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Perifusion of Human Precision-Cut Liver Slices in a Microfluidic Device for Metabolism and Toxicology Studies

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Abstract

Early information on metabolism and toxicity properties of new drug candidates is crucial for selection of the proper candidate for further testing. *In vitro* tests are used to gain information about new chemical entities (NCE) with minimal use of animals. Due to species differences in metabolism and toxicity, *in vitro* systems are needed which permit the incorporation of human material. Knowledge of how *in vitro* animal data extrapolates to *in vivo* data allows, under some circumstances, the extrapolation of *in vitro* data gained using human material back to the human *in vivo* situation. We and others showed that rat and human precision-cut liver slices (PCLS) can be used to perform these studies. These slices are commonly incubated in well plates or dynamic organ culture systems. Recently, we reported a novel microfluidic-based (biochip) perfusion system for rat liver slices, also with metabolism and toxicity studies in mind. This report describes the verification of the microfluidic system for studies involving human liver slices. Slice viability has been tested by morphological evaluation and measurement of the leakage of liver-specific enzymes. All experiments were verified by comparison with well plates, which is an excellent benchmark for these experiments. The metabolism of four different substrates, lidocaine, 7-hydroxycoumarin (7-HC), 7-ethoxycoumarin (7-EC) and testosterone, was also tested in both systems. Finally, the ability to form phase I and II metabolites after 24 hour of incubation was assessed by measuring the metabolism of 7-EC and 7-HC. For all the experiments performed, the results in both systems were not significantly different. Human liver slices were as viable and metabolically active in the biochip as in the well plates. The biochip system can thus be used in addition to the well plate system for human liver tissue incubation, thereby enabling to questions related to the effect of flow on metabolism to be addressed. We believe this system will yield more information about new drug candidates in an early stage of development compared to well plates alone.
Introduction

Only one in nine newly developed drugs entering clinical trial will be approved by European and/or the US regulatory authorities. Many of the drugs that fail are withdrawn due to unforeseen toxicity or lack of efficacy. This means that preclinical tests, which include both in vitro and in vivo methods, are not able to adequately predict how new drugs will behave in the human body. There is thus a continuing demand to develop in vitro systems which are able to better mimic the in vivo environment. Preclinical tests normally use animals (in vivo tests) and human and animal tissue or cells (in vitro tests). By extrapolating results obtained in vitro to those obtained in vivo for the tested animals, a prediction of the human in vivo behavior of a drug can be made based on the in vitro data of human material. However, as interspecies differences are often evident with respect to the behavior of animal and human cells or tissue under culture conditions, each in vitro system developed with animal material should be verified for its applicability with human material. If an in vitro system based on human material with excellent correlation to the in vivo situation were to exist, animal experiments could be reduced and replaced. To develop an in vitro system with the ability to correlate accurately to the in vivo situation, attention should be paid to the selection of a biological system which represents the organ as closely as possible.

While there are various models to determine ADME-Tox profiles, only a few exhibit a good correlation to the in vivo situation, among them primary hepatocytes and precision-cut liver slices (PCLS). The main advantage of PCLS compared to primary hepatocytes is that PCLS contain all liver cell types in their native environment. This model is thus also very useful for toxicity studies which are mediated via multicellular processes. Normally PCLS are incubated in well plates or dynamic roller culture systems. In both systems, the slices are cultured in an excess of medium which is refreshed only once every 24 h. This leads to an accumulation of waste products and depletion of medium components. By using microfluidic technologies to maintain a constant flow to perifuse slices in microchambers, the environment around the slices remains unchanged. The presence of a constant flow over the tissue has also been suggested to be more consistent with the in vivo situation. We therefore recently developed a new in vitro system based on microfluidic technologies for the incorporation of precision-cut tissue slices. The polydimethylsiloxane (PDMS) device contains a microchamber of 25 µL into which liver slices and intestinal slices can be incorporated. The chamber is then continuously perfused with fresh medium to ensure the stability of the slice environment during incubations. The system has been verified for rat liver and intestinal slices with respect to the optimized conventional well plate system, in which slices retain their in vivo metabolic rate at least for 8 h (intestine) and 24 h (liver). In the case of intestinal slices, only short incubations of 3 h have been performed. However, liver slices retain their viability.
for at least 72 h in the microfluidic device (see Chapter 5).

