ARTICLE

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Hydrogel Embedding of Precision-Cut Liver Slices in a Microfluidic Device Improves Drug Metabolic Activity

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ABSTRACT: A microfluidic-based biochip made of poly-(dimethylsiloxane) was recently reported for the first time by us for the incubation of precision-cut liver slices (PCLS). In this system, PCLS are continuously exposed to flow, to keep the incubation environment stable over time. Slice behavior in the biochip was compared with that of slices incubated in well plates, and verified for 24 h. The goal of the present study was to extend this incubation time. The viability and metabolic activity of precision-cut rat liver slices cultured in our novel microflow system was examined for 72 h. Slices were incubated for 1, 24, 48, and 72 h, and tested for viability (enzyme leakage (lactate dehydrogenase)) and metabolic activity (7-hydroxycoumarin (phase II) and 7-ethoxycoumarin (phase I and II)). Results show that liver slices retained a higher viability in the biochip when embedded in a hydrogel (Matrigel) over 72 h. This embedding prevented the slices from attaching to the upper polycarbonate surface in the microchamber, which occurred during prolonged (>24 h) incubation in the absence of hydrogel. Phase II metabolism was completely retained in hydrogel-embedded slices when medium supplemented with dexamethasone, insulin, and calf serum was used. However, phase I metabolism was significantly decreased with respect to the initial values in gel-embedded slices with medium supplements. Slices were still able to produce phase I metabolites after 72 h, but at only about ~10% of the initial value. The same decrease in metabolic rate was observed in slices incubated in well plates, indicating that this decrease is due to the slices and medium rather than the incubation system. In conclusion, the biochip model was significantly improved by embedding slices in Matrigel and using proper medium supplements. This is important for in vitro testing of drug metabolism, drug–drug interactions, and (chronic) toxicity.

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KEYWORDS: perifusion culture; Matrigel; rat liver slices; microfluidics; metabolism

Introduction

Various in vitro models exist which are able to mimic in vivo liver metabolism (Brandon et al., 2003; Kramer and Tracy, 2008). Of these, primary hepatocytes and precision-cut liver slices (PCLS) exhibit the best correlation with the in vivo situation, with each model having its own particular advantages and disadvantages (Brandon et al., 2003). The main advantage of PCLS is that the tissue architecture remains intact even when cut into thin slices (Farkas and Tannenbaum, 2005). All cell types are present in their original matrix, making PCLS a good model to study multicellular processes. For example, the role of stellate cells in the development of liver fibrosis, (Van de Bovenkamp et al., 2008; Vickers et al., 2004) or the role of Kupffer cells in mediating hepatic toxicity can be investigated (Roberts et al., 2007). PCLS are usually incubated in well plates or in a dynamic organ culture (DOC) system (De Graaf et al., 2007). Although slices are generally incubated for a short period of time (<24 h), incubations of 72 h are possible in well plates or in a DOC system (Hashemi et al., 1999; Toutain et al., 1998; Vickers et al., 2004). Slices retained their viability over a 72 h period in both systems, although a rapid decrease in phase I metabolism was observed (De Graaf et al., 2007).

Recently, we developed a microfluidics-based incubation system incorporating rat PCLS with continuous perfusion (Van Midwoud et al., 2010a). Tissue slices were incubated in

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small microchambers made of poly(dimethylsiloxane) (PDMS), in which the incubation environment could be well controlled. The continuous flow of medium ensures that the incubation environment is kept stable over time, with a continuous influx of nutrients and removal of waste products. In this way, no depletion of nutrients or accumulation of waste products occurs, which is the case in well plates and DOC systems. Moreover, microfluidic technologies enable very precise control of medium flow and composition. The advantages of this microfluidic device are diverse, including (i) the ability to study the effect of flow on liver metabolism, (ii) easier on-line analysis to enable the measurement of unstable metabolites, (Van Midwoud et al., 2011), and (iii) the chambers containing different organ slices can be coupled and perfused sequentially to study interorgan interactions (Van Midwoud et al., 2010b). Previously, we showed that rat liver slices remain viable and metabolically functional for at least 24 h (Van Midwoud et al., 2010a, 2011). However, no studies have been performed to date with incubations longer than 24 h, though this would be beneficial for studying the effect of prolonged exposure to drugs on liver metabolism, toxicity, and fibrosis.

