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Membrane bioreactor using pig hepatocytes for in vitro evaluation of anti-inflammatory drugs

Loredana De Bartolo [a,*], Simona Salerno [b], Lidietta Giorno [a], Sabrina Morelli [a], Giuseppe Barbieri [a], Efrem Curcio [c], Maria Rende [a], Enrico Drioli [a,c]

[a] *Institute on Membrane Technology, National Research Council of Italy, ITM-CNR, C/o University of Calabria, via P. Bucci cubo 17/C, I-87030 Rende (CS), Italy*
[b] *Department of Pharmacobiology, University of Calabria, Rende (CS), Italy*
[c] *Department of Chemical Engineering and Materials of University of Calabria, via P. Bucci cubo 45/A, 87030 Rende (CS), Italy*

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Abstract

Membrane bioreactor using isolated hepatocytes is a good system for in vitro studies of cell drug metabolism. In this system cells are cultured on modified polyethersulfone membrane under homogeneous, stable and three-dimensional conditions in a similar in vivo microenvironment. The aim of this study was to evaluate liver specific functions of pig hepatocytes cultured in the bioreactor at increasing drug concentration of rofecoxib. Rofecoxib is a potent and highly inhibitor of cyclooxygenase-2 (COX-2), an inducible isoform of cyclooxygenase that plays a key role in inflammatory processes. The bioreactor performance was evaluated by assessing the ability of liver cells to eliminate rofecoxib. Biochemical activity of cells was assessed in terms of urea and albumin synthesis.

Pig liver cells cultured in the membrane bioreactor exhibited viability and liver specific functions at higher levels than those of cells cultured in batch systems. The rate of rofecoxib elimination by cells increased with increasing rofecoxib concentration in the range 0–400 nM. Hepatocytes exhibited highest rates of albumin production and urea synthesis at 300–400 nM rofecoxib concentration.

Keywords: Hepatocytes; Membrane bioreactor; Rofecoxib; Liver specific functions; Drug metabolism

1. Introduction

Isolated hepatocytes may be able to undertake the full range of known in vivo biotransformation [1–3]. They are capable of supporting all essential hepatic functions, including synthetic and drug biotransformation functions and may supply biologically active substances that promote the regeneration and repair of the damaged liver being supported [4]. A limiting problem is that in vitro hepatocytes lose their metabolic functions with time, especially when they are cultured in traditional static systems characterised by an unstirred medium layer overlying cells attached usually to a gas impermeable substratum. In batch system, cells are exposed to continuous changes of nutrient concentration and catabolite accumulation as function of time. The development of dynamic culture systems such as the membrane bioreactor, permits to culture cells in a similar in vivo microenvironment and to overcome the limitations of batch culture systems. In recent years numerous bioreactor hepatocytes devices using different materials and configurations have been developed [5–11]. Among these devices membrane bioreactor is particularly attractive because membranes of suitable molecular weight cut-off allow the specific transport of metabolites and nutrients to cells and the removal of catabolites and specific products from cells. Furthermore, membranes act also as support for adhesion of anchorage-dependent cells [12–14]. However, still, not all of the important liver functions can yet replicated at desired levels. Hepatocyte culture systems that appear to offer longevity also add a layer of complexity to interpretation of metabolic studies where outcomes can depend on matrix nature, fluid dynamics, mass transport, etc., prompting to continue the development of dynamic systems.

The request to have a reliable in vitro physiological model with great control of variables for studying disease drug

* Corresponding author. Tel.: +39 0984 492036; fax: +39 0984 402103.
E-mail address: l.debartolo@itm.cnr.it (L. De Bartolo).

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infection and molecular therapeutics led us to develop a flat membrane bioreactor for hepatocytes culture. A distinguishing feature of the developed bioreactor is that it has a modelled fluid dynamics that ensure a uniform concentration of metabolites at the cell site owing to well mixing. The bioreactor behaviour is similar to that an ideal continuous stirred tank reactor (CSTR) whose fluid dynamics provide a direct estimate of the rate at which a metabolite is produced or consumed by cells. Therefore, the metabolite concentration at the cell site is easily measured and controlled. The aim of this study was to evaluate the effect of a specific anti-inflammatory drug used as a model to evaluate liver specific functions of pig hepatocytes cultured in a membrane bioreactor, under homogeneous conditions as a similar in vivo microenvironment. Pig hepatocytes were used since they perform liver specific functions comparable to those of a human liver [15].

