kit
P450 activity	Promega Kit
Human α1-Antitrypsin	ELISA (Assay Pro)
Human alpha-Fetoprotein (AFP)	ELISA (R&D)



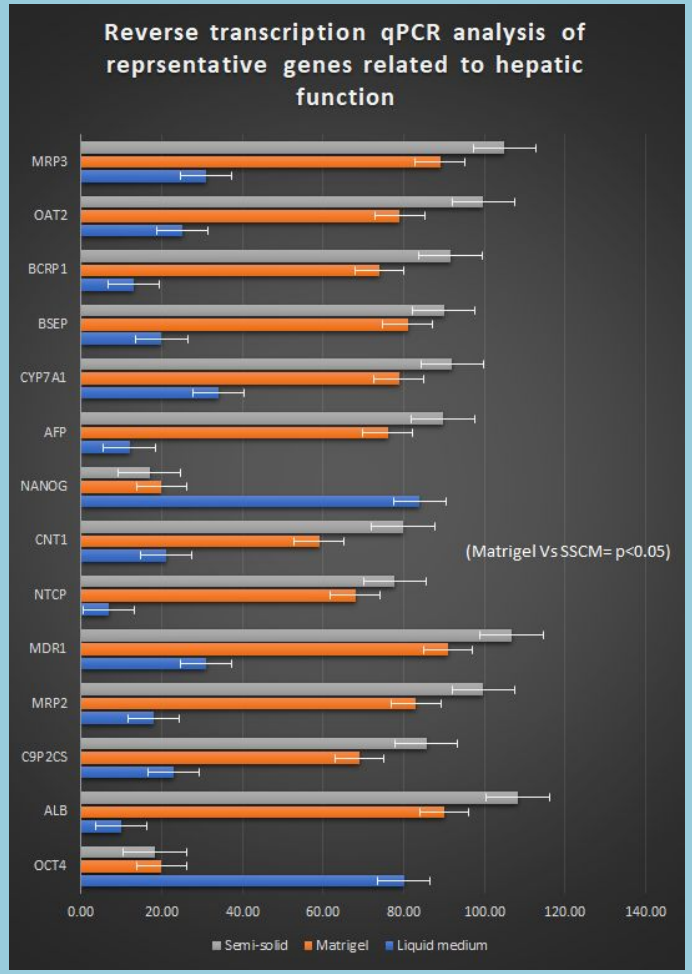
Functional assessment of liver organoids showed (n=20) better results in semi-solid culture medium in comparison with matrigel. They were statistically significant ($p<0.05$)

Liquid culture medium caused poor function of organoids

Results-Gene expressions of liver organoid

- Gene expression profiles of liver organoid by qPCR

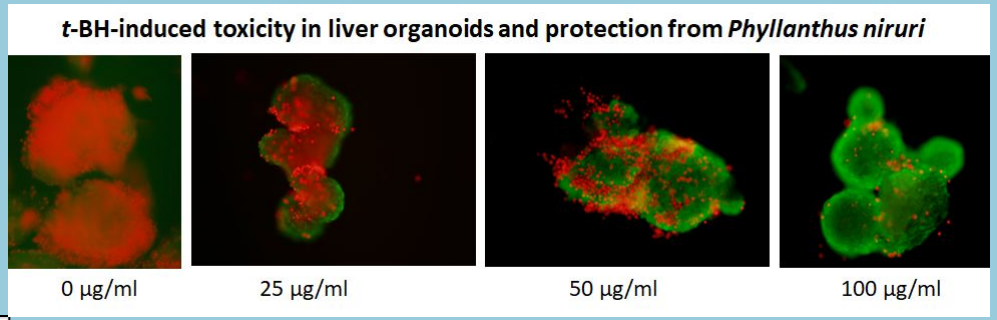
- Hepatic marker genes:
  - albumin (ALB)
  - a-fetoprotein (AFP)
  - Cytochrome P (CYP)
  - 450 family 2 subfamily C member 9 (CYP2C9) and CYP7A1
- Genes related to polarity and transporter activities (increase in expression levels):
  - Multiple drug resistance 1 (MDR1)
  - Multidrug resistance-associated protein (MRP2 and MRP3)
  - Bile salt export pump (BSEP)
  - BCRP1
  - Na^+-taurocholate co-transporting polypeptide (NTCP)
  - Organic anion transporter 2 (OAT2)
  - Concentrative nucleoside transporter 1 (CNT1)
- Decrease in proliferative markers (undifferentiated state):
  - NANOG
  - OCT4