The aim of this study was to monitor and improve the maintenance of viability and metabolic activity of rat PCLS incubated in our microfluidic device over an extended period of 72 h. The viability was assessed by measuring the leakage of the enzyme, lactate dehydrogenase (LDH). LDH is mainly present in the hepatocytes, and leaks out of cells which have been injured (Naik et al., 2004). Phase I metabolism was determined by incubating PCLS with 7-ethoxycoumarin (7-EC). This substrate is converted mainly by CYP1A1 and CYP2B of the cytochrome P450 (CYP) enzyme complex into 7-hydroxycoumarin (7-HC) (Kuhn et al., 1998). After formation of 7-HC, this compound is further converted into the phase II products 7-hydroxycoumarin glucuronide (7-HC-G) by UDP-glucuronosyltransferase, and 7-hydroxycoumarin sulfate (7-HC-S) by sulfotransferase (Van Midwoud et al., 2010a). This phase II metabolic activity was also assessed directly by incubating slices with 7-HC. The stability of phase I and II metabolite formation was assessed daily, and compared to well plates as control. As mentioned above, a decrease in phase I metabolism was observed in conventional systems over time, with phase II metabolism proving to be more stable than phase I (De Graaf et al., 2007).

Pilot experiments demonstrated that the viability of PCLS decreased with time when incubated in our microfluidic device, and that they lose their metabolic activity as well. It was observed that PCLS were attached to the upper polycarbonate membrane forming the ceiling of the microchamber after incubations longer than 24 h. As we hypothesized that this might be the cause of the decreased viability, it was decided to embed the liver slices in a hydrogel to prevent attachment to the membrane to improve the viability and stability of metabolite formation. A hydrogel is colloidal gel (mixture with properties between those of a solution and fine suspension) in which water is the dispersion medium. Thus slices embedded in hydrogel are still sufficiently wetted by medium. The hydrogel used in this study was Matrigel, which is normally used as culture overlay or to create three-dimensional hepatocyte cultures (LeCluyse et al., 1996). It is a basement membrane consisting mainly of laminin, collagen IV, heparin sulfate proteoglycan, and entactin, (Arnold et al., 2001) and is therefore very similar to the native extracellular matrix in the rat liver (LeCluyse et al., 1996). The use of Matrigel for hepatocyte cultures and hepatoma cells (HepG2/C3A) has been shown to substantially improve morphology and functionality (Dixit et al., 1992; Page et al., 2007; Schuetz et al., 1990; Sung and Shuler, 2009). Various supplements were also added to the medium to further improve the metabolic stability of the PCLS during culturing, as mentioned previously (De Graaf et al., 2007).

Materials and Methods

Chemicals

William's medium E (WME) supplemented with Glutamax I was purchased from Gibco (Paisley, UK). D-glucose, 7-EC, 7-HC, 7-HC-G, 7-HC-S, stabilized antibiotic antimycotic solution (100x), and insulin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Matrigel^{TM} was obtained from BD Biosciences (Bedford, MA, USA). Dexamethasone was purchased from Genfarma BV (Maarssen, The Netherlands). Hyclone heat-inactivated bovine calf serum was supplied by Thermo Scientific (Logan, UT, USA).

Preparation of Precision-Cut Liver Slices

Male Wistar rats (300–350 g) obtained from Harlan (Horst, The Netherlands) were used for all experiments. Liver slices were prepared as described previously (De Graaf et al., 2010; Van Midwoud et al., 2010a). Briefly, the liver was excised after anesthetizing the rat with isoflurane/oxygen. Subsequently, cylindrical cores of liver tissues with a diameter of 4 mm were made by utilizing a hollow drill bit. Cores were placed in a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) to produce reproducible PCLS with a thickness of $\sim$250 μm and a wet weight of approximately 5 mg.

Incubation of Precision-Cut Liver Slices

Slices were pre-incubated for 1 h in individual wells of a 12-well plate, each of which contained 1.3 mL WME with Glutamax I. Medium was supplemented with 25 mM D-glucose, 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (solution has both antibiotic and antimycotic properties). This pre-incubation removes cell debris and enzymes originating from damaged cells at the cutting surfaces. The damaged cells result during

preparation of the tissue slices, and are only present in the outer layers of the slice. The energy status of the cells also recovers during pre-incubation, as the intracellular adenosine triphosphate (ATP) is restored to the value of fresh tissue after being reduced during the slicing procedure at 4°C (De Kanter et al., 2002; Obatomi et al., 1998).

After pre-incubation, slices were incubated in two different incubation systems: (i) well plates and (ii) the microfluidic biochip. When incubated in well plates, the slices were placed individually in 1.3 mL medium, as described in detail elsewhere (De Graaf et al., 2010). The well plates were placed in humidified plastic containers and shaken back and forward 90 times per minute in an incubator set at 37°C. The containers were continuously supplied with humidified carbogen gas (95% O_2/5% CO_2). Unless otherwise stated, the medium was supplemented with D-glucose, penicillin, streptomycin, and amphotericin B at the same concentrations as used for the pre-incubation. In well plates, the medium was refreshed every 24 h by transferring the slices to a new well plate containing fresh medium.

