

Grade: 1.2

Lab Report

Schenk Lab

M.Sc. BMT, Implantology
University of Tübingen

May, 5th 2022

Supervisor: Maria Seitzer

1 Introduction

Liver disease and the subsequent loss of liver function is a frequent pathology caused by hepatitis, tumors, or alcohol abuse. Currently, the only therapy for end-stage liver disease is liver-transplantation [1]. Since the number of organ donors is not sufficient there is an urgent necessity regarding other treatment options [1]. At present, much hope is placed in the generation of fully functional tissue engineered liver implants [1]. These artificial structures are created by e.g. allocating adult hepatocytes or stem cell derived hepatocyte-like cells within a 3D structure scaffold [2]. However, the complexity in the liver's microarchitecture and especially in its functionality are currently limiting the success in developing such an implant [3].

Since it is known that the function of liver cells is influenced by their surrounding structure, the aim of this practical was to compare different liver parameters measured for human hepatocellular (HepG2) cells seeded on a monolayer, as well as for cells seeded on a 3D matrix, functioning as scaffold (tissue). The four liver parameters, one of them observed by each practical group, are briefly explained below.

2 Methods

2.1 Liver Parameters

Aspartate Aminotransferase (ASAT) is a housekeeping enzyme of hepatocytes. While catalyzing the transfer of amino groups, NADH is oxidated to NAD as a side reaction. As the rate of oxidation can be measured by a decrease in light absorbance at 340 nm, proportionally to the ASAT activity, this method gives reliable information about the functionality of this important enzyme. Urea is a major end product of protein catabolism. Since hepatocytes eliminate toxic ammonium by building urea, its production out of ammonium chloride is used for measuring the detoxifying capacity of liver cells. Hepatocytes take up and transfer D-galactose into NADH whose amount can be related stoichiometrically to the amount of D-galactose, and thus, giving information about the activity of hepatocytes by measuring the light absorbance at 340 nm. Thiazolyl blue (MTT) enables measurement of the cellular metabolic activity, and is therefore used as an indicator of cell viability. MTT is a tetrazolium salt, appearing yellow, that is reduced by metabolic active cells to formazan crystals that can be dissolved in isopropanol. The resulting colored solution can be evaluated with a photometer at 570 nm, where a darker solution indicates more viable active metabolic cells [4].

2.2 Preparation of Extracellular Matrix (ECM)

The ECM was prepared by the supervisor in the previous week for subsequent usage in this practical. Therefore, a full liver from the OR was taken, and perfused with 1 % of the detergent sodiumdodecylsulfate (SDS) for 24 h to wash the liver cells out of the organ. SDS generally enables elimination of cell nuclei out of dense cells and organs. Furthermore, it dissolves cell membranes and separates DNA from proteins. Next, aqua dest was added to the decellularized liver for 6 h to elute the detergent. The decellularized liver was stored at - 80 °C until further usage. On Friday, the liver was cut in thin slices that were each given into a 24 tissue well-plate. 1 ml of medium was added on each slice, and the well-plate was incubated over the weekend.

On Monday, HepG2 cells, growing in the incubator at 37 °C with 5 % carbondioxide over the weekend, were adhered on the ground. The growth medium RPMI was supplemented with 10 % foetal calf and antibiotica. Medium was removed from the decellularized liver matrix, and 200 000 HepG2 cells in 1 ml of medium were seeded in each well containing a monolayer or 3D matrix.

2.3 Elimination of Ammonium Chloride

To simulate the elimination of ammonium by hepatocytes building urea in vitro, a 2 mM ammonium chloride solution was added to both, the wells containing monolayers and 3D matrices with HepG2 cells, that there were two monolayer and two tissue samples to be further investigated. For subsequent evaluation of time-dependent urea production in the different structures, samples of 100 µl were taken after 1, 2 and 4 h each. This step was done one day before the practical.