In the present study, the incorporation of human material in the microfluidic biochip is demonstrated. Though metabolism studies have been successfully performed with rat liver slices, it is not a given that human liver slices will also be metabolically active in the microfluidic device, due to the interspecies differences that exist. This study thus set out to verify that human tissue could also be tested in the microfluidic system, using well-plate experiments as controls, as these serve as an excellent benchmark. To assess the viability of the slices, slice morphology was assessed after incubation, and the leakage of liver-specific enzymes was measured. Since the main purpose of this system is to perform metabolism studies, the ability of human material to convert substrates into metabolites was also measured. 7-Ethoxycoumarin, 7-hydroxycoumarin, testosterone, and lidocaine were chosen as substrates, since they are involved in various phase I (hydroxylation, oxidation, N-deethylation, and O-deethylation) and phase II (glucuronidation and sulfation) metabolic routes. 7-Hydroxycoumarin (7-HC) is a phase I metabolite of 7-ethoxycoumarin (7-EC), which is further converted into 7-hydroxycoumarin glucuronide (7-HC-G) and 7-hydroxycoumarin sulfate (7-HC-S), both phase II products. Lidocaine is converted by the liver mainly into monoethylglycinexylidide (MEGX). Finally, testosterone (TT) is converted into several hydroxytestosterone metabolites and into androstenedione (TT-A), which is the major metabolite of TT. In this study, only the metabolites 6β-hydroxytestosterone (6β-OH), 2β-hydroxytestosterone (2β-OH), and TT-A were measured.

Materials and Methods

Human liver tissue

Pieces of human liver tissue were obtained from redundant donor tissue as surgical waste remaining after split-liver transplantation. The pieces of human liver were perfused with and stored in ice-cold UW solution (liver preservation solution). The research protocols were approved by the Medical Ethical Committee of the University Medical Center in Groningen, The Netherlands.

Preparation of precision-cut liver slices

The preparation of precision-cut tissue slices followed exactly the same procedure as described previously for rat liver tissue. In short, a hollow drill bit was utilized to obtain Ø4 mm cylindrical samples of liver tissue (cores). These cores were placed in a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold, oxygenated Krebs-Henseleit buffer. After slicing, the slices were stored in ice-cold UW solution until incubation. Slices were approximately 4 mm in diameter and 250 µm thick, and had a wet weight of about 5.0 mg.
Incubation

Tissue slices were pre-incubated individually in wells of a 12-well plate (Greiner Bio-One GmbH, Frickenhausen, Austria) containing William’s medium E with Glutamax-I (Gibco, Paisley, UK) without xenobiotics. For all described experiments, William’s Medium E was supplemented with 25 mM D-glucose (Sigma-Aldrich, St. Louis, MO, USA) and 50 µg/mL gentamicin (Gibco). Slices were pre-incubated for 1 hour to remove cell debris and enzymes present in damaged cells at the edges and surfaces of the slice, due to the slicing procedure. Thereafter, slices were removed from the well plate and introduced either into another well plate or into the microfluidic biochip for incubation.

For the well plates, the slices were individually placed in wells of a 12-well plate containing fresh 1.3 mL medium. The well plates were placed in humidified plastic containers with continuous exchange of carbogen (95% oxygen / 5% carbon dioxide) in a 37°C incubator. The plastic containers were in turn placed on a shaker with a speed of 90 cycles per minute. In the biochip, slices were placed individually into microchambers (25 µL) which were continuously perfused with medium using syringe pumps (10 µL/min, New Era Pump Systems Inc., Farmingdale, NY, USA). The fabrication process and verification of the biochip for metabolism studies with rat liver slices have been elaborated elsewhere. Two porous polycarbonate membranes formed the floor and ceiling of the PDMS microchamber to create a well-

Figure 1. A comparison of two incubation systems, well plates and the microfluidic biochip, for tissue slice incubations to perform metabolism and toxicity studies.
characterized medium flow around the tissue slice. Two PDMS membranes served as aeration windows for the exchange of oxygen and carbon dioxide into the chamber when the biochip was placed in a humidified plastic container filled with carbogen gas, to maintain an optimal incubation environment. Both the plastic container and syringe pumps were placed in an incubator set at 37°C. A comparison of the two systems with their characteristics is given in Figure 1.