Slices incubated in the biochip were placed in small microchambers made of PDMS. An extensive description of the fabrication process of the biochip was published recently (Van Midwoud et al., 2010a). In the PDMS device, each slice was incubated in a 25 μL microchamber (2 mm × Ø4 mm) in which polycarbonate membranes (10 μm thick, Ø8 μm pore size, Millipore, Bedford, MA, USA) formed the top and bottom surfaces of the chamber. Integration of these membranes into the device ensured a well-controlled, even flow of medium from the bottom of the chamber around the tissue slices and out the top. PDMS membranes were integrated above and below these polycarbonate membranes to act as "breathing" membranes. Dissolved CO_2 and O_2 concentrations in the medium could be regulated by exchange of these gases through these membranes when the chip was placed in an environment with a controlled atmosphere (humidified carbogen supply consisting of 95% O_2 and 5% CO_2). Medium was introduced by a syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA) using syringes filled with WME supplemented with glucose, penicillin, streptomycin, and amphotericin B, which had been pre-equilibrated with carbogen gas at 37°C. The syringes were connected to the biochip with PEEK tubing (DaVinci Europe, Rotterdam, The Netherlands). PTFE tubing (Polyfluor Plastics, Oosterhout, The Netherlands) was connected to the outlet of the biochip to collect fractions for further studies. The flow rate was set at 10 μL/min for all experiments.

Slices were incubated either directly in medium or embedded in Matrigel. The procedure to embed the slices in Matrigel is shown in Figure 1. The Matrigel was diluted 1:3 with WME to obtain a porous structure which could be perfused with medium, since concentrated Matrigel results in a dense polymer through which medium does not easily pass. Ten microliters of diluted, ice-cold Matrigel (4°C) was introduced into a microchamber. Subsequently, the slice was placed on top of this first layer of Matrigel and covered with another 10 μL of ice-cold Matrigel. The whole device was then closed by assembling the gel-filled bottom half with the top half of the chamber. The insertion of liver slices into the PDMS device occurred in an incubator set at 37°C. Hence, the polymer immediately polymerized (Kibbey, 1994). The flow (10 μL/min) was started approximately 10 min after insertion of the liver slices.

Viability Testing

The viability of liver slices incubated for 72 h in both well plates and the biochip was assessed by measuring the leakage of the enzyme, LDH, over time. Analysis was performed on a Roche/Hitachi Modular System (Roche, Mannheim, Germany), according to a routine laboratory procedure performed at the University Medical Center of Groningen at the Clinical Chemistry Laboratory. The analysis is an enzymatic reaction with photometric detection. PCLS were pre-incubated for 1 h; subsequently, fractions of medium were continuously collected over an initial 3-h period (1.8 mL), from 3 to 24 h (12.6 mL), from 24 to 48 h (14.4 mL), and from 48 to 72 h (14.4 mL). Preliminary experiments demonstrated a stable LDH activity when samples were kept at 37°C, with no enzyme degradation observed. Therefore, samples collected for 24 h were kept in the incubator and after collection stored at −80°C until analysis. To assess the total LDH content in fresh liver slices, three liver slices were collected after pre-incubation. These were homogenized in 1 mL of medium, which was then centrifuged for 3 min at 16,000 *g*, and 4°C. The supernatant was then analyzed to determine the initial LDH content in fresh liver slices. The supernatant was stored at −80°C until analysis. Each experiment was performed in triplicate using slices from 3 different rats.

Metabolism Studies

The metabolic activity of liver slices was assessed by addition of substrate after 1 h pre-incubation (0 h), and after 24, 48, and 72 h of incubation, by measuring the metabolite formation in medium containing 100 μM 7-EC or 100 μM 7-HC. Slices were exposed to these substrates for 3 h starting at the indicated time points. Pilot experiments showed that liver slices could be exposed repeatedly to 7-HC without affecting the metabolic rate. In well-plate experiments, liver slices were transferred directly after the pre-incubation to well plates containing fresh medium with 100 μM 7-HC and exposed to 7-HC for 3 h. Subsequently, the slices were transferred to a new well plate containing fresh medium and no substrate for further incubation for 21 h. This procedure was repeated and the slices were exposed to 7-HC four times during the 75-h time period (72 + 3 h incubation with 7-HC). In the biochip experiment, slices were transferred to the biochip after 1 h pre-incubation in a well plate and perifused with medium

