

Grade 1,2

Protocol for the experiment

**Effectiveness of HepG2 cell integration in a
decellularized 3D-matrix (AG Schenk)**

May 12, 2022

Date

1 Introduction

Liver failure is a life-threatening condition in which liver tissue has been damaged by viral, toxic or autoimmune causes, resulting in 1 million deaths worldwide each year [1] [2]. Currently, the only effective method is considered to be liver transplantation, which, however, cannot always be used due to the limited number of liver donations [3].

Alternatives to liver transplantation could be the use of decellularized scaffolds. For this purpose, the livers of animals or humans are taken and decellularized so that new cells such as stem cells or hepatocytes can subsequently colonize the resulting liver matrix. The advantage of these scaffolds is that they already have the structure of a liver and can serve as a reservoir for growth factors and other molecules that promote cell growth [1]. In this experiment, a decellularized liver matrix was seeded with hepG2 cells derived from a hepatocellular carcinoma and the viability and growth of the cells were examined based on the metabolic activity. As a comparison, hepG2 were also seeded in parallel in a monolayer traditionally used for cell culture. To investigate the viability and metabolic activity of the cells, four independent experiments were performed that determined the thiazolyl blue formazan (MTT) cleavage, the aspartate amino transferase (ASAT) activity, the urea production, and the D-galactose elimination by the cells. The aim was to determine whether the viability of cells in the decellularized matrix was higher than those seeded in the monolayer [4].

2 Methods

Prior to the labwork, a decellularized liver matrix was prepared into which the HepG2 cells were subsequently introduced. For this purpose, a complete liver was harvested in the operating theatre and the cells were washed out of it with a 1% sodium dodecyl sulphate (SDS) solution. This process lasted for 24 hours during which cell nuclei were washed out, deoxyribonucleic acid (DNA) and proteins were separated and cell membranes were dissolved. After this time, the decellularized liver was rinsed with aqua dest and left in it for 6 hours to elute the SDS detergent. The liver was then stored at -80° until further use. On the Friday before the experiment, the liver matrix was cut into thin slices, the pieces were each placed in a 24-tissue well plate and 1 ml of medium was added to them. The slices were incubated over the weekend.

In the next step, the HepG2 cells were prepared by incubating them in the incubator at 37 °C and a carbon dioxide content of 5 % in the growth medium. The medium was Roswell Park Memorial Institute (RPMI) medium supplemented with 10 % of proteinaceous fetal calf, as well as antibiotics and antimycotics.

On the Monday before the experiment, the medium was removed from the decellularized matrix slices. The cells that settled at the bottom of the vessel after incubation were seeded into the decellularized matrices, as well as into the traditionally used monolayers. For this purpose, 1 ml of cell-containing liquid with a concentration of 200,000 cells/ml was added to each well. The cells had time to proliferate in the 3D-matrices and the monolayers until the experiment on Thursday.

In the scope of the experiment, the elimination of D-galactose in the prepared 3D-matrices was investigated in comparison to the traditionally used monolayers. Living HepG2 are able to take up D-galactose, making this method ideal for the cell viability analysis. In parallel, studies of the MTT cleavage, the ASAT activity and the urea production of the seeded cells were performed, the implementation of which is not addressed in this protocol. For the analysis of the galactose elimination, 1 ml of a 1 mmol/l D-galactose solution was first added to each of four wells in preparation. In two of the wells, decellularized matrices were added in advance and in the other two the monolayers. After addition of the D-galactose, 4 hours were waited so that the D-galactose could be taken up by the cells. Subsequently, 100 µl of each sample was transferred to tubes and stored at room temperature.

To check the galactose content, 10 µl quadruplets of each of the two prepared matrices and monolayers were added to a 96 well plate. In addition, 10 µl quadruplets of a blank, a 0.25 mmol/l, 0.5 mmol and 1 mmol/l galactose dilutions were added to the plate as references. To the samples and standards 10 µl of a solution containing citrate buffer and nicotinamide adenine dinucleotide (NAD) was added and all samples were incubated for 20 minutes at room temperature on an enzyme-linked immunosorbent assay (ELISA) shaker. Subsequently, 92.5 µl of aqua dest and 50 µl of a solution containing potassium diphosphate buffer were added and the mixtures were incubated for another 3 minutes on the ELISA shaker. The samples and standards were transilluminated with light of wavelength 340 nm using a photometer and the resulting extinction values were stored. Then, the reaction was started in which galactose and NAD react to form D-galactonic acid and NADH. For this, 5 µl of galactose dehydrogenase suspension was added to

catalyse the reaction. The mixture was incubated for 20 minutes at room temperature on an ELISA Shaker. In the final step, a photometric measurement of the samples and standards was again performed with light of wavelength 340 nm, where the resulting NADH, which is stoichiometric to the galactose content of the sample, absorbed light. The resulting extinction values were stored in tabular form [4].