Viability testing

The viability of human liver slices was assessed in well plates and biochip by morphological evaluation, and by measuring the leakage of liver-specific enzymes after 4 h and 24 h. For morphological evaluation, slices were fixed in 4% formaldehyde in phosphate-buffered saline solution for 24 h at 4°C. Subsequently, they were placed in 70% ethanol until paraffin embedding, sectioning and haematoxylin and eosin staining (HE staining) as described previously. The leakage of the enzymes lactate dehydrogenase (LDH), alanine aminotransferase (ALAT), and aspartate aminotransferase (ASAT) were measured using the Roche/Hitachi Modular System (Roche, Mannheim, Germany). Analysis was performed according to a routine laboratory procedure. Leaked enzymes were measured in medium fractions collected after 4 and 24 hours, and compared to the total enzyme content present in intact fresh tissue slice (after 1 h pre-incubation).

Metabolism of 7-EC, 7-HC, TT and Li

The metabolic activity of the human tissue slices was assessed by measuring the metabolites formed from four different substrates, 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC), testosterone (TT) and lidocaine (Li). Liver slices were incubated with 500 µM 7-HC and 500 µM 7-EC for 3 hours, and with 5 mM lidocaine for two hours. In the biochip case, the medium outflow containing the metabolites from 7-HC, 7-EC, and Li was continuously collected and stored at -20°C until analysis. Medium samples were obtained from the well plates after incubation was completed. Earlier experiments showed that the percentage of metabolites retained in the liver slices were negligible (data not shown). The HPLC-UV analysis of 7-EC and 7-HC was performed as described previously, and the analysis of Li with its metabolite was the same as described by Olinga et al. The incubation of PCLS with testosterone was performed at a concentration of 250 µM, for an incubation period of 2 hours. In this study, only the metabolites 6β-hydroxytestosterone (6β-OH), 2β-hydroxytestosterone (2β-OH) and TT-A were measured. Since the amounts of metabolites retained in the liver slices for testosterone were not negligible, the slice together with medium was stored after incubation at -20°C to assess metabolite formation. 11β-hydroxytestosterone was used as an internal standard for sample preparation. Tissue was homogenized, and the metabolites and testosterone were
extracted from the medium with liquid-liquid extraction using dichloromethane. After evaporating the dichloromethane, the residue was reconstituted in 50% methanol and 50% water; a 50-µL aliquot was injected onto an HPLC system as described previously.13

After 24 h incubation in both systems, PCLS which had not yet been exposed to xenobiotics were incubated with 500 µM 7-ethoxycoumarin or 500 µM 7-hydroxy-coumarin to assess the stability of phase I and phase II metabolic activity. All experiments were performed using slices prepared from tissue originating from three human livers, using at least three slices per treatment.

Metabolite formation was expressed per mg protein (pmol/min/mg protein). Protein amounts in control tissue slices were determined in five representative slices using the Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) using bovine serum albumin as standard.

Statistical evaluation
Significant differences between slices were determined using the Student’s t-test, with p < 0.05 considered as significantly different. The measured metabolic rates were statistically evaluated.

Results and Discussion
Viability
Human liver slices were less fragile than rat liver slices. The removal of human liver slices after incubation from the biochip was thus easier compared to rat slices. When opening the biochip, extra preheated medium was added into the microchamber from the top. This resulted in the slices floating at the surface of the medium in the chamber, and made it possible to remove the slice with a spatula without damaging it for further processing. Figure 2a) and b) show the morphology of liver slices incubated for four hours in well plates and biochip, respectively. No difference in liver morphology was obtained between the two systems. Figure 2c) and d) shows the morphology of the liver slices incubated for 24 h in well plates and biochip, respectively. Here also no differences in morphology were observed, indicating that human liver slices remained intact in both systems for at least 24 h.

