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**Metabolic characterization of primary rat hepatocytes cultivated in parallel  
microfluidic biochips**

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## **Abstract**

The functionality of primary rat hepatocytes was assessed in an Integrated Dynamic Cell Cultures in Microsystem (IDCCM) device. We characterized the hepatocytes over 96h of culture and evaluated the impact of dynamic cell culture on their viability, inducibility and metabolic activity. RTqPCR were performed on selected genes : liver transcription factors (*HNF4 $\alpha$* , *CEBP*), nuclear receptors sensitive to xenobiotics (*AhR*, *PXR*, *CAR*, *FXR*), cytochromes P450 (CYPs) (*1A2*, *3A2*, *3A23/3A1*, *7A1*, *2B1*, *2C6*, *2C*, *2D1*, *2D2*, *2E1*), phase II metabolism enzymes (*GSTA2*, *SULT1A1*, *UGT1A6*), ABC transporters (*ABCB1b*, *ABCC2*) and oxidative stress related enzymes (*HMOX1*, *NQO1*). Microperfused-cultured hepatocytes remained viable and differentiated with *in vivo*-like phenotype and genotype. In contrast with post adhesion gene levels, the first 48h of perfusion enhanced expression of xenosensors, and their target CYPs. Furthermore, *CYP3A1*, *CYP2B1*, *GSTA2*, *SULT1A1*, *UGT1A1*, *ABCB1b* and *ABCC2* were upregulated in IDCCM and reached above post extraction levels all along the duration of culture. Metabolic activities were also confirmed with the detection of metabolism rate and induced mRNAs after exposure to several inducers: 3-methylcholanthrene, caffeine, phenacetin, paracetamol, rifampicin and midazolam. Finally, this metabolic characterization confirms that IDCCM is able to maintain rat hepatocytes functional in order to investigate drug metabolism and predictive toxicology.

**Keywords:** hepatocytes; hepatic metabolism; drug delivery system; Cytochrome P450; phase II metabolism; Efflux Pumps; *in vitro* models; microfluidic biochip

## 1 Introduction

Hepatic metabolism is a major pathway to improve or inhibit toxic effect of some chemical compounds. Several researches aim at developing new *in vitro* powerful methods to predict the liver xenobiotic metabolism and toxicity. New systems based on microscale bioartificial organs have been proposed using recent developments in the field of micro technology and allow designing *in vitro* systems on a very microenvironment.<sup>1-6</sup> Previous works in our laboratory developed microfluidic biochip based approach that create a specific microenvironment where the surface to volume ratio and the micro structuration of cell culture chamber contribute to enhance specific tissue properties.<sup>7</sup> Thus, when hepatocytes (human primary cells or HepG2/C3A cell lines) are cultivated in microenvironment, activities and mRNA levels of cytochromes P450 (CYPs) and drug transporters, related to the drug metabolism and multi-drug resistance phenotype, respectively, were upregulated compared to static cultures.<sup>4,8-10</sup>

Moreover, dynamic perfusion circuit provides a continuous cell supplying and/or xenobiotic exposure in biochip.<sup>2,11,12</sup> Indeed, perfusion can reproduce some liver zonation aspects *via* nutrient gradients along the tissue and *via* specific liver differentiation according to the cell position in the biochip.<sup>13-15</sup> Furthermore, *in vitro* data issued from microfluidic systems can be used for *in silico* pharmacokinetic model to extrapolate *in vivo* exposure.<sup>5,16</sup> Finally, a first attempt to use the liver microfluidic biochip for biomarker researches and predictive toxicology applications has been proposed.<sup>17,18</sup> In order to use these microfluidic biochips for high-throughput screening, parallelized systems were created by integrating conventional multiwell plates formats which are compatible with others emerging or conventional technologies of bioanalyses (such as microscopes, robotic handlers,<sup>6</sup> integrated peristaltic actuators<sup>19</sup> or external fluid pumping system<sup>20</sup>). In addition, when compared to our first generation of biochips<sup>10,21</sup>, the extension of the number of cell culture conditions allowed testing simultaneously different concentrations of chemical compounds.

In this paper, we have evaluated the performance of such parallelized biochips with the culture of primary rat hepatocytes. Indeed, primary human hepatocytes is preferentially used to predict *in vivo* toxicity but is limited by availability of donor material and the large variability between the donors. Therefore, primary hepatocytes

isolated from other mammals (mouse, rats) are used as an alternative to human tissue.<sup>22</sup> Nevertheless, the maintenance of differentiation of primary hepatocytes is a major requirement to achieve physiological metabolism and toxicity.<sup>23,24</sup> We characterized phenotype and genotype of hepatocytes cultivated in an “Integrated Dynamic Cell Cultures in Microsystems” (IDCCM) to demonstrate the advantages of this new parallelized system applied to toxicology assessment.