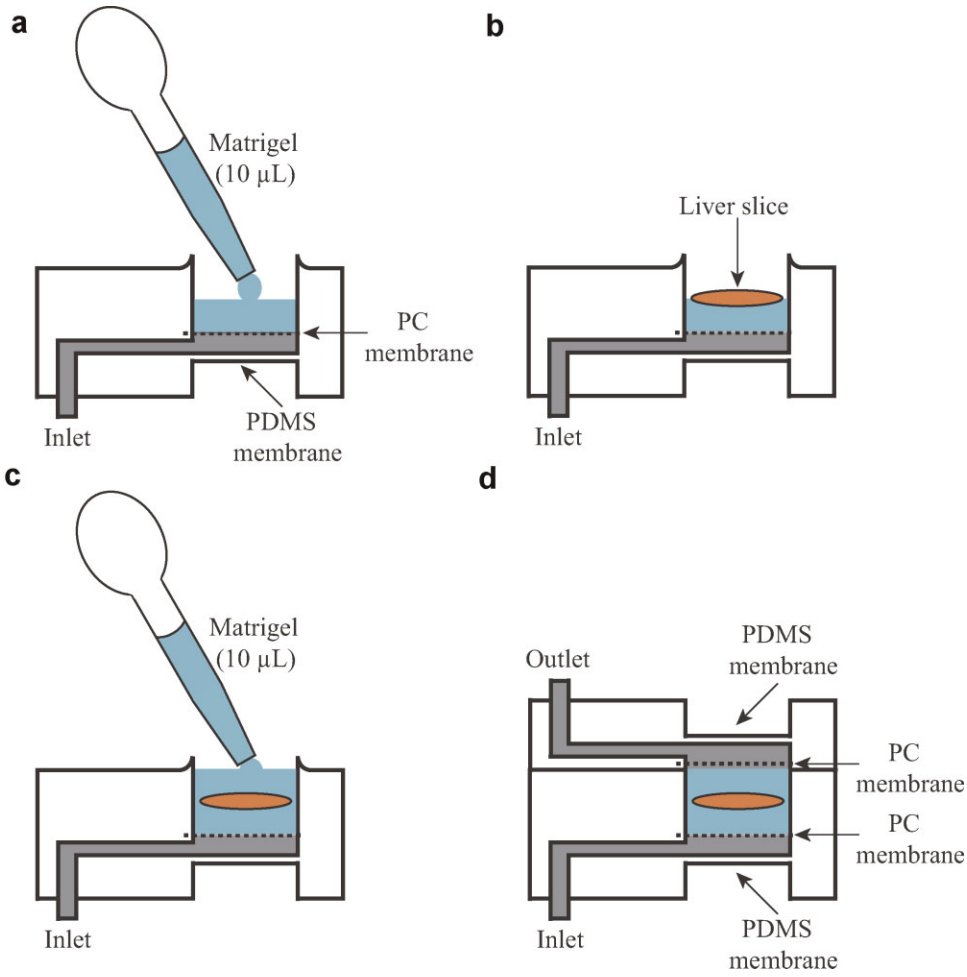

Figure 1. Schematic diagram of the procedure for embedding liver slices in Matrigel in the microfluidic device. **a**: 10 μL Matrigel (diluted with medium in a 1:3 ratio) is added to an open chamber. Subsequently, (**b**) the slice is placed on top of the Matrigel, and is covered with (**c**) another 10 μL Matrigel. Finally, (**d**) the device is assembled and ready for use. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

containing 100 μM 7-HC. The slices were perifused for 30 min to correct for delay times in the system, after which the medium was collected as a fraction of 1.5 mL (2.5 h) for metabolite analysis. After 3 h of exposure to 7-HC, the syringes filled with 100 μM 7-HC in medium were replaced with syringes filled with medium only and perifusion was continued. After 21 h, a switch back to medium containing 7-HC was made, and slices were perifused for a second period of 3 h, with a 1.5-mL fraction collected for analysis as described above. This was repeated until the slices had been exposed four times in total to 7-HC.

When 7-EC was the selected substrate, each slice was only exposed once over a period of 3 h, after a pre-incubation in medium without 7-EC of 1, 24, 48, or 72 h, respectively. This is because earlier experiments showed that the metabolite formation decreased rapidly upon repeated exposure to 100 μM 7-EC, indicating that this concentration is most likely toxic for the cells.

Medium fractions collected after all 7-EC and 7-HC exposure experiments were stored at −20°C until analysis. Experiments were performed with four rats, with three to four slices per treatment per rat.

The amounts of metabolite formed from 7-EC and 7-HC were determined using an HPLC with UV detection, as described earlier, using 7-EC, 7-HC, 7-HC-G, and 7-HC-S as standards (Van Midwoud et al., 2010a).

The metabolic rate was expressed per milligram protein. Slices pre-incubated for 1 h were used to determine the protein amount. The Bio-Rad protein assay (Bio-Rad, Munich, Germany) was employed according to the manufacturer's protocol using bovine serum albumin as standard.