The enzymes LDH, ALAT and ASAT are present in very different amounts in human liver slices. While all three enzymes may be found localized in parenchymal cells, only ALAT and LDH are present in the cytosol of hepatocytes. Of the three enzymes, only ASAT is present in the mitochondria.19-21 However, the leakage of these enzymes as a percentage of the total amount measured over time was very comparable in well plates and biochip, as shown in Figure 3. After four hours, only ~5% of the enzyme had leaked out of the tissue slices for all three enzymes, and in both incubation systems. This means that 95% of cells remained viable after 4 hours. Even
after an incubation period of 24 h, the cumulative leakage of enzymes was still low. Less than 10% of the total ALAT had leaked out in both well plates and biochip, whereas about 15% leakage of ASAT in both well plates and biochip had occurred. About 10% LDH had leaked from the slices in the biochip, while this amount was higher at
15% in well plates. According to this data, about 85% of cells present in the human slices were still intact after 24 hours of incubation. This correlates well with the intact morphology observed for the slices. It could therefore be concluded that liver slices remain viable for at least 24 hours in both systems. It was demonstrated previously that human liver slices maintained their viability when incubated for 24 h in well plates. These experiments have verified that liver slices also remain viable when incubated in the biochip under flow conditions.

**Metabolism studies**

As expected, large differences in metabolism were observed between livers from different patients, which is probably due to different diets and medications. Since rat liver slices incubated in well plates exhibit a good correlation to the rat situation in vivo, results obtained for human slices in the biochip were compared to results obtained for human slices in well plates. In order to do this, the metabolite formation in the biochip was normalized with respect to that obtained in well plates. Figure 4 presents the metabolism of four substrates in fresh tissue slices.

![Figure 4. Metabolic activity of (a) 500 µM 7-ethoxycoumarin, (b) 500 µM 7-hydroxycoumarin, (c) 5 mM lidocaine, and (d) 250 µM testosterone in fresh human liver slices. White bars represent results obtained in well plates, while gray bars represent results obtained in the biochip. Results are mean of three organs ± SEM, with three slices per organ per incubation system per experiment. Significant differences of biochip results with respect to the well plates are indicated with an asterisk (P<0.05)](image)
Human liver slices were able to form both phase I and phase II metabolites from 7-EC (see Figure 4a), as shown previously. These results indicate O-deethylation and the activity of sulfotransferase and UDP-glucuronosyltransferase in human liver slices. Though the metabolite formation in the biochip appeared to be slightly higher for all three metabolites compared to the well plates, the differences were not significant. The metabolism of 500 µM 7-HC into 7-HC-G also appeared to yield a slightly higher mean compared to well plates (see Figure 4b), although this difference proved not to be significant. Sulfation rates, on the other hand, were very similar. The formation of MEGX from lidocaine (see Figure 4c) demonstrated the ability of the human liver to N-deethylate a substrate, and was very comparable both in well plates and biochip for all three livers tested. Only in the case of testosterone metabolite formation was a significant difference between well plates and biochip observed (see Figure 4d). The means of the values for the measured formation rates for the three metabolites of interest were in all cases lower when compared to well plate results. Of these, however, only androstenedione and 2β-hydroxytestosterone exhibited significantly different behavior in the two systems. These two metabolites were also the most hydrophobic formed from testosterone, and are more hydrophobic than any of the other metabolites studied for 7-HC, 7-EC, and Li. One of the disadvantages of using PDMS is its tendency to absorb and adsorb hydrophobic compounds. To verify if adsorption/absorption occurred in the PDMS device, medium containing testosterone metabolites was flushed through a PDMS device which did not contain a liver slice. As expected, recoveries of circa 80% of the most hydrophobic compounds were obtained (see Chapter 8). This correlates well with the lower amounts of metabolites observed in the biochip when compared to well plates. It was therefore concluded that the metabolite formation for testosterone was also similar in well plates and biochip, but that 10 to 20% of the metabolites formed were adsorbed or absorbed onto or into the PDMS. The hydrophobicity of PDMS limits the applicability of the biochip and dictates that the possible adsorption or absorption of substrates and metabolites be taken into account when planning studies with this system.

The ability to form metabolites after 24 hours of incubation in well plates and the biochip was assessed by measuring the metabolism of 7-EC and 7-HC (see Figure 5a and b, respectively). The results were normalized to those obtained for fresh slices in well plates. After 24 h, the human slices were still able to form all three metabolites from 7-EC (see Figure 5a). A substantial amount of free 7-HC was also detected after 24 hours, while this was hardly detectable when incubating rat PCLS after 24 hours of incubation in both systems (see Chapter 5). However, the metabolite formation rates in human PCLS were only 35 to 40% of the formation rates measured for fresh slices. This decrease in metabolism is probably due to the decrease in CYP activity over time, something which is well known. The loss in CYP activity does not
Figure 5. Phase I and phase II metabolic activity of slices incubated for 24 h in well plates (white bars) and biochip (gray bars). (a) 7-EC was used to assess phase I metabolism and (b) 7-HC to assess phase II metabolism. Results are the mean of three organs ± SEM, with three slices per organ per incubation system per experiment. Significant differences with respect to fresh slices incubated in well plates (black bars) are indicated with an asterisk (P<0.05). No significant differences were obtained between well plates and biochip after 24 h incubation.