## 2 Material and methods

### 2.1 Microfluidic biochips and IDCCM box

The entire set up was called the IDCCM for “Integrated Dynamical Cell Culture in Microsystems”. The biochips design and microfabrication were described in Baudoin *et al.*, 2011.<sup>25</sup> The biochips are made in polydimethylsiloxane (PDMS) conventional moulding (**Figure 1A**). The concept and detail of the IDCCM box are presented in detail in our previous work.<sup>20,26</sup> Briefly, the PDMS biochips were connected at the bottom of the IDCCM box by a simple series of “plugging” ports. This format allowed an “easy plug and display” of the biochips to external set up such as microscopes. IDCCM is a manufactured polycarbonate box using the conventional format of 24-well plate. Each microfluidic biochip was connected between two wells. The 24 wells were used as entrance and outlet reservoir leading to the parallelized culture of 12 biochips (**Figure 1B, C**). A specific cover was designed in order to allow the hermetic closure of the polycarbonate box for continuous flow perfusion. The cover includes ports for fluid perfusion and sampling. The hermetic closure of the IDCCM box and the pressure in the IDCCM box avoid any leakage or reservoir drain (**Figure 1C**).

### 2.2 Primary rat hepatocytes culture in IDCCM

The experiments were performed during 5 days. It included three different phases: the day of hepatocyte extraction (day-0), the adhesion phase (day-1) and 72h of perfusion phase (from day-2 to day-4). The sterilization of the biochips, of the IDCCM box and the perfusion circuit was done by autoclaving all the set up. The biochips were then connected to the box under sterile condition.

#### 2.2.1 Primary rat hepatocytes isolation

Primary hepatocytes from 5-week-old male Sprague-Dawley rats weighing about 250g were isolated by a modification of the two-step *in situ* collagenase perfusion according to the method of Seglen.<sup>27</sup> Animals were provided by the Janvier Elevage Animal Center (Le Genest Saint Isle, France). They were housed at the University of Compiègne with a 12h light / dark cycle at 22°C with food and water *ad libitum*. All experiments were approved by the Animal Experimental Committee of

the University of Compiègne. The animal was anesthetized by intra peritoneal injection of sodium pentobarbital (Centravet, Gondreville, France). Briefly, after *in situ* washing and perfusion with collagenase solution at 30ml/min, the liver was extracted and the digested liver tissues were filtered through cotton gauze, then 400 and 100µm filters. The cell suspension was centrifuged and washed three times. Percoll isogradient centrifugation was performed to isolated dead cells and a significant portion of the non-parenchymal cells in a floating top layer that was discarded. The resulting cells were finally suspended in the culture medium as described below. Cell viability was assessed by trypan blue dye exclusion and hepatocytes with a viability of greater than 90% were used.

Freshly isolated hepatocytes were cultured in first 24 hours in seeding medium composed by William's E Glutamax medium (Fisher Scientific, Illkirch, France) supplemented with bovine insulin (5µg/ml, Sigma-Aldrich, Saint-Quentin Fallavier, France) and serum bovine fetal (10%). To enhance cell adhesion, the inner surface of the biochips was coated with rat tail type 1 (0.3mg/ml, BD Biosciences) prepared in phosphate-buffered saline (PBS) for 1 hour at 37°C in a humidified atmosphere supplied with 5% CO<sub>2</sub>. After washing with seeding medium, the cells were inoculated at a density of  $0.2 \times 10^6$  cells per biochip ( $0.1 \cdot 10^5$  cells per cm<sup>2</sup>) inside the biochips with the aid of a pipette and each well was completed by 2ml of seeding medium. After 24 hours, cells were cultured in William's E GLutamax medium supplemented with 3mg/ml Bovin Serum Albumin (Sigma-Aldrich), 5ng/ml dexamethasone (Sigma-Aldrich), 6.25µg/ml insulin-transferrin-selenium solution (Becton Dickinson, Biosciences, Le Pont de Claix, France), 100units/ml penicillin and 100mg/ml streptomycin (Fisher Scientific, Illkirch, France). During cell adhesion and cell culture, primary hepatocytes were incubated at 37°C in a humidified atmosphere supplied with 5% CO<sub>2</sub>.

### **2.2.2 Dynamic culture in IDCCM**

After the 24h of adhesion, IDCCM is prepared for the perfusion phase. Medium reservoirs at the entrance and outlet were filled with a volume of 2mL of culture medium to ensure the correct medium supply during dynamic culture. It resulted in a total volume of 4mL for each biochip. Then, the polytetrafluoroethylene (PTFE) tubes were connected to the fluidic cover of the IDCCM box and to the

peristaltic pump (**Figure 1D**). PTFE tubes were used to prevent loss of chemical compounds to the tubing used in the fluidic circuit. The entire system (pump and IDCCM box with the biochips) was placed into the incubator (37°C, 5% CO<sub>2</sub>) and the perfusion started with a flow rate of 25µl/min. After adhesion phase and 24h, 48h and 72h of perfusion, culture medium was stocked at -20°C for posterior assays. Cytochrome assays and cell counting were performed on biochips (see below). Cells were photographed using a Leica DMI 6000B microscope together with the Leica camera and LAS life software (Leica Microsystems). Six independent experiments (i.e 6 euthanized rats and 6\*12 = 72 biochips) were assessed and each analyze was repeated at least three times in triplicate biochips. Data were plotted as mean ± standard error of the mean (SEM).