3 Results

The extinction values of the quadruplet recorded during the experiment were evaluated for further analysis using Excel [5]. For this purpose, the values from each well of the first photometric measurement were subtracted from those of the second photometric measurement. Subsequently, the resulting differences ΔE of the standards were displayed in a diagram and their galactose content was plotted as a function of these (see figure 1). Using the diagram, the relationship between absorbance and galactose content of the samples could be determined from the slope of the trend line.

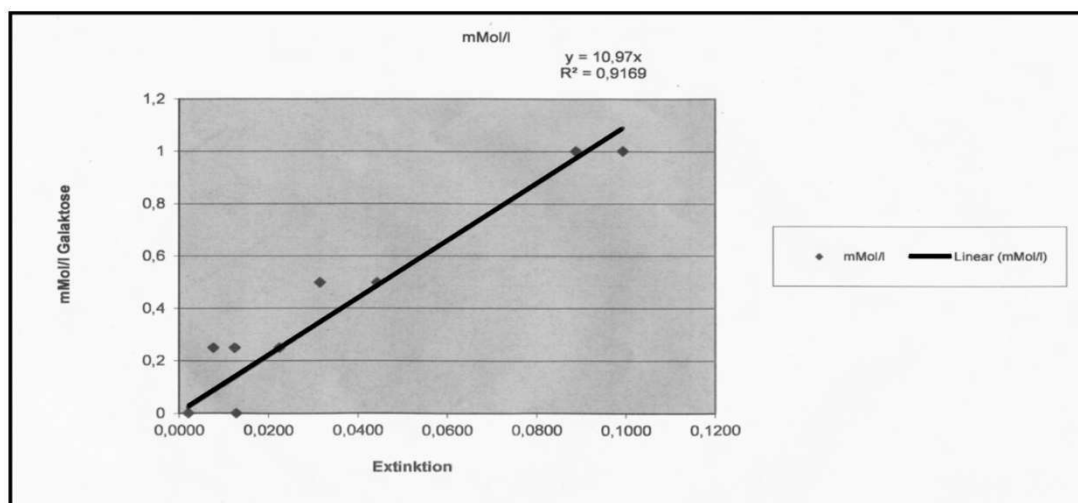


Figure 1: Galactose concentration of the standards as a function of the measured extinction

Next, the mean value E was calculated from the determined differences ΔE of each quadruplet and the average of the blank was subtracted from it. Based on this value and the determined slope m of the trend line, the galactose concentration G in the individual samples could be determined as follows (see formula 1):

$$G = m \cdot E = 10.97 \cdot E \quad (1)$$

The determined galactose concentrations were then related to the galactose concentration of 1 mmol/l, as this was the initial concentration added to the 3D-matrices and monolayers. Subtracting the resulting percentages from 100 % gave the final galactose elimination values (see table 1).

Table 1: Calculated data of the samples and standards

	Mean ΔE	Mean ΔE – Blank ΔE	Galactose concentra- tion [mmol/l]	Galactose concentra- tion [%]	Galactose elimination [%]
Blank	0.0075	(0)	0.08	(0)	(100)
Galactose 0.25 mmol/l	0.0151	0.0076	0.17	16.51	83.49
Galactose 0.5 mmol/l	0.0315	0.0241	0.35	34.56	65.44
Galactose 1 mmol/l	0.0887	0.0813	0.97	97	0
Monolayer No.1	0.0933	0.0859	1.02	102.35	-2.35
Monolayer No. 2	0.0552	0.0478	0.61	60.59	39.41
3D-matrix No. 1	0.1164	0.1089	1.28	127.64	-27.64
3D-matrix No. 2	0.1179	0.1105	1.29	129.36	-29.36

Figure 2A shows the graphs plotting the galactose elimination values. It can be seen that in the sample of the first monolayer there was a slight increase in the galactose content by 2.35 %, whereas in the second monolayer there was a significant decrease in the concentration by 39.41 %. In both samples containing the 3D-matrices, there was an increase in the galactose content, with the sample of the first having an increase of 27.64 %, while the second had one of 29.36 %.

In addition, the mean values were calculated from the galactose elimination values of the two monolayers and the 3D-matrice (see figure 2B). The monolayers had a mean galactose elimination of 20.88 %. In contrast, the two 3D-matrices showed an average increase in galactose of 28.5 %.