Statistical Evaluation

The results were analyzed for significant differences using the Student *t*-test, with $P < 0.05$ considered as significantly different.

Results and Discussion

In our previous studies, slices were incubated in the biochip for a maximum of 24 h (Van Midwoud et al., 2010a, 2011). To be able to measure the effect of prolonged exposure of xenobiotics on liver metabolism and toxicity, this incubation period should be increased to at least a few days, and preferably to weeks. However, pilot experiments showed that increasing the exposure time resulted in an even more decreased metabolic activity compared to PCLS incubated in the well plates. We hypothesized that this might be due to attachment of the slices to the polycarbonate ceiling of the microchamber which was observed at exposure times >24 h, as mentioned above. To avoid this damage, it was decided to embed slices in Matrigel, a matrix with hydrogel properties. Indeed, the embedding of slices in Matrigel prevented the slices from coming in contact with the polycarbonate surface.

Viability Testing

The leakage of the enzyme, LDH, was determined in slices incubated in well plates, the biochip without gel embedding, and in the biochip with slices embedded in Matrigel for 72 h. Pilot experiments showed that Matrigel did not adsorb the enzyme, LDH. LDH was incubated with 40 μL of Matrigel for 24 h, at which time LDH recovery was still 100% when compared to enzyme activity at 0 h. This experiment also indicated that the enzyme LDH was stable at 37°C for at least 24 h.

The cumulative leakage is given in Figure 2. The results indicated that liver slices retained a higher viability in the biochip with Matrigel over 72 h. Around 90% of the cells remained intact after the incubation period. The cumulative leakage of LDH in liver slices which were not embedded in gel reached a maximum value of 15% at 48 h, with no further increase observed afterwards. This was surprising, given the fact that after the experiment slices were found attached to the microchamber ceiling, and it was impossible to remove them intact from the device. Though earlier experiments indicated that the metabolic activity decreased strongly, the LDH leakage was relatively limited for non-gel-embedded slices in the chip. This indicated that the cell membranes of the hepatocytes remained intact, despite attachment of the slices to the upper polycarbonate surface of the microchamber. In the case of gel-embedded slices, there was no problem removing slices after 72-h incubation in the biochip, although they were more fragile compared to fresh tissue slices. Slices could also be removed from the wells without damaging them, although they were also more fragile than fresh tissue slices.

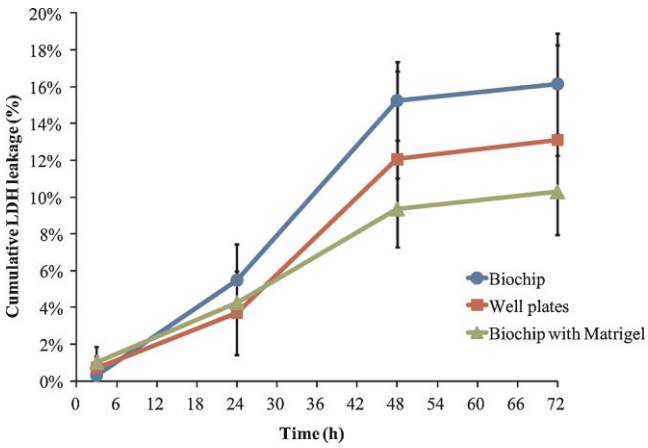

Figure 2. Cumulative leakage of the enzyme LDH as a percentage of the total LDH present in a typical slice, measured over a period of 72 h. The results presented are the mean ± standard error of the mean (SEM) of three rats, with three slices per rat per experiment. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

Phase II Metabolism with and without Matrigel-Embedding

The metabolism studies were performed with a 7-HC concentration below the concentration required to achieve a maximum metabolic rate (Van Midwoud et al., 2010b), in order to limit the consumption of co-factors during the course of the experiments. Liver slices were exposed daily to 100 μM 7-HC in the biochip with and without gel-embedding of slices (Fig. 3). Both 7-HC-G and 7-HC-S were formed. As expected, no significant differences were obtained for fresh slices with or without gel-embedding on the first day. The metabolic rate was around 190 pmol/min/mg protein for 7-HC-G and 25 pmol/min/mg protein for 7-HC-S, which was similar to data obtained previously (Van Midwoud et al., 2011). After 24 h, similar metabolic rates were again observed. This indicated very stable phase II metabolism for 24 h both with and without gel embedding, as observed previously in medium alone by us in the biochip (Van Midwoud et al., 2011), and in Erlenmeyer flasks by others (Pissowotzki et al., 2003). However, at 48 h, the metabolite formation in slices without Matrigel significantly decreased to a value which was 7% of the initial value for 7-HC-G and 14% for 7-HC-S. In contrast, gel-embedded slices retained a much higher metabolic rate, though a decrease in phase II metabolism was observed in this case as well. The 7-HC-G formation rate decreased to 32% of the initial value, while that of 7-HC-S dropped to 52% of its initial value after 48 h of incubation. This was also the case after 72 h of incubation, with a significantly higher amount of 7-HC-G being formed in gel-embedded slices compared to slices without gel. The formation rates of 7-HC-G decreased to 2% and 25% of the initial value for slices without gel and with gel, respectively. The 7-HC-S formation rates decreased to 8% and 31%, respectively, for the gel and no-gel situations.