### **2.2.3 Cell counting and viability**

Cell counting was performed on Malassez cell after cell detachment with trypsin-EDTA (Fisher Scientific). Cell viability was quantitatively analysed by trypan blue staining. In addition, the cell viability was qualitatively assessed at the end of the perfusion in the liver biochips (n = 4). After washing, a fresh solution consisting of 50nM propidium iodide (PI) and 2µM calcein-AM (Invitrogen, Saint Aubin, France) was incubated in PBS for 15min at 37°C, CO<sub>2</sub> 5%. After this incubation the biochip were rinsed twice in PBS, pH 7.4, and maintained in PBS during imaging. The liver microfluidic biochip was directly observed using a conventional fluorescence microscope (Leica DMI 6000B, LAS-AF software, Leica Microsystems) : 494 nm excitation filter and 517 nm emission filter (calcein AM), 536 nm excitation filter and 617-nm emission filter (PI). Viable and dead cells appeared green and red, respectively.

### **2.2.4 Cytochrome 1A2 assay**

We measured both basal and induced levels of metabolic enzymatic activities after incubation of 3-methylchlorentrene (5µM) or Rifampicin (25µM) during 72h of perfusion. CYP1A2 activities were determined using 5-ethoxyresorufin (10µM) as substrate. Resorufin formation by 7-ethoxyresorufin O-deethylation (EROD) was quantified with a fluorescence micro-plate reader (TECAN, Spectrafluor plus) after 1 hour's incubation at 37°C, CO<sub>2</sub> 5% in the presence of salicylamide (3mM) in order to inhibit phase II enzymes. All products were purchased from Sigma-Aldrich.



## **2.3 Metabolism analysis**

### **2.3.1 Chemicals and solvents**

The reference standards and their metabolites were obtained from Sigma-Aldrich (St Quentin Fallavier, France): midazolam maleate salt (CAS 59467-94-6), 1-OH-midazolam (CAS 59468-90-5), phenacetin (CAS 62-44-2), paracetamol (CAS 103-90-2), paracetamol-glucuronide (CAS 120595-80-4), paracetamol-sulfate (CAS 10066-90-7).

HPLC-grade acetonitrile (ACN) and formic acid (HCOOH, content > 99%) were purchased from VWR International (Fontenay-sous-Bois, France). HPLC-grade methanol (MeOH) was purchased from Carlo Erba reagents (Val de Reuil, France). HPLC-grade water was obtained by purifying dematerialized water in a Milli-Q system (Millipore, Molsheim, France).

Stock solution (1mg/mL) of each individual standard was prepared by dissolving an appropriate amount of each substance in HPLC grade MeOH or ACN (depending on the solubility of each compound) and further diluted as necessary to obtain the required concentration. To minimize degradation of standards, stock and working standards solutions were stored at  $-18^{\circ}\text{C}$  immediately after preparation.

### **2.3.2 Sample preparation**

The incubation medium containing the each substrate at  $1\mu\text{M}$  was incubated for 24h (from J1 to J2) and sampled after 1h, 2h, 3h, 4h, 5h and 24h. Since serum was not used in the experiments, there was no binding of the drugs in the culture medium. To check non-specific binding in the perfusion system we evaluated the adsorption of each substrate.<sup>26</sup> For that purpose the peristaltic pump was started at a flow rate of  $25\mu\text{L}/\text{min}$ . Each sampled volumes were collected and a volume of  $50\mu\text{L}$  of sample from biochip was mixed with  $100\mu\text{L}$  of methanol to precipitate proteins. The mixture was then centrifuged (10min at 20 000 g). The supernatant was extracted and evaporated to dryness under gentle nitrogen flow and warming in water bath,  $30^{\circ}\text{C}$ , and resuspended in  $50\mu\text{L}$  of 5/95 (v:v) ACN/water solution. Finally, the samples were stored in the dark at  $4^{\circ}\text{C}$  before analysis.

### 2.3.3 Liquid Chromatography

The chromatographic separations were performed with a Nexera LC-30AD (Shimadzu, Marne-la-Vallée, France), equipped with a binary solvent manager, a sample manager and a thermostatic oven. Compound separation was carried out using a Xterra C18 column, 5 $\mu$ m, 150mm  $\times$  2.1mm I.D. (Waters, Guyancourt, France) with a Javelin column filter 0.5 $\mu$ m (ThermoScientific, Les Ulis, France). The column was kept at 30°C and the sample manager was maintained at 4°C. The mobile phase was composed of solvents A (water) and B (acetonitrile) both with 0.1% HCOOH. The gradient for all samples was as follows: 5% B (0-2min), 5-100% B (2-22min), 100% B (22-26min), 100-5% B (26-26.1min) and 5%B (26.1-30min). The flow rate of the mobile phase was 0.3 mL/min and the injection volume was 10 $\mu$ L.

### 2.3.4 Mass spectrometry

The analyses were performed using an Exactive (ThermoScientific, Les Ulis, France) in alternative switch mode ESI(+) and ESI(-). Mass resolution of the instrument was set to 25 000 (FWMH). The capillary voltages were 4800 V and 2800 V for ESI(+) and ESI(-) respectively. The desolvation gas flow of N<sub>2</sub> was 28 and 9 (arbitrary units for ion max source) for sheath gas and auxiliary gas respectively. The capillary temperature was 280°C. The measured mass range ( $m/z$ ) was 80-1000 in profile mode. To assure accuracy of the mass measurements, the instrument was calibrated every week in both polarities with a Calmix solution (ThermoScientific, Les Ulis, France) to obtain a mass precision less than 2ppm. Mass windows of 5ppm were used to extract mass traces.