The anti-inflammatory drug used as model was Rofecoxib o (3-phenyl-4-[4-(methylsulfonyl)phenyl]-2-(5*H*)-furanone), which is a potent and highly inhibitor of cyclooxygenase-2 (COX-2), an inducible isoform of cyclooxygenase that plays a key role in inflammatory process and that is over expressed in many kind of tumour. Despite of numerous studies conducted in vitro to evaluate rofecoxib activity on human and animal tumour cell line [16–22], only few papers are present in literature about rofecoxib activity on primary cells. Callejas et al. reported the effect of an analogue of rofecoxib on primary hepatocytes and on a hepatoma cell line, demonstrating that this compound does not alter in vitro cell viability [23]. Many in vivo studies were conducted to evaluate the disposition and metabolism of rofecoxib in laboratory animals and humans [24–26]. The three metabolic pathways of rofecoxib biotransformation observed in vivo (oxydative, reductive and back reduction) were replicated in vitro using only liver subcellular fraction and redox cofactors [27,28].

Few information is present in literature concerning the elimination of rofecoxib and its effects on hepatocytes metabolism in a culture system. We evaluated the effect of rofecoxib that was used as a model of anti-inflammatory cyclooxygenase inhibitor drug, on liver specific functions with respect to albumin and urea synthesis as well as secretion of total proteins in a membrane bioreactor.

2. Experimental

2.1. Membranes for cell culture

Flat polyethersulfone (PES) membranes with 0.1 μm pore size (PALL Corporation, Michigan, USA) modified by plasma process and immobilization of galactonic acid were used for pig hepatocyte culture in the bioreactor. The membrane morphology was characterised with respect to average pore size, porosity and thickness. The wettability of the membrane surface was characterized by means of water contact angle (WCA) measurements. Contact angle of water droplets were measured at room temperature with a CAM 200 contact angle meters (KSV Instruments Ltd., Helsinki, Finland). WCA measurements were performed in standardised conditions, which take into account various parameters (e.g., temperature, clean of sample, drop volume). The instrument supported by video camera and software permits precise drop measurements and evolution in the time.

2.2. Porcine hepatocyte isolation

Liver cells were isolated from slaughtered pigs weighing from 20 to 25 kg at a local slaughterhouse. The liver piece was removed from animal and pre-perfused with 1 L of Ringer lactate at 4 °C. After transportation to the laboratory in an ice-cold sterile Ringer lactate, the liver piece was perfused in single-pass at 80 mL min^{-1} with 1 L of oxygenated buffer I consisting of 8.3 g L^{-1} NaCl, 0.5 g L^{-1} KCl, 2.4 g L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.19 g L^{-1} ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA) (Sigma, St. Louis, MO, USA) at 37 °C. This was followed by perfusion with 1 L of oxygenated buffer II containing 8.3 g L^{-1} NaCl, 0.5 g L^{-1} KCl, and 2.4 g L^{-1} HEPES (Sigma, St. Louis, MO, USA) at 37 °C. Thereafter, 400 mL of oxygenated buffer III containing 3.9 g L^{-1} NaCl, 0.5 g L^{-1} KCl, 2.4 g L^{-1} HEPES and 0.7 g L^{-1} $CaCl_2$ (Sigma, St. Louis, MO, USA), supplemented with 130 U mL^{-1} collagenase type IV (Biochrom, Berlin, Germany) was recirculated for 18 min at 70 mL min^{-1}, as described previously [3]. Upon disruption of the liver capsule, the cells were liberated in an ice cold buffer IV containing of 9.91 g L^{-1} Hanks buffered salt without calcium and magnesium, 2.4 g L^{-1} HEPES and 2.0 g L^{-1} bovine serum albumin. The resulting cell suspension was filtered through a nylon mesh with 100 μm pore size and then centrifuged at 800 rpm for 10 min. The cell pellet at the bottom of the tubes was washed three times with buffer IV at 4 °C. The viability of the hepatocytes ranged between 90% and 95% and was assessed by trypan blue exclusion. An average of 3×10^9 cells was obtained from one isolation procedure.