In summary, even though metabolic rate decreased over time, this decrease was less in liver slices embedded in Matrigel compared to slices without gel. Slices were not

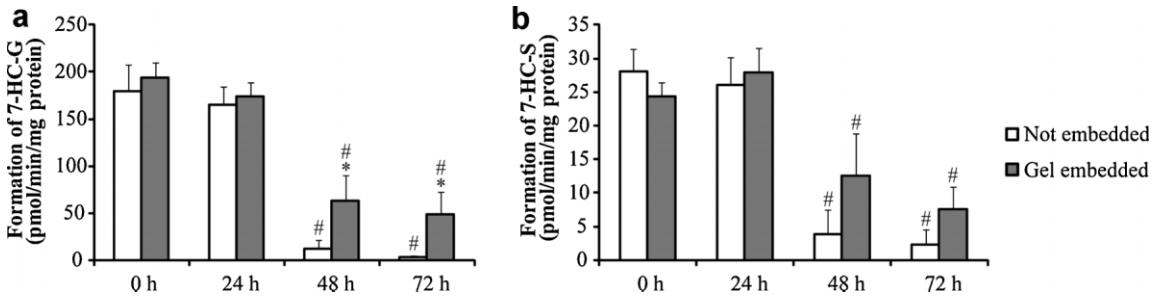

Figure 3. Liver metabolism of 100 μM 7-HC over a period of 72 h with unsupplemented medium, measured once a day in the biochip for slices embedded in Matrigel (gray bars) and without Matrigel (white bars). Slices were exposed for 3 h to 7-HC starting at each time point indicated along the horizontal axis. **a**: Formation rates for 7-HC-G. **b**: Formation rates for 7-HC-S. Results are the mean ± SEM of four rats with four slices per rat per experiment. Significant differences with respect to 0 h are indicated with # $P < 0.05$. Significant differences between slices embedded in Matrigel and without Matrigel are indicated with * $P < 0.05$.

attached to the microchamber ceiling after 72 h of incubation, and were able to produce metabolites. Embedding PCLS in Matrigel in the microfluidic device thus improves viability and metabolic functionality.

Phase II Metabolism with Medium Supplements

Liver slices incubated in well plates in unsupplemented medium exhibited a decrease in phase II metabolic rates during incubation which resembled the behavior of Matrigel-embedded slices in the biochip (data not shown). This indicated that the decrease was independent of the incubation system, provided slices did not adhere to the ceiling of the microchamber. Various medium supplements have been shown to improve the maintenance of the metabolic rate for longer periods of time (Agius et al., 1986; Li et al., 2007). It was therefore decided to supplement the medium with 5% v/v heat-inactivated calf serum, as has commonly been done by others when liver slices were incubated longer than 48 h (De Graaf et al., 2007). Insulin (1 μM) was also added to improve glucose uptake (Buettner et al., 2005) and 0.1 μM dexamethasone as glucocorticosteroid to reduce inflammatory reactions (Liu et al., 1996; Turncliff et al., 2004). Phase II metabolism was again assessed by incubating liver slices with 7-HC, comparing slices incubated in well plates and gel-embedded in the biochip.

As can be seen in Figure 4, very stable phase II metabolism was observed over the 72 h measured using supplemented medium. There was no significant difference between fresh slices and slices incubated for 72 h both in the biochip and in the well plates. These results were in line with those of the LDH leakage (Fig. 2), which indicated that tissue slices remained intact for at least 72 h. On the other hand, when liver slices were incubated in the biochip with supplemented medium but without Matrigel, a decrease in metabolic rate was observed (data not shown). This can be ascribed to attachment of the liver slices to the upper filter, as mentioned above. Hashemi et al. (1999) also measured phase II metabolism in well plates for 72 h. In contrast to the results presented here, they reported a decrease in UDP-glucuronosyltransferase (UGT) activity over time, using medium supplemented with insulin, serum, and a glucocorticosteroid (hydrocortisone 21-hemisuccinate). A decrease in sulfotransferase activity was also reported, albeit a decrease which was slower over time compared to UGT. The