### 2.3.5 Data processing

The instrument was operated with Xcalibur 2.1 software (ThermoScientific). Data were processed with two application managers of Xcalibur 2.1: Qualbrowser to extract mass traces and Quanbrowser to determine peak area.

Signal identification was based on two criteria: (1) retention time of the analyte as compared to the retention time of standard compound in the time window of  $\pm$  0.25min and (2) the difference of calculated exact mass and measured accurate mass of the analyte less than 5ppm. The exact mass of protonated molecule [M+H]<sup>+</sup>

and the exact mass of deprotonated molecule  $[M-H]^-$  were used as the identification and quantification trace in ESI(+) and ESI(-), respectively.

To achieve internal quality criteria, the quantification of positively identified analytes was performed using standard addition method. Eight standard additions corresponding to concentrations of 1ng/mL, 5ng/mL, 10ng/mL, 20ng/mL, 50ng/mL, 100ng/mL, 200ng/mL and 500ng/mL with three replicated injections were carried out. The quantification was considered valid only inside the measured linear range. The limits of detection were 1ng/ml for phenacetine, paracetamol, midazolam; 5ng/ml for caffeine, paraxanthine, 1-hydroxymidazolam and 100 ng/ml for paracetamol-sulfate.

### 2.3.6 Metabolic ratio

The metabolic ratio (MR) was defined by the ratio of the area under the curve (AUC) of the reaction products divided by the sum of all the AUC of the parent substrate and its metabolites<sup>9</sup>. The metabolic production over time was calculated as follow:

$$MR_t = 100 \times \frac{AUC(P)_t}{AUC(P)_t + AUC(S)_t - MR_0}$$

In which

MR<sub>t</sub> is the metabolic ratio at time t

AUC is the area under the curve of the P product of the enzymatic reaction or the S substrate

MR<sub>0</sub> is the initial value of metabolic ratio before the incubation.

### 2.4 Transcriptomic analysis

Total RNA was extracted using the Nucleospin® RNA XS isolation kit (Macherey-Nagel EURL, Hoerdt, France). The quantity of RNA was assessed with a Nanodrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). The quality of the RNA extracted was evaluated using the Agilent 2100 Bioanalyzer (using the Agilent RNA 6000 Nano and Pico kits). All RNA samples had an RNA Integrity Number of more than 8 (between 9.3 and 10).

cDNA were synthesized from 500ng of total RNA with oligodT Primer, using SuperScript II (Life Technologies), according to the manufacturer's protocol. Quantitative RT-PCR was performed using the LightCycler 480 Sybr Green I Master and the LightCycler 480 Probes Master (Roche), for the housekeeping genes and the targets genes respectively, and the Real Time PCR system LC480 (Roche). Pairs of primers for each of the 23 targets genes were designed using the Roche RealTime ready (UPL probes) Configurator tool (**Table 1**). Couples of primers for housekeeping genes (GAPDH, HPRT1, PPIB and SDHA) were designed with Primer3, and tested for efficiency and specificity. The expression stability of these reference genes was assayed using GeNorm software application, which allows selecting the two most stable housekeeping genes in our model. All the couples were used in duplicate. Data from target genes were normalized with the two reference genes (GAPDH and PPIB). Data were analyzed using the  $\Delta C_t$  method<sup>28</sup> after validation of each duplicate (i.e. elimination of outliers' measures from duplicate with standard deviation above 0.4).

### 3 Results

#### Cell morphology and viability of primary rat hepatocytes cultivated in IDCCM

We performed hepatocytes culture over 96h of cultures. The cells successfully attached on the biochip surface since three hours of culture. The inoculated density contributed to create a confluent tissue as shown in **Figure 2A to C**. The hepatocytes were singular and spherical after inoculation (**Figure 2A**) whereas the cells were refringent between their cell junctions, with a typical cuboid shape at the end of perfusion (**Figure 2B**). Indeed, hepatocyte-like morphology was conserved over time and the hepatocytes were still located in the biochips forming a dense tissue: some binucleated hepatocytes were observed with the formation of structures resembling bile canaliculi (light areas between cells) (**Figure 2C**). The cell counting showed the decrease of the percentage of counted cells in comparison with the number of initial density (200 000 cells / biochip or 100 000 cells / cm<sup>2</sup>) at 76.7 ± 21.7% and 53.5 ± 4.1%, after 24h post adhesion and during perfusion, respectively. However the cell density remained stable during the 72h of perfusion around  $1.10^5 \pm 2.10^4$  cells/biochip and confirmed that primary hepatocytes were not in proliferation (**Figure 2D**). Viability tests performed by trypan blue after 24h, 48h and 72h of perfusion have demonstrated that the cell viability was maintained during the cultures and few dead cells was detected (data not shown). These results were also confirmed by Calcein AM® and PI® staining (**Figure 3**).

#### Gene expressions of primary rat hepatocytes cultivated in IDCCM

In order to quantify the level of gene expressions of genes representative of hepatic functionality, we performed RTqPCR analysis after hepatocyte extraction, after hepatocyte adhesion, and during the 72h of the perfusion.

The levels of gene expression linked to hepatic functionality were assayed on hepatic transcription factors, xenosensors, CYPs and phase II metabolism enzymes. ABC transporters and genes implicated in inflammation and / or oxidative stress were equally studied. Details of selected genes were listed in **Table 1**.