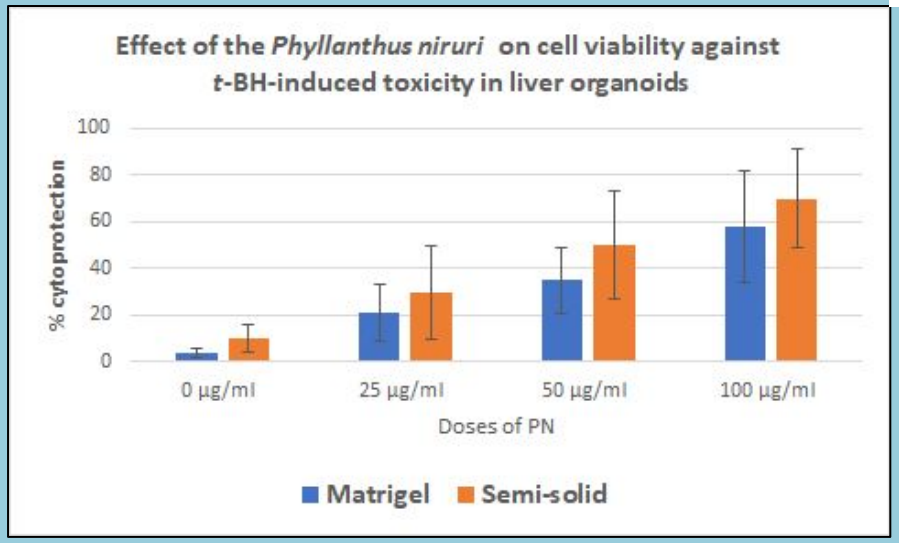


Gene expression profiles assessed in reverse transcription qPCR (n=20) showed improved hepatic marker genes in semi-solid culture medium ($p<0.05$) compared to matrigel

t-BH induced toxicity in liver organoids and protection from Phyllanthus niruri

- Comparing matrigel cultured and semi-solid medium cultured organoids showed significant difference (P<0.05) in t-BH toxicity study





- *t*-BH-induced severe toxicity in liver organoids in dose dependent manner. Read-dead cells; Green-viable cells.

- Supplementation of *Phyllanthus niruri* extract protected liver organoids from t-BH induced cell death.

Summary

To summarize this study, I found the best ways to culture liver organoids and create one. Through my research I found that a semi-solid culture medium results were superior to the current matrigel culture. In a semi-solid medium it was easy to retrieve the organoid from the semi-solid culture condition which is something that matrigel lacks. In comparison liquid medium is not ideal for preserving a liver organoid.

Finally,when I tested the Effect of the Phyllanthus niruri on cell viability against t-BH-induced toxicity in liver organoids after culturing in my semi-solid culture medium and obtained better results compared to matrigel.

Conclusion: Overall a semi-solid culture medium is a novel method to culture organoids. PN provides protection from liver toxicity and consumption of this plant extract will protect liver cirrhosis.

Future Direction: I will plan to test this medium for preserving other type of larger cells like cancer cells, other types of organoids, and smaller organs.

References:

Wu, L., Chen, Z., Wang, Y., Zhao, J., Xie, X., & Chen, G. (2019, April 28). Organoids of liver diseases: From bench to bedside. Retrieved February 24, 2021, from https://www.wjgnet.com/1007-9327/full/v25/i16/1913.htm

Prior, N., Inacio, P., & Huch, M. (2019, December 01). Liver organoids: From basic research to therapeutic applications. Retrieved February 24, 2021, from https://gut.bmj.com/content/68/12/2228

Broutier, L., Mastrogiovanni, G., Verstegen, M., Francies, H., Gavarró, L., Bradshaw, C., . . . Huch, M. (2017, December). Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. Retrieved February 24, 2021, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5722201/