2.3. Liver cell culture

The isolated hepatocytes were seeded on modified PES membranes to give a surface concentration of 7×10^4 cells cm^{-2} and were incubated in Williams medium (Sigma, USA) supplemented with 5% foetal bovine serum, prednisolone 0.76 μg mL^{-1}, glucagone 0.133 μg mL^{-1} insulin 0.18 U mL^{-1} (Sigma, USA), penicillin 200 U mL^{-1}, L-glutamine 250 U mL^{-1} and streptomycin 200 μg mL^{-1} (Biochrom, Berlin, Germany). The isolated hepatocytes were seeded in the membrane bioreactor and were incubated in Williams medium supplemented with 5% foetal bovine serum. After the liver cells were adhered, the bioreactor was perfused with oxygenated medium containing rofecoxib without serum. Hepatocytes were cultured also in batch system on membrane and on collagen. This latter was used as reference substratum. Type I lyophilised collagen from rat tail (Roche Diagnostics, Mannheim, Germany) was dissolved with sterile acetic acid to the final concentration of 2 mg mL^{-1}; pH was adjusted to 7.4 with 10× concentrate Dulbecco's modified Eagle medium diluted 1:10

with the collagen solution. Solution of collagen gel was added to obtain a coating density of 5 μg cm^{-2}. Cells and controls were incubated at 37 °C in a 5% CO_2; 20% O_2 atmosphere (v/v) with 95% relative humidity for the duration of the experiments.

Experiments were performed at increasing drug concentrations in the culture medium ranging from 0 to 800 nM.

The effect of rofecoxib on liver cell metabolism was evaluated in terms of albumin production, urea synthesis and secretion of total proteins.

2.4. Membrane bioreactor

The membrane bioreactor inserted in the perfusion system was used for pig liver cell culture. The bioreactor consists of a circular acrylic housing (volume 65 cm^3) fitted with inlet and outlet ports. At the bottom of chamber put up a semipermeable membrane with the active area of 41.8 cm^2. The bioreactor was connected to the perfusion system consisting of a glass medium reservoir, tubing, oxygenator and two microperistaltic pumps (Fig. 1). The medium from reservoir enters before the oxygenator where it is heated and oxygenated, then the medium flows into the membrane bioreactor. The oxygenated medium containing rofecoxib was continuously fed to the cells adhered on the membrane in the bioreactor with flow rate Q of 0.6 mL min^{-1} that was set on the basis of average retention time; a fraction of the stream leaving the bioreactor Qr of 4.5 mL min^{-1} was recycled to mix with the stream of fresh medium, Q, before entering the bioreactor. The investigation was aimed to establish the operating conditions that ensure uniform metabolite concentration in the bioreactor whose fluid dynamics resemble that of a continuous stirred thank reactor. The bioreactor fluid dynamics were characterised by tracer experiments. The bioreactor without cells was challenged by changing the tracer concentration stepwise in the feed stream (C_{in}) and the outlet concentration (C_{out}) was continuously monitored by on-line spectrophotometer (UV Cord Pharmacia, Uppsala, Sweden). As tracer we used trypan blue dissolved in bidistilled water. The bioreactor fluid dynamics was characterised in terms of the cumulative residence time distribution (RTD) to a step inputs:

$$F\left(\frac{t}{\tau}\right) = \frac{C_{out}}{C_{in}} \tag{1}$$

where t is the actual time and τ is the mean residence time.

The agreement of the tracer response with that of a CSTR was assessed by a comparison of the experimental data with the theoretical curve:

$$F\left(\frac{t}{\tau}\right) = 1 - e^{-t/\tau} \tag{2}$$

Under the chosen operating conditions the bioreactor is well mixed: as a result the metabolite concentration in the bioreactor is uniform and equal to that in the stream leaving the bioreactor. The metabolic rate of a given metabolite by the hepatocytes cultured in the membrane bioreactor was estimated directly from the metabolite concentration in the inlet and outlet streams according to the steady state mass balance about the bioreactor as the following equation:

$$-r = \frac{Q(C_{in} - C_{out})}{V} \tag{3}$$

where Q is the flow rate, V is the volume of the bioreactor, C_{in} is the metabolite concentration in the feed stream and C_{out} in the outlet stream.