After hepatocytes adhesion, among 74% of analyzed genes have a reduction of their expression levels when compared to post extraction situation (**Table 2, column A**). The levels of gene expressions of hepatic transcription factors, such as *HNF4 $\alpha$*  and *CEPB $\beta$* , were reduced by 65% after the adhesion step in comparison to

post extraction. Other expressed genes (*AhR*, *PXR*, *GSTA2*, *CYP2D1* and *CYP1A2*) represent at least 50% of their post extraction levels. On contrary, the level of some PXR target genes (*MDR1* > *CYP2B1* > *UGT1A6* > *CYP3A23/3A1* > *MRP2*) and oxidative stress-related genes (*HMOX1* > *NQO1*) were found upregulated.

When the perfusion started, we detected at least 50% of post extraction levels for 39% and 44% of analyzed genes after 24h and 48h of perfusion, respectively (**Table 2, column B**). We observed that all expressions are decreased after the first 24 h of perfusion except for *AhR*, *CAR*, *CYP3A23/3A1*, *GSTA2*, *SULT1A1*, *ABCB1b*, *ABCC2* and *NQO1*. During the first 48h of perfusion, 91% of selected genes are induced at a maximum of their expressions in comparison to post extraction. Particularly, the levels of mRNAs of *AhR*, *CAR*, *CYP3A23/3A1*, *CYP7A1*, *CYP2B1*, *GSTA2*, *SULT1A1*, *UGT1A6*, *ABCB1b*, *ABCC2*, *HMOX1* and *NQO1* increased around or above 100% of the post adhesion levels. After 72h of perfusion, all mRNA expressions decreased except the expression of some genes (*CEBP*, *FXR*, *CYP1A2*, *GSTA2*, *UGT1A6* and *NQO1*) which remain induce by perfusion. Moreover, only the xenosensor, *CAR* and this target gene, *CYP2E1* decreased during the 72h of perfusion.

### **Liver-specific activity and inducibility of primary rat hepatocytes cultivated in IDCCM**

Liver-specific activity was checked at each experiment using albumin secretion assay. The secretion of hepatic specific marker was confirmed and reached  $342.9 \pm 109.9$  and  $138.1 \pm 31.2$  ng/ $10^6$  cells/hour after 24 and 72h of perfusion, respectively (data not shown).

To check the inducibility of hepatocytes, the cell response to prototypical inducers (3MC, rifampicin) was evaluated after 72h of perfusion in IDCCM. First, we measured the resorufin production by EROD in uninduced and 3MC-induced rat hepatocytes. *CYP1A2* activity increased during the perfusion and is about 3 times higher at 72h of perfusion in comparison with the activity after adhesion of rat hepatocytes in biochips (**Figure 4**). After 72h of induction, 3MC increased *CYP* activity at  $2.1 \pm 0.5$  times which illustrate the inducible properties and functionality of *CYP1A2* in the microfluidic biochips (data not shown). In addition, RT qPCR analysis reported that the level of *AhR* and *CYP1A2* mRNA were upregulated in comparison

with uninduced cell culture as shown in **Figure 5A**. Moreover, these inductions contributed to maintain the levels of mRNAs up to 102 and 136 %, respectively for *AhR* and *CYP1A2*, in comparison with the post extracted levels. We also detected that hepatic transcription factor gene, *HNF4 $\alpha$* , and P-glycoprotein (Pgp) related gene, *ABCB1b*, were upregulated by 3-MC induction.

After rifampicin induction, we observed upregulation of *GSTA2* gene expression after 72h of perfusion (**Figure 5B**) whereas this expression remained at the same level between 48h and 72h in uninduced culture (**Table 2, column B**). Concerning *ABCB1b* expression gene, rifampicin induced a fold change of  $81.8 \pm 27.3$  (**Figure 5B**) even though this expression fall down after 72h of perfusion in control cell culture (**Table 2, column B**). No induction of *CYP1A2* activity was observed after EROD test.

### **Kinetics of metabolism of primary rat hepatocytes cultivated in IDCCM**

To analyze the functionality of the hepatocytes in IDCCM, we measured the kinetics of metabolites production from biotransformation of prototypical substrates (phenacetin, paracetamol, caffeine, midazolam). The analyses were performed during 24h after 48h of cultures in biochips (24h of adhesion and 24h of perfusion).

Metabolic ratio showed kinetics of paracetamol from 2h to 24h of perfusion with phenacetin produced by *CYP1A2* activity (**Figure 6A**). In contrast with paracetamol, paracetamol-sulfate metabolites were detected only after 24h of  $1\mu\text{M}$  phenacetin exposure. After 24h of exposure, metabolic ratio is 40 times higher for paracetamol ( $\text{MR} = 10.3 \pm 1.7$ ) in comparison with paracetamol-sulfate metabolites ( $\text{MR} = 0.3 \pm 0.1$ ) (**Table 3**). After  $1\mu\text{M}$  Paracetamol exposure, we also observed paracetamol-sulfate formation only after 24 h of perfusion with a metabolic ratio of  $1 \pm 0.2$  (**Table 3**). These results show that sulfotransferase 1A (*ST1A1*) is 3.3 times more performant after paracetamol exposure in comparison with results of phenacetin metabolism. Moreover, after phenacetin and paracetamol exposures, we did not detect paracetamol-glucuronide metabolites linked to UDP-glucuronosyltransferase 1A1 (*UGT1A1*) activity.