On practical day, a serial dilution consisting of 5 mg/dL urea nitrogen standard solution and aqua dest was prepared first. The exact composition of the different samples is listed in Table 1. The samples prepared the day before, containing either monolayer or tissue and ammonium chloride were diluted with aqua dest by a ratio of 1:2. Next, 40 µl of all standards, samples, 2 mM ammonium chloride, and ammonium chloride diluted with aqua dest by a factor of 1:2, were pipetted into a 96-well plate duplicated to be further able to calculate a mean value of each probe. All tubes were vortexed before the solutions inside were pipetted into the well-plate. A working reagent was prepared by mixing 5 ml of color reagent A and 5 ml of color reagent B (urea with diacetylmonoxime gives a red-purple chromogen in presence of thiosemicarbazide [4]). 160 µl of this mixture were added into each well containing liquid. Finally, the well-

plate was put on an ELISA shaker for 50 min at room temperature to mix the solutions inside the wells. The mixed solutions were measured with a photometer at a wavelength of 520 nm.

Table 1: Serial dilution of urea nitrogen standard solution with aqua dest

Standards	Serial Delution	Aqua Dest
S7 5.0 mg/dL	150 µL	-
S6 4.0 mg/dL	120 µL	30 µL
S5 3.0 mg/dL	90 µL	60 µL
S4 2.0 mg/dL	60 µL	90 µL
S3 1.0 mg/dL	30 µL	120 µL
S2 0.5 mg/dL	15 µL	135 µL
S1 0.0 mg/dL	-	150 µL
Blank	-	150 µL

3 Results

3.1 Production of Urea

The absorbance measured for the serial dilution can be seen in Figure 1. With this curve, the unknown urea concentrations in the samples could be determined.

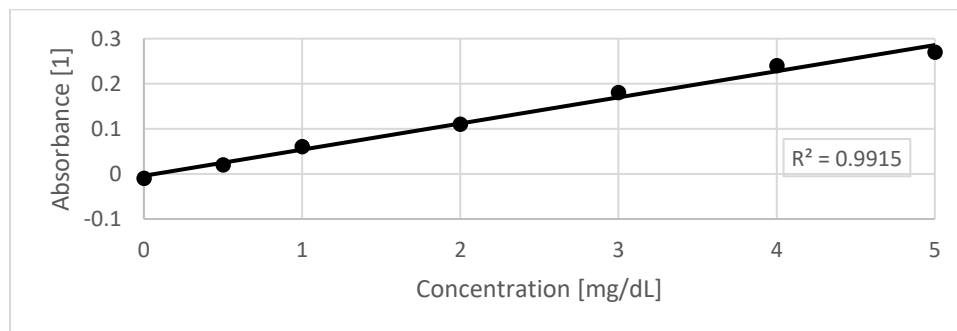


Figure 1: Calibration curve obtained by measuring absorbance of serial dilution

Table 2 shows the concentration of urea produced by HepG2 cells after 1, 2, and 4 h of measurement in the monolayer and the tissue. It can be investigated that HepG2 cells in tissue are able to transform ammonium chloride into urea successfully over time, but not in a monolayer. Figure 2 depicts the determined urea concentration graphically over time.

Table 2: Production of urea by HepG2 cells for monolayer and tissue

Time [h]	Urea Concentration Monolayer [$\mu\text{g/dL}$]	Urea Concentration Tissue [$\mu\text{g/dL}$]
1	5.69	5.34
2	5.62	5.89
4	5.46	5.86
Average Growth (at 4h)	-0.08 [$\mu\text{g/dL/h}$]	0.19 [$\mu\text{g/dL/h}$]