affect every isoenzyme equally. However, Vandenbranden et al. found a decrease in coumarin 7-hydroxylase activity (CYP2A6) of 60 to 90% after 24 hours in three human livers. This is in line with our results, which reveal a decrease of 60 to 65% in 24 h. The reduction in CYP enzyme activity is not limited to slices, as primary hepatocytes also exhibit this drop. When metabolism in well plates and biochip are compared after 24 h of incubation, no significant difference can be discerned. Therefore, it can be concluded that the lower metabolic rates are due to the tissue and not the incubation system. This decrease can also be minimized by adding the right supplements, such as the inducer, dexamethasone, to the medium. However, phase II metabolism after 24 hours is well maintained in human slices, at levels which are not significantly different from fresh slices in both well plates and biochip (see Figure 5b). This also indicates that human liver slices are viable for 24 hours, independent of the incubation system. Since the conversion of 7-HC into 7-HC-G and 7-HC-S was comparable after 24 hours of incubation to that observed in fresh slices in well plates, the decrease in 7-EC metabolism after 24 hours can only be due to the low CYP activity.

Conclusion
Before using a new incubation system to test new chemical entities, it is important to verify that the system will yield results which are reliable. Previously, we developed a microfluidic-based incubation system for the perifusion of PCLS. This system has been verified with respect to the well-plate system for the incubation of rat liver slices. However, the ultimate goal is to incubate human tissue to predict the metabolic
pathway of xenobiotics without the need to sacrifice animals and with better extrapolation to the *in vivo* situation in humans. The microfluidic-based perifusion system was therefore also tested in conjunction with human tissue in this study. We have shown that it is possible to incubate viable human tissue in the biochip while maintaining its metabolic function. Slice viability was tested after 24 hours of incubation. The results showed intact structure and a low leakage of liver-specific enzymes, indicating viable slices. The metabolic function was also maintained in the biochip, as revealed by tests with four different model substrates (7-EC, 7-HC, Li, TT). Even after 24 h, the metabolic rate in the biochip was very comparable to the well-plate system, the benchmark for these kinds of experiments. Therefore, it can be concluded that the biochip system is appropriate for metabolism and toxicity studies using both rat and human liver slices.

Even though the results generated in the two systems were comparable, the biochip has several advantages compared to well plates. One of the advantages enabled by the continuous flow is that the chip outlet can be directly coupled to an HPLC injector for on-line analysis of metabolites (see Chapter 4). It becomes possible to measure unstable metabolites formed by liver slices, such as acyl glucuronide, a metabolite of diclofenac, as shown previously for rat slices. These types of metabolites often remain undetectable in well plates due to their rapid degradation. A second advantage is the very efficient use of scarce tissue that can be achieved. We have demonstrated previously, for example, that it is possible to perform a complete on-line inhibition study using only three rat liver slices. Another advantage is the ability to measure the effect of flow on the metabolic pathways of xenobiotics, thereby mimicking the blood flow *in vitro*. Finally, it is possible to mimic first-pass metabolism with this system by exploiting the continuous flow feature. This can be accomplished by connecting the outlet of one chamber containing an intestinal slice to the inlet of a different chamber containing a liver slice from a different organ (see Chapter 7). In this way, it becomes also possible to investigate the effect of products formed by one organ on a different organ, the so-called interorgan effects. In well plates, this type of experiment involves placing two slices in one well; however, mutual interaction between slices can take place, and it is never clear which organ is responsible for the effect. In the biochip, the two organ slices are isolated from each other, making it far easier to characterize interorgan interactions. The preservation of human organs is a crucial issue for this type of experiment, since the chance of receiving surgical waste from two different organs on the same day is rather low.

This system, then, has the potential to contribute to drug metabolism and toxicology studies of novel chemical entities. Though it will not replace incubation in well plates, it will provide supplementary information about xenobiotics in an early stage of development.
References