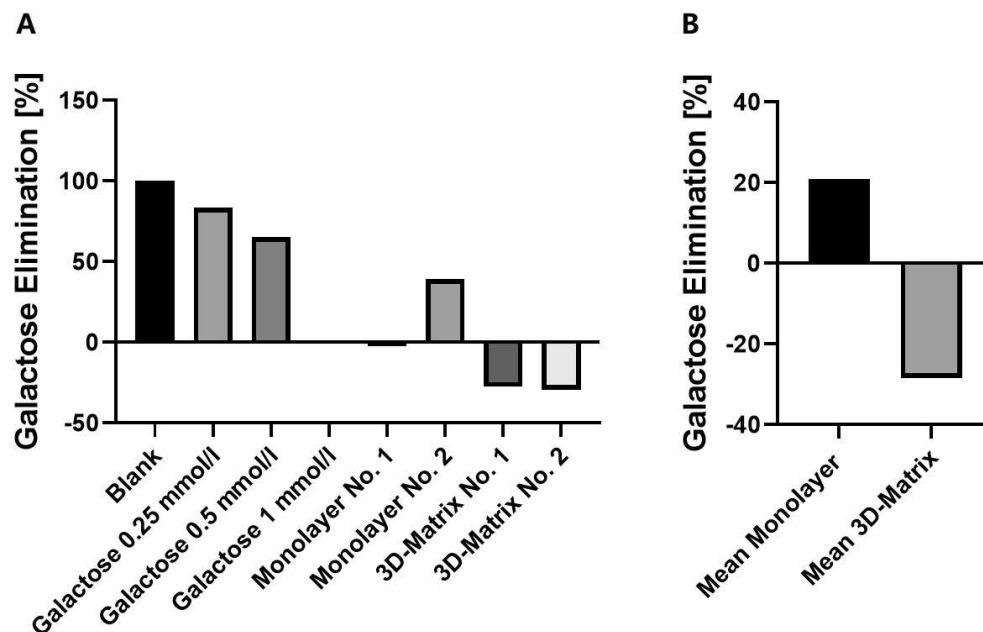


Figure 2: Galactose elimination of samples and standards (A) and average values of galactose elimination of the samples (B)

After completion of the laboratory work, the results of all experiments were entered in table 2.

Table 2: Data of the experiments: MTT cleavage, ASAT activity, urea production, and D-galactose elimination

	Monolayer		3D-Matrix/Tissue	
	Absolute	Relative to MTT	Absolute	Relative to MTT
MTT optical density	0.67	-	0.0915	-
ASAT activity [Units/l]	45.7	68.21	2.01	21.97
Urea (4 h) [mg/dl]	-0.08	-0.12	0.19	2.1
Galactose elimination (4 h) [%]	-20	-30	28	295

4 Discussion

It can be seen in table 2 that the values of the MTT cell viability test were low in both the monolayer and the 3D-matrix, indicating low cell viability. Compared to the monolayer, the viability in the 3D-matrix was lower by a factor of 7. Thus, it can be assumed that in this experiment the monolayer led to better cell viability and better growth of the cells.

The measurement of ASAT activity showed a similar result to the MTT test. Again, a higher cell viability can be assumed in the monolayer, since the ASAT activity in the monolayer was about 23 times higher than in the 3D-matrix.

In contrast, when analysing the urea production of the cells, a higher content of urea was found in the 3D-matrix compared to the monolayer. However, since the values were very similar, it is highly likely that this difference was the origin of statistical fluctuations. As both urea values were low, it is also assumed that there was hardly any metabolic activity in the monolayer and 3D-matrix and therefore cell viability can be presumed to be low.

The measurement of galactose elimination showed an uptake of galactose from the monolayers and a resulting elimination of about 20 %, whereas in the 3D-matrices there was a secretion of galactose, resulting in an increase in galactose content of about 28 %. Hence, this experiment also shows that seeding the cells into the monolayer resulted in higher metabolic activity and thus cell viability.

The values of the samples representing the ratio to the MTT assay showed completely different values, therefore it can be assumed that there were large differences in metabolic activity in both the monolayer and the 3D-matrix in each assay. The differences between the individual tests may be due to measurement errors during preparation, as well as statistical fluctuations, since only a few samples were prepared. In addition, the samples were supposed to be protected from direct sunlight during the tests, which was not always the case and thus influenced the outcomes.

Since three of the assays showed higher metabolic activity and thus higher cell viability of HepG2 cells in the monolayer, it can be assumed that the monolayer is more suitable for seeding and integrating cells compared to the 3D-matrix. This refutes the initial assumption that the 3D-matrix would be a better alternative for cell seeding and growth.

5 References

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