We also analyzed caffeine metabolism by *CYP1A2* and *2C11* of rat hepatocytes cultivated in IDCCM and we performed detection of one metabolites, paraxanthine, which was detected only after 24h exposure ( $\text{MR} = 0.1 \pm 0.1$ ) (**Table**

3). The effect of caffeine on rat hepatocytes was equally analyzed on gene expressions linked to xenobiotic metabolism (**Figure 7A**). After 24h of perfusion, no induction of *CYP1A2* genes was observed but up-regulation on PXR nuclear receptor and these target genes (*CYP3A2*, *CYP3A23*, *CYP2B1*, *CYP2C*, *SULT1A1*, *ABCB1b*) were detected. The genes related to cholesterol, steroids and bile acid biosynthesis (*FXR* and *CYP7A1*) and oxidative stress (*HMOX1*) were also stimulated with caffeine induction.

Furthermore, in order to quantify the activity of CYP3A1, 2B1/2, 2E, 2C6 implicated in midazolam biotransformation, we observed kinetic formation of 1-OH midazolam metabolites from 1h to 24h of exposure (**Figure 7B**). Metabolic ratio was  $2.1 \pm 0.3$  (**Table 3**). In addition, midazolam induction was detected on levels of genes related to PXR and oxidative stress (*CYP1A2*, *CYP3A2*, *CYP3A23*, *CYP2B1*, *GSTA2*, *UGT1A6*, *ABCB1b*, *HMOX1*, *NQO1*) (**Figure 7B**).



## 4 Discussion

Evaluation of drug and xenobiotic toxicity stimulate the development of *new in vitro* alternative methods based on the reconstruction of major organ implicated in metabolism, the liver. Recently developed in our laboratory, original parallelized microfluidic biochip box (named IDCCM) have already demonstrated this capacity to increase liver phenotype of hepatocytes cell lines (HepG2 C3A)<sup>20</sup> and human cryopreserved hepatocytes.<sup>26</sup> Briefly, the IDCCM contributed to reduce the time of culture preparation and of biochip manipulation, and to increase the number of parallel cultures. In this work we analyzed the behavior of freshly isolated primary rat hepatocytes which is a cell culture model largely used in research due to low disponibility of human tissue. In contrast with plated Petri cultures,<sup>29</sup> rat hepatocytes culture are enhanced in IDCCM because no dedifferentiation of hepatocytes after isolation was observed along the 72h of perfusion which is a strong criteria for quality culture.

To investigate the metabolic competence of biochip-cultured rat hepatocytes in more details, gene expression profiles from *AhR*, *PXR*, *CAR*, *FXR* and selected drug metabolizing proteins, such as phase I and II biotransformation enzymes and ABC transporters, were evaluated during the cultivation period. Even within the short period of 24h after isolation, the expression levels of many CYPs are known to decrease tremendously in rat hepatocytes.<sup>30</sup> In our study, we demonstrated that the gene expression levels of the same enzymes showed a slower decrease over time indicating that rat hepatocytes remain more stable and inducible in IDCCM. Indeed, we found that the levels of mRNAs (among 74% of analyzed genes) were reduced after the hepatocytes adhesion when compared to post extraction situation. Perfusion stimulated the increase of the expression levels of all selected genes (except *CAR*, and *CYP2E1*) until the 48h of perfusion. Nevertheless, the fall down of mRNA expression observed after 72h of perfusion may be more explored by continuing the perfused-culture over several days.

The HNF family is involved in specific liver functions as hepatocytes differentiation and in the regulation of CYPs *via* the modulation of nuclear receptors such as *PXR* and *CAR*.<sup>31,32</sup> Among them, we observed that the transcription factor *HNF4 $\alpha$*  and the nuclear receptors *PXR*, *CAR* and *AhR* reached levels corresponding from 24% to 80% of the post extraction values after 48h of perfusion at 25 $\mu$ L/min.

Similarly to our results, Sivaraman *et al.* showed that the transcription factors genes in their silicon biochips reached values closed to *in vivo* and freshly isolated levels.<sup>4</sup>

Our results evidenced that PXR pathway and particularly, *CYP3A1* and *CYP2B1* genes, are the most upregulated genes in our biochips. Consistently with Sivaraman *et al.*, 2005, we found that the levels of CYP2B1 enzymes remained upregulated above post extraction level over the 96h of culture.<sup>4</sup> CYP2B1 has been related to zonal activity in both *in vivo* conditions and *in vitro* dynamic cultures.<sup>13,14,33</sup> In fact, our previous work evidenced a periportal like phenotype for HepG2/C3a cell lines cultivated in biochip,<sup>13</sup> which could be more explored for IDCCM cultures with primary rat hepatocytes. Phase II (*GST1A2* > *UGT1A6* > *SULT1A1*) and ABC transporters (*ABCB1b* > *ABCC2*) targeted by PXR genes were also induced and were generally higher in culture compared to post extraction. Interestingly, the rise of *ABCB1b* expression over time was already observed in sandwiched-cultured primary rat hepatocytes culture<sup>34</sup> but equally in combined static monolayer's culture with dexamethasone induction.<sup>35,36</sup> These upregulations suggest the functionality of biliary canaliculi observed in our culture because MDR1 and MRP2, coded by *ABCB1b* and *ABCC2*, respectively, are known to be implicated in drug transporters as canalicular export proteins.<sup>37</sup> Concerning the phase II enzymes, we agree with previous works which demonstrated that phase II enzymes are better preserved in culture than CYPs.<sup>38</sup> On contrary to sandwich-cultured hepatocytes, the gene expression level of phase II enzymes didn't decrease after 72h of perfusion in IDCCM under post extraction level.<sup>39</sup>