2.5. HPLC analysis of Rofecoxib

The HPLC method with fluorescence detector was used to analyse rofecoxib elimination [29]. A C_{18}-RP Purosphere Star 5 μm, 250 mm × 4.6 mm column equipped with a precolumn was used. The samples from the bioreactor were mixed with acetonitrile (ACN) (1:1) and vortexed for 20 min at 9500 rpm. The supernatant was filtered through a 0.2 μm membrane and

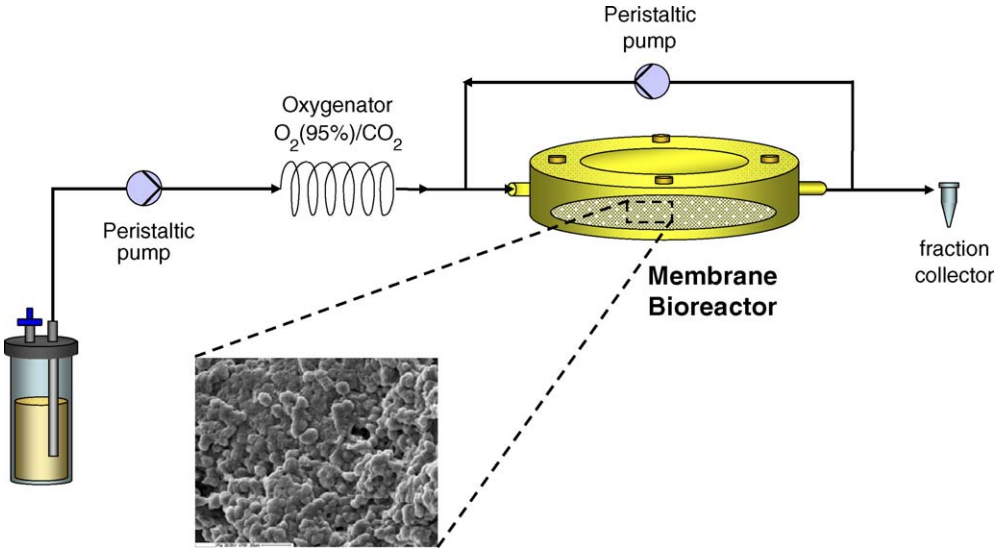

Fig. 1. Scheme of the membrane bioreactor connected to the perfusion system.

irradiated at 254 nm before injection in column. Upon exposure to UV light, the analyte was found to undergo a stilibenephenanthrene-like photocyclisation reaction with the resulting formation of a highly fluorescent species. The sample injection volume was 150 μL. The mobile phase consisted of a mixture of ACN–water (35:65, v/v) was delivered at a flow rate of 1.2 mL min^{-1}. The column was operated at ambient temperature. The fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 375 nm.

2.6. Gel electrophoresis of proteins

Proteins from medium collected by hepatocytes treated with rofecoxib were separated by one-dimensional native-PAGE on an 8–25% PhastGel™ gradient using buffer strips. The 8/1 μL sample applicator was used (Amersham Biosciences, UK). The gel has a continuous 8–25% gradient gel zone with 2% crosslinking. The buffer system in the PhastGel Native Buffer Strip is of 0.88 M L-alanine and 0.25 M Tris, pH 8.8. Each sample was loaded onto separate lane of the gel containing 1 μL of sample. The gels were stained with Coomassie blue and then destained with 30% methanol and 10% acetic acid in distilled water. The solution for preserving the gels contained 10% glycerol and 10% acetic acid in water.

The gel images captured by a scanner were analysed by Image Quant TL Software (Amersham Biosciences, UK), which permitted band molecular weights (MWs) and concentration to be identified. The estimation of protein MW was calculated by using the molecular size calibration mode in a gel image containing standard MWs. The MWs of the proteins contained in the culture medium were calculated from the logarithm curve fitting, which relates the standard MWs with the relative mobility as pixel position by using calibration Kit proteins (HMW and LMW, Amersham Biosciences, UK). The amounts of proteins identified in the gels were calculated from the quantitative calibration curve, which relates the band volume and the image intensity to protein amount by using standard proteins.

2.7. Biochemical assays

Samples of the culture medium were collected from bioreactor and batch cultures in pre-chilled tubes and stored at −20 °C until assayed. The protein content in the samples was determined by protein assay using bicinchoninic acid solution (Sigma, St. Louis, MO, USA) by spectrophotometer analysis. The urea concentration was assayed by the enzymatic urease method (Sentinel, Milano, Italy).

Albumin secretion was measured in the samples by immunoenzymatic method (ELISA) [30] with the modification that antibodies against pig albumin and pig albumin were used (Sigma, St. Louis, MO, USA). ELISAs were done on cells of six different isolations. Chromatographically purified porcine albumin and the monoclonal antibody for porcine albumin were from Bethyl (Bethyl Laboratories Inc., USA). Ninety-six well plates were coated with 50 μg mL^{-1} of albumin and left overnight at 4 °C. After washing the Plates four times with phosphate buffer solution (PBS) and Tween 20 (TPBS), 100 μL of cell culture supernatant was added to each well and incubated with 100 μL of anti pig albumin antibody conjugated with horseradish peroxidase (Bethyl Laboratories Inc.). After 24 h at 4 °C, the substrate buffer containing Tetramethylbenzidine and H_2O_2 (Sigma, St. Louis, MO, USA) was added for 7 min. The reaction was stopped with 100 μL of 8 N H_2SO_4. Absorbance was measured at 450 nm using a Multiskan Ex (Thermo Lab. Systems).

The statistical significance of the experimental results was established according to the Unpaired Statistical Student's *t*-test ($p < 0.05$) and ANOVA test.

3. Results

Tracer experiments were performed to investigate the bioreactor fluid dynamics closely resemble that of a CSTR. An average residence time of the fluid inside the bioreactor was chosen in order to obtain a cell metabolic conversion appreciable by using the available analytic instruments. At the residence time of 1.8 h corresponded an inlet flow rate significantly low that provided an inadequate degree of mixing inside the bioreactor. For this reason we recycled part of the stream leaving the bioreactor back to the inlet where it mixed with fresh medium before entering the bioreactor. Tracer experiments were performed at increasing recycle ratios (*R*) to establish the value of *R* that would ensure a uniform metabolite concentration in the bioreactor. As is shown in Fig. 2, for a trypan blue challenge, after a relatively short transient, the dye concentration in the stream leaving the bioreactor attained a steady state that remained constant through out the duration of experiment. The operation conditions chosen with a value of *R* of 7.5 gave a good mixing without any chanelling or stagnant

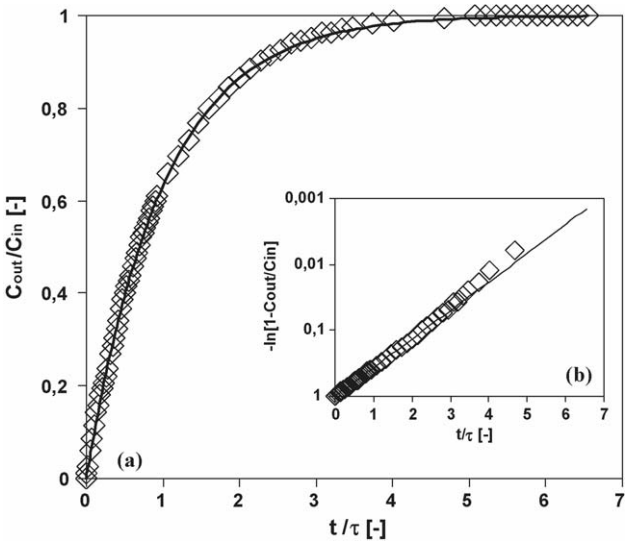

Fig. 2. Cumulative RTD of the bioreactor following a step change of trypan blue concentration in the perfusion medium. (a) Approach to a steady state of the fractional exit concentration for $Q = 0.6$ mL min^{-1} and $Qr = 4.5$ mL min^{-1}: (◇) experimental data; (-) prediction for a CSTR of equal volume. (b) Cumulative RTD data rearranged according to Eq. (2) with a straight line.