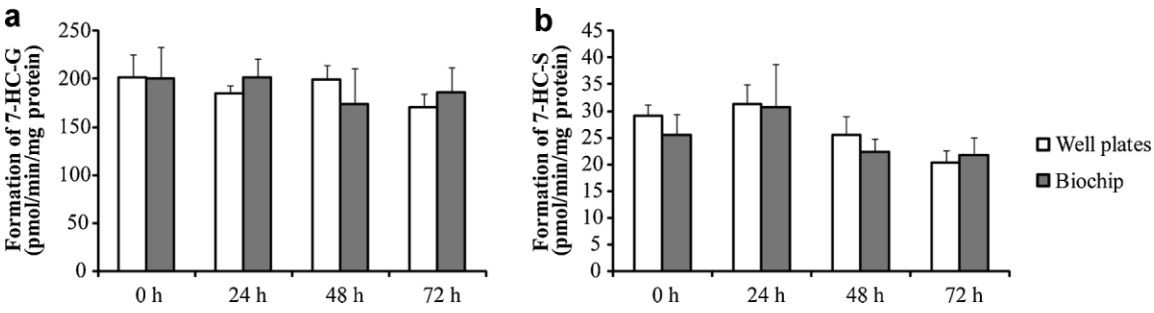

Figure 4. Liver metabolism of 100 μM 7-HC over a period of 72 h with supplemented medium measured every day for slices in well plates (white bars) and Matrigel-embedded slices in the biochip (gray bars). **a**: Formation rates for 7-HC-.G. **b**: Formation rates for 7-HC-S. Results are mean ± SEM of four rats with three slices per rat per experiment. No significant differences were observed between results obtained in well plates and results in the biochip, or between fresh slices and those incubated for 24, 48, and 72 h.

difference in observed metabolic rates between this study and that of Hashemi et al. (1999) might be explained by the low concentration of oxygen used in the latter case. They incubated the slices in an environment consisting of 5% CO_2 in air, while it is known that slices need an oxygen concentration >70% to culture them longer than 24 h (De Graaf et al., 2007; Fisher et al., 2001; Toutain et al., 1998).

Khong et al. (2007), on the other hand, demonstrated an increase in metabolic rate for phase II metabolism with their intra-tissue perfusion system. The slices incubated in a static environment showed a decrease in metabolic rate, while perfusion through the tissue resulted in an increase. We did not find a difference in phase II metabolic rates between flow (biochip) and no flow (well plates) conditions. On the contrary, a stable formation of metabolites over 72 h was found in both systems. It should be noted that the well plates were not cultured under static conditions in this study, since the plates were moved back and forward 90 times per minute. In neither situation, however, was upregulation of UGT activity found, as reported by Khong et al. (2007).

Phase I Metabolism with Medium Supplements

The phase I metabolism in gel-embedded slices in the biochip and in slices incubated in well plates was assessed by determining the metabolism of 7-EC. All three metabolites 7-HC, 7-HC-G, and 7-HC-S, were formed. The total phase I metabolism is the sum of 7-HC, 7-HC-G, and 7-HC-S produced by the slices. However, the concentration of 7-HC was low and hardly detectable after 24 h of incubation, and negligible compared to 7-HC-S and 7-HC-G, suggesting that phase I metabolism is the rate-limiting step in 7-EC metabolism. This is the reason only the formation rates of 7-HC-G and 7-HC-S are given in Figure 5. Unfortunately, the metabolism decreased upon culturing. A significant decrease in phase I metabolism was observed after 24 h of incubation in both well plates and biochip. Only 60%–70% of cell activity was retained at this point, which further decreased to 25%–30% after 48 h of incubation in both systems. After 72 h a significant difference between well plates and biochip was observed, with slices incubated in the well plates retaining 15% of their cell activity while the biochip exhibited only 5% of the initial value. Overall, the decrease in formation of 7-HC-S during culturing occurred at a slower rate than that of 7-HC-G. This is probably due to the lower concentrations of 7-HC formed over time, as the ratio of 7-HC-G to 7-HC-S formed decreases as 7-HC concentrations decrease (Conway et al., 1982).