AhR pathway is also induced in our perfused biochips. Whereas *AhR* level is maximal at 48h of perfusion, the level of *CYP1A2* keep a weak basal level which is continuously enhanced during the 72h of perfusion. Moreover, other *AhR* target genes, coded to phase II enzymes (*GSTA2* and *UGT1A6*) were also upregulated. Stress genes linked to AhR and NrF2 pathway, *HMOX1* and *NQO1* were initially induced after cell adhesion and remained elevated until 48h of perfusion as observed for HMOX1 genes in static sandwich-cultured hepatocytes cultures.<sup>34</sup> Furthermore, we have investigated the levels of *FXR* and *CYP7A1* that are reported to regulate the synthesis cholesterol, steroids and the bile acid metabolism. The level of those genes appeared downregulated in our biochip configuration which appeared similar to the observations in silicon biochips.<sup>4</sup>

In comparison with our previous work performed on cryopreserved human hepatocytes cultivated in IDCCM <sup>26</sup>, some similitudes between rat and human genotype were observed. In fact, same profiles before and after perfusion were detected for hepatic transcription factors *HNF4 $\alpha$*  and for more active CYPs genes, *CYP1A2* and *CYP3A23/3A1* (human/rat). In contrast with human hepatocytes, rat hepatocytes expressed great levels of others CYPs, phase II enzymes and ABC transporters genes when cells are cultivated in IDCCM, consistently with high level of *AhR* gene observed during the 72h of perfusion.

Potential of inducibility of rat hepatocytes in IDCCM was also evaluated with prototypical inducers: 3MC, phenacetin, paracetamol, caffeine, rifampicin and midazolam. Firstly, we used 3MC which is an aromatic hydrocarbon that induces by activating the AhR and then, the expression of *CYP1* family genes. <sup>40,41</sup> Our results confirm that the most inducible cytochrome, <sup>42</sup> *CYP1A2*, was inducible by 3MC in our cultures and reached mRNA values closed to the post extraction levels as already observed. <sup>4</sup> Interestingly, in comparison with HepG2C3A cell lines cultivated in IDCCM <sup>20</sup>, the level of basal *CYP1A2* activity measured by EROD test showed that primary rat hepatocytes have great activity (x 100) which confirm the interest of primary rat culture in metabolism studies. In addition, we confirmed the possibility to evaluate kinetics of metabolism in our hepatic cultures <sup>26</sup> by detecting the activity of *CYP1A2* and *ST1A1*, to produce paracetamol from phenacetin <sup>43</sup> and paracetamol-sulfate from paracetamol, <sup>44</sup> respectively,

After caffeine exposure, we evidenced paraxanthine metabolites only after 24h of exposure. Paraxanthine provided from 3-N demethylation of caffeine catalyzed by *CYP1A2* and *CYP2C11*, the pivotal metabolism pathway of caffeine. <sup>45,46</sup> RTqPCR analysis evidenced an upregulation of some *PXR* target genes, as *CYP3A1* and *CYP2C*, all implicated in metabolism caffeine pathways <sup>45,46</sup>, but we didn't detected *CYP1A2* induction. Further LC-MS analyses need to be performed to detect other caffeine metabolites, as theobromine, theophylline and 1,3,7-trimethyluric acids and conclude on the kinetics of caffeine metabolism linked to *CYP1A2* and *CYP2C11* activities.

Rifampicin is an inducer of *CYP3A4* and *PXR* genes in human hepatocytes and consistently with literature, we didn't detect this induction in rat hepatocytes culture. <sup>47,48</sup> We evidenced that Rifampicin induced Pgp efflux pump expression in our biochip. As suggested by Schuetz *et al.*, Pgp-mediated transport can influence

the hepatocellular concentration of xenobiotics that are modulators and substrates of CYPs (CYP3A in human).<sup>49</sup> We suggest that *ABCB1b* induction may be linked to another pathway than CYP3A in rat hepatocytes.

Nevertheless, midazolam exposure was performed to confirm CYP3A inducibility in our culture. Midazolam is mainly metabolized in rats by CYP3A isoforms (CYP3A2 in majority), leading to 1'-hydroxy-, 4-hydroxy- and 1',4-dihydroxymidazolam, the later representing a minor metabolite<sup>50</sup>. In IDCCM, basal levels of *CYP3A1* remain highly over expressed in compared with *CYP3A2* during all times of cultures and *versus* post extraction levels (up to 20 fold after 48h of perfusion). Midazolam exposure confirmed the capacity of rat hepatocytes to metabolize midazolam into 1-hydroxymidzalom along the 24h of treatment. RTqPCR analysis evidenced the effect of the induction on mRNA levels of *CYP3A1*, *2B1*, *2C* as suggested by Hoen *et al.*<sup>51</sup> Consequently, these results illustrate the CYP3A1 activity and inducibility in primary rat hepatocytes cultivated in our biochips.

Together these results indicate that the IDCCM-cultured primary rat hepatocytes system is robust and seems to maintain its metabolic competence (CYPs and phase II enzyme activities). These results confirm the advantages to cultivate primary rat hepatocytes in microfluidic biochip which were already investigated under various flow rate and cell densities and in comparison to static-cultured rat hepatocytes (Baudoin *et al*, unpublished results). Furthermore, as previously demonstrated,<sup>26</sup> intrinsic *in vitro* clearances may be extracted using a Physiologically based pharmacokinetic (PBPK) model for predictions and comparisons to literature *in vitro* data and *in vivo* situations. However, the levels of biotransformation in the biochips were not obtained at the substrate concentration corresponding to the  $V_{max}$  value which normally leads to the maximal disappearance rate of the drugs. As future steps, the  $V_{max}/K_m$  couple values must be investigated in our next development in order to complete and to status on the performance analysis of our biochips.

## 5 Conclusions

The relevance of the rat *in vitro* model is obvious since this is one of the most widely applied species in regulatory toxicity testing. Indeed, primary cells retained specific hepatocyte properties closest to physiological levels, although it cannot

reproduce the *in vivo* state perfectly. More similarities in genotype and phenotype of primary rat hepatocytes cultivated in IDCCM were observed with sandwiched cultures and other microperfused systems but we illustrated the advantages to couple perfusion method and time-lapse samplings for metabolism analysis. Nevertheless, IDCCM could be use, such as cell suspension and monolayer cultures, for short-term Drug-Drug Investigations with a maximum culture time of 72 h after single exposure. Based on our genotype and phenotype analyses, we recommend applying a 24-h recovery period before using the IDCCM-cultured rat hepatocytes as for the use of human hepatocytes. Applications of IDCCM with primary hepatocytes from various species, including human, offers the opportunity to detect species-specific effects and would contribute to extrapolate results to man. Finally, this new tool could be use, in the drug discovery process, to identify potential drugs and thereby reduce late stage failures or animal testing.

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## Figure Legends

**Figure 1: The IDCCM box** with (A) PDMS biochip, (B) Three compartments of IDCCM, from top to bottom: (a) the fluidic cover, (b) the silicon joints and (c) the 12 biochips connected under the 24-well polycarbonate plate, (C) The side view of IDCCM, (D) two IDCCMs connected to the peristaltic pump with 24 PTFE tubes. Corresponding bars were indicated in mm in views A & C.

**Figure 2 : Morphology and cell counting of primary rat hepatocytes in IDCCM.** (A) Hepatocytes after inoculation and (B,C) at the end of the perfusion experiments (72 hours), (C) High magnification of primary rat hepatocytes. Cells are confluent with a typical cuboid shape and binucleated cells were detected. (Inset) Arrows indicate bile canaliculi-like structures. Corresponding bars were indicated in  $\mu\text{m}$ . (D) Evolution of cell number per biochip counted after cell adhesion and during the perfusion experiments (mean and SEM of three independent experiments with minimum  $n=3$  biochips).

**Figure 3 : Cell viability of primary rat hepatocytes in IDCCM.** (A) morphology of hepatocytes after 48 hours of perfusion (phase contrast) and (B) after calcein AM and propidium iodide staining. Viable cells appeared green, while dead cells appeared, red. (C) Overlay of phase and fluorescence views. Corresponding bars were 100  $\mu\text{m}$  in all views.

**Figure 4 : CYP1A2 activity in IDCCM.** Comparison of the resorufin production by 7-ethoxyresorufin O-deethylation (EROD) in control cell culture (uninduced) of rat hepatocytes cultivated during 72h in IDCCM (mean $\pm$ SEM;  $n=4$  biochips).

**Figure 5 : Mean fold changes in gene expression induced by 3-MC and rifampicin after 72h of primary rat hepatocytes exposure in IDCCM.** Only fold changes greater than 1.5 were selected in these figures. (A) 5 $\mu\text{M}$  3-methylcholanthrene (B) 25  $\mu\text{M}$  rifampicin (mean $\pm$ SEM;  $n=3$  biochips).

**Figure 6 : Metabolite ratio of phenacetin and midazolam after 24h of exposure in IDCCM.** (A) Kinetics of paracetamol production from phenacetin; (B) Kinetics of 1-OH midazolam production from midazolam (mean±SEM; n=3 biochips).

**Figure 7:** Mean fold changes in gene expression induced by midazolam and caffeine after 24h of primary rat hepatocytes exposure in IDCCM. **Only fold changes greater than 1.5 were selected in these figures.** (A) 1µM midazolam (B) 1µM caffeine (mean±SEM; n=3 biochips).

### Table Legends

**Table 1: List of 23 target genes studied using the RealTime ready probes from Roche.**

**Table 2 : Comparative study of expression of selected mRNA of genes implicated in xenobiotic metabolism.** Results are expressed as % compared to the levels in post-extracted rat hepatocytes : 24h after adhesion (column A) and after 24, 48 and 72h of perfusion (column B) (mean ± SEM; number of replicates are indicated in each column).

**Table 3 : Substrates and metabolites investigated in IDCCM after 24h of perfusion.** n.d : not detected.

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