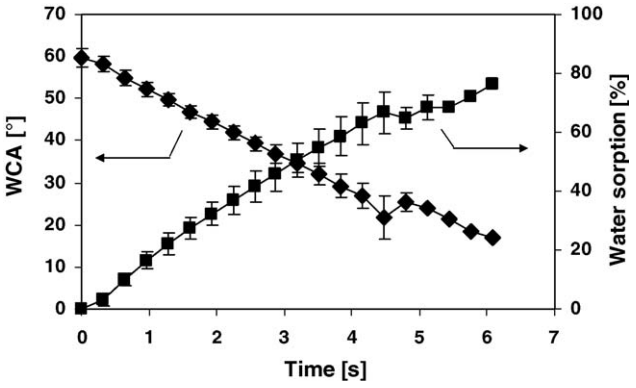

Fig. 3. Time-related water contact angles (◆) and water sorption (■) measured on modified membranes. The reported values are the mean of 30 measurements of different droplets on different surface regions of each sample ± standard deviation.

zone. For a trypan blue challenge the cumulative RTD agreed well with that theoretically predicted for a CSTR (Eq. (1)) as is confirmed also by the agreement of experimental data rearranged according to Eq. (2) with a straight line. On the basis of this result the characterisation of cell metabolic reactions was performed by operating the bioreactor in recycle mode with an inlet flow rate of 0.6 mL min^{-1} and a recycle flow rate of 4.5 mL min^{-1}.

Before culture experiments morphological and physico-chemical characterisation of modified PES membrane was carried out in order to establish the surface properties of the membrane which are important for cell attachment and consequent reorganisation. The modified PES membrane surface has pores with a mean diameter of 0.1 μm regularly distributed on the surface. The symmetric membrane has 72.5 ± 0.19% porosity and a thickness of 190 ± 15 μm.

Time-related contact angle and water sorption measurements (Fig. 3), show that modified PES is a very wettable surface in fact the water contact angle measured on this membrane was 59.7° ± 2.3° at $t = 0$ s, and the increase of water sorption was about 76% after 6 s.

These results validated its use for hepatocyte culture because the small pores present on the surface offer points for cell anchorage and the high porosity increased the surface available for cell adhesion.

In the membrane bioreactor isolated cells rapidly attached and formed small clusters. They spread within 24 h. After 1 day hepatocytes established cell-to-cell contacts with near cells and exhibited a polygonal shape.

The ability of hepatocytes to perform metabolic functions, in terms of albumin and urea synthesis, as well as secretion of total proteins, was evaluated in both membrane bioreactor and batch system. In batch system, the metabolic activity of pig hepatocytes was estimated on collagen gel and on the membrane surfaces (Fig. 4a and b). The ability of hepatocytes to synthesise urea was higher on the membranes at the drug concentration of 400 nM (difference statistically significant: $p < 0.05$, Student's t-test) with respect to the control and the samples treated with rofecoxib 800 nM. Fig. 4a shows a comparison of urea synthesis of hepatocytes after 2 days of

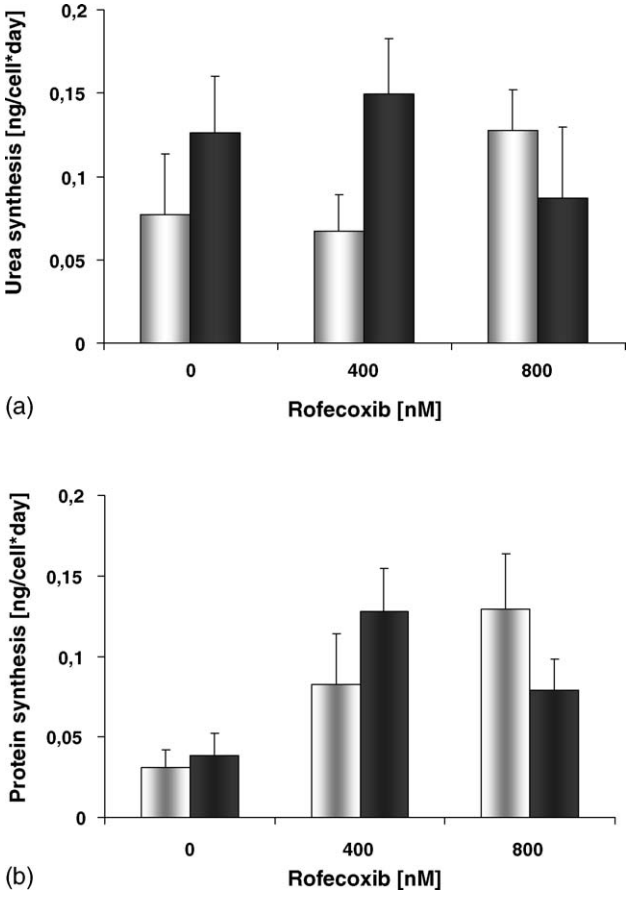

Fig. 4. Effect of rofecoxib on Urea synthesis (a) and total protein synthesis (b) of pig hepatocytes after 2 days of culture in batch system on collagen (shade bar) and on modified PES membranes (full bar). The values are the mean of six experiments ± standard deviation.

culture on PES membranes and on collagen gel: the ability of hepatocytes to synthesise urea was higher on the membrane than that exhibited by cells on collagen treated and untreated with rofecoxib 400 nM.

The exploration of metabolic activity of pig hepatocytes was continued also in terms of total protein synthesis. Cells incubated with rofecoxib 400 and 800 nM secreted proteins to a larger extent with respect to those cultured without drug. On collagen gel total protein synthesis had a proportional increasing with increasing concentration of rofecoxib in the culture medium with a significant level of $p = 0.003$ (ANOVA test). A comparison of total proteins secreted by cells on collagen and on the membranes after 2 days of culture evidenced the different behaviour of cells on the different substrates (Fig. 4b). Identification of the proteins secreted by cells cultured on membrane and on collagen gel by native-PAGE gel electrophoresis is reported in Fig. 5a and b.

The separation of proteins synthesised by cells cultured on the membrane through native-PAGE gel (Fig. 5a) evidenced a marked band with 66 kDa corresponding to MW of albumin. Other bands with higher MW in the range of 120–440 kDa were observed. The intensity of these bands was very marked for cells incubated with rofecoxib 400–800 nM. Quantitative analysis confirmed that albumin was the protein to a large

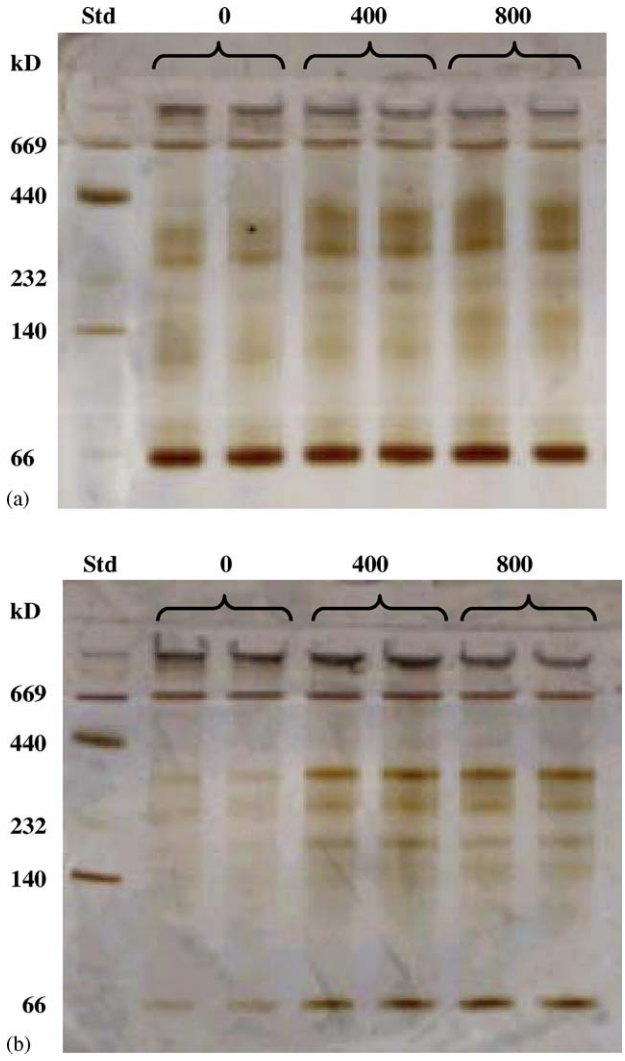

Fig. 5. Native-PAGE with PhastGel gradient 8–25% of medium samples collected by pig hepatocytes cultured on the membrane (a) and on Collagen gel (b) in batch system in absence and in presence of rofecoxib 400 and 800 nM at day 2 of culture.

extent synthesised by cells. Proteins with high MW (281 and 300 kDa) increased with increasing concentration of rofecoxib. Also native-PAGE gel electrophoresis of cell samples on collagen evidenced an increase of bands which identified proteins with MW ranging from 120 to 440 kDa at high concentration of rofecoxib (Fig. 5b). Quantitative analysis evidenced as these bands were very marked with increasing concentration of rofecoxib while on membrane there were no differences between drug-treated and untreated samples.

Liver specific functions of pig hepatocytes cultured in the membrane bioreactor on modified PES membrane were investigated at increasing drug concentration.

As shown in Fig. 6, the rate of albumin synthesis increased with increasing doses of rofecoxib, and reached maximal values of 0.047 pg h^{-1} $cell^{-1}$ at the concentration of 400 nM. The effect of rofecoxib was evaluated also on urea synthesis. Hepatocytes displayed similar behaviour with respect to urea synthesis: cells synthesise urea with rates that increased with increasing drug concentration passing through a maximal value

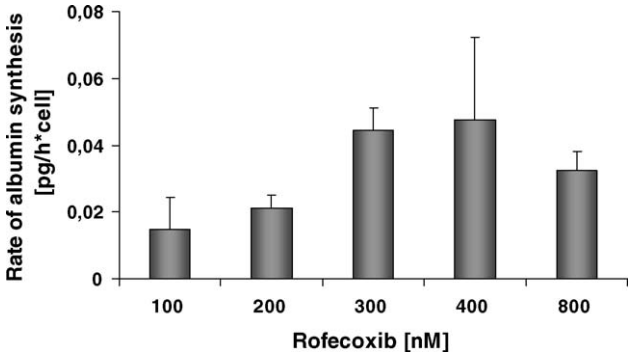

Fig. 6. Rate of albumin synthesis by pig hepatocytes cultured in membrane bioreactor related to the rofecoxib concentrations. The values are the mean of six experiments ± standard deviation.

of 0.65 ng h^{-1} $cell^{-1}$ corresponding to the concentration of 400 nM (Fig. 7).

The changes of total proteins found in the culture medium when cells were cultured at increasing concentration of rofecoxib revealed an effect of drug also on protein secretion function. Hepatocytes secreted proteins with highest rates reaching values of 4.65 ng h^{-1} $cell^{-1}$ when they were cultured at concentration of 300 nM and metabolic rates decreased markedly at drug concentration of 800 nM (Fig. 8). This metabolic function takes into account all proteins secreted by

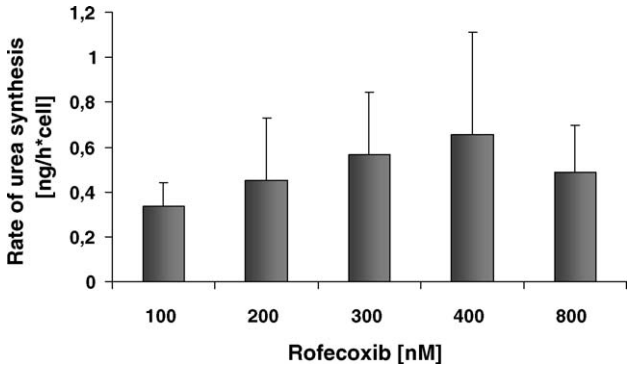

Fig. 7. Rate of urea synthesis by pig hepatocytes cultured in membrane bioreactor related to the rofecoxib concentrations. The values are the mean of six experiments ± standard deviation.

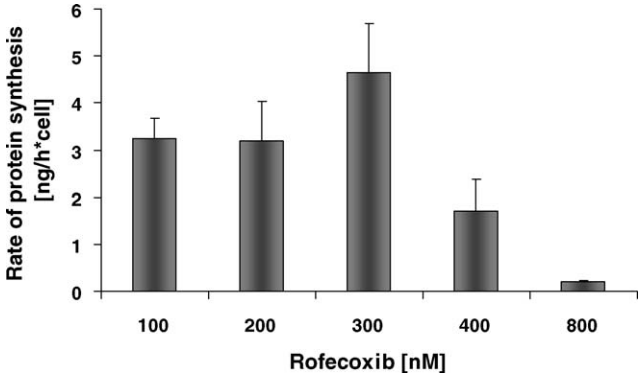

Fig. 8. Rate of total protein synthesis by pig hepatocytes cultured in membrane bioreactor related to the rofecoxib concentrations. The values are the mean of six experiments ± standard deviation.