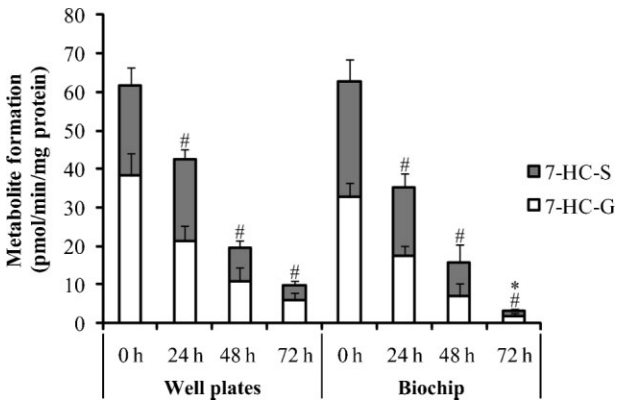

Figure 5. Liver metabolism of 100 μM 7-EC over a period of 72 h with supplemented medium, measured once a day in well plates and in the biochip. White bars represent the formation of 7-HC-G, and gray bars the formation of 7-HC-S. Slices were exposed only once to 7-EC for a 3-h period, starting at the time point indicated along the horizontal axis, due to the toxicity of 7-EC for the slices. Results are mean ± SEM of four rats with three slices per rat per experiment. Significant differences of total phase I metabolism with respect to 0 h are indicated with # $P < 0.05$. Significant differences of total phase I metabolism between well plates and biochip are indicated with * $P < 0.05$.

The decrease of phase I metabolism in liver slices over time has been previously reported (De Graaf et al., 2007). In isolated hepatocytes, CYP-mediated metabolism also declines during culturing (Rogiers et al., 1990; Wortelboer et al., 1990). Hashemi et al. (2000) demonstrated a 90% reduction in the phase I metabolism of ethoxyresorufin in rat liver slices within 24 h, with hardly any metabolites formed after 72 h. In our study, a decrease of 30%–40% in metabolic activity was observed after 24 h, and slices were still able to produce metabolites after 72 h (5%–15%). The differences in results are probably due to differing experimental conditions in the two studies. The rats used by Hashemi et al. (2000) were treated with inducing agents, and the oxygen concentration during incubation was relatively low, as mentioned above, which might explain the discrepancy in the two sets of results. Another important issue is that the loss in CYP activity does not affect every isoenzyme equally. Our results are more in line with data shown by VandenBranden et al. (1998) who demonstrated a decrease in coumarin 7-hydroxylase activity to about 10%–40% of the initial value after 24 h. However, VandenBranden et al. (1998) used human liver slices instead of rat liver slices. Apparently, our incubation medium lacks components that maintain the expression or activity of CYP1A1 and 2B in the liver slices at their physiological level.

Conclusion

In this study it was demonstrated that the biochip model for liver-slice incubation was significantly improved by embedding the liver slices in Matrigel, allowing liver slices to remain viable up to 72 h. The Matrigel embedding prevents the attachment of PCLS to the upper polycarbonate membrane of the biochip's microchamber during incubations longer than 24 h. Results show that liver slices retained a higher viability in the biochip with Matrigel over 72 h, and that ~90% of cells were intact after 72 h. With the proper medium supplements, the metabolic phase II activity could also be retained in PCLS for over 72 h. No significant decrease in the metabolic rates for 7-HC-G and 7-HC-S production was observed over this period of time. However, the phase I metabolic rate did fall within 24 h to a value

which was 30%–40% less than the initial rate, which though considerable, was less than that found by Hashemi et al. (2000) The same results for phase I and phase II metabolism were obtained in well plates, which is the benchmark for these experiments. It should also be noted that the flow conditions under which incubations were carried out did not influence the observed metabolic behavior of slices, contrary to what was reported by Khong et al. (2007) We thus hypothesize that the lowered metabolic rates measured were not linked to the incubation system, but rather due to the combination of PCLS and incubation media. More research has to be done to better maintain metabolic phase I activity using supplemented medium. Key to this would be the selection of medium supplements which influence the transcription factors that might be responsible for the decline in CYP isoforms, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and farnesoid x receptor (FXR). However, the current system can be used to assess phase II metabolism. The induction of phase I and phase II metabolism can also be studied in biochips for 72 h.

This is the first example of embedding liver slices in Matrigel. This is also the first example of incubating rat liver slices in a microfluidic device for a period longer than 24 h. We do believe that this perifusion system could be a good addition to the conventional well-plate system. As mentioned above, this perfusion system can also address questions related to the effect of flow on metabolism. The flow rate can be varied between 4 and 50 μL/min, while maintaining a high metabolic activity during the first 3 h (Van Midwoud et al., 2010b). Unstable metabolites which are difficult to detect in well plates can be analyzed by coupling the microfluidic culture systems directly to an HPLC (Van Midwoud et al., 2011). Finally, interorgan interactions can be studied by coupling microchambers containing different organ slices and perfusing them sequentially, as demonstrated by the interplay of liver and intestine in the regulation of bile acid synthesis published by our labs recently (Van Midwoud et al., 2010b). We therefore believe that this improved system has the potential to significantly contribute to drug metabolism and toxicology studies of novel chemical entities.

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