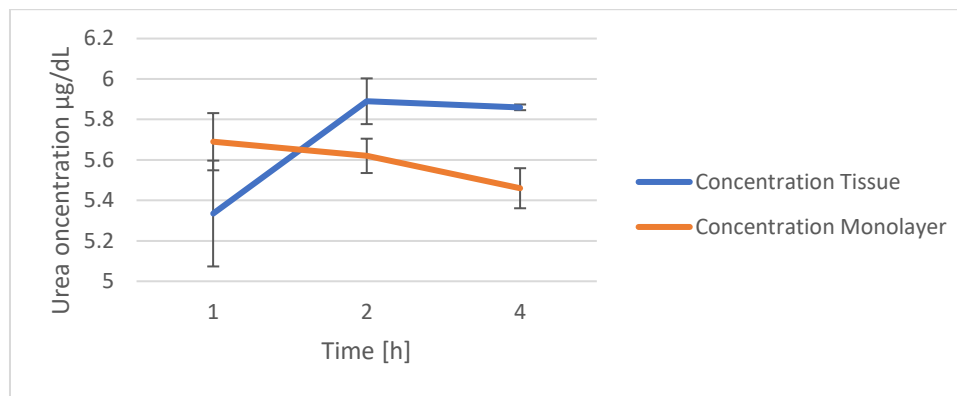


Figure 2: Course of urea concentration in monolayer (orange) and tissue (blue)

3.2 Summary of All Parameters Measured

Table 3 shows the results for all parameters observed by the different groups. The parameters are listed absolutely and relatively to the amount of MTT, thus, to the viable cells, showing an appr. six times higher viability in the monolayer than in the tissue.

Table 3: Absolute and relative parameters related to MTT

	Monolayer		Tissue	
	Absolut	Relative to MTT	Absolut	Relative to MTT
MTT optical density [1]	0.67	1	0.09	1
ASAT activity [units/L]	45.70	68.21	2.01	21.97
Urea (at 4h) [$\mu\text{g/dL/h}$]	-0.08	-0.12	0.19	2.11
Galactose elimination (at 4h) [%]	-20	-30	28	295

4 Discussion

In this practical different liver parameters were observed regarding their functionality when seeding HepG2 cells on either a monolayer or a 3D matrix (tissue).

The overall cell viability was six times higher on the monolayer than in the tissue, indicating that the viability of cells is significantly dependent on the surrounding structure. Therefore, all parameters further observed were related to the MTT results. This huge difference in viability is exceptionally high, as it is usually expected between a third and a half. ASAT level determination revealed a much higher cell activity in the monolayer than in the tissue. However, the production of urea was much higher by cells seeded in the tissue than in the monolayer. Here, no urea was produced at all. The small decrease of $-0.08 \mu\text{g/dL/h}$ might be due to statistical reasons. It would be necessary to observe the production of urea in tissue over a longer time period as the urea concentration produced between 1 h and 2 h, and 2 h and 4 h strongly deviates. While observing galactose metabolization, it could be detected that the monolayer eliminated the galactose as expected, and spread out. The tissue however, seemed as it secreted galactose in a huge amount rather than eliminating it, and was not spread out.

Regarding the challenge of developing a fully functional liver implant there is still a long way to go. Nevertheless, in this practical it could be demonstrated that some functions can already be recreated as e.g. the urea production out of ammonium chloride by HepG2 cells seeded in a scaffold structure. Others, as e.g. the cell activity, cannot be satisfyingly reproduced yet. Furthermore, enhancing the overall cell viability of cells seeded on a 3D matrix is an important need to be overcome.

5 Literature

1. Yang, G., et al., *Engineered Liver Tissue Culture in an In Vitro Tubular Perfusion System*. Tissue Eng Part A, 2020. **26**(23-24): p. 1369-1377.
2. Mazza, G., et al., *Liver tissue engineering: From implantable tissue to whole organ engineering*. Hepatol Commun, 2018. **2**(2): p. 131-141.
3. Agarwal, T., B. Subramanian, and T.K. Maiti, *Liver Tissue Engineering: Challenges and Opportunities*. ACS Biomater Sci Eng, 2019. **5**(9): p. 4167-4182.
4. Schenk, M., *Function follows form*. University of Tübingen, 2022.

I hereby declare that my elaboration was made independently and is not a copy of an existing protocol.

Date: May, 11th 2022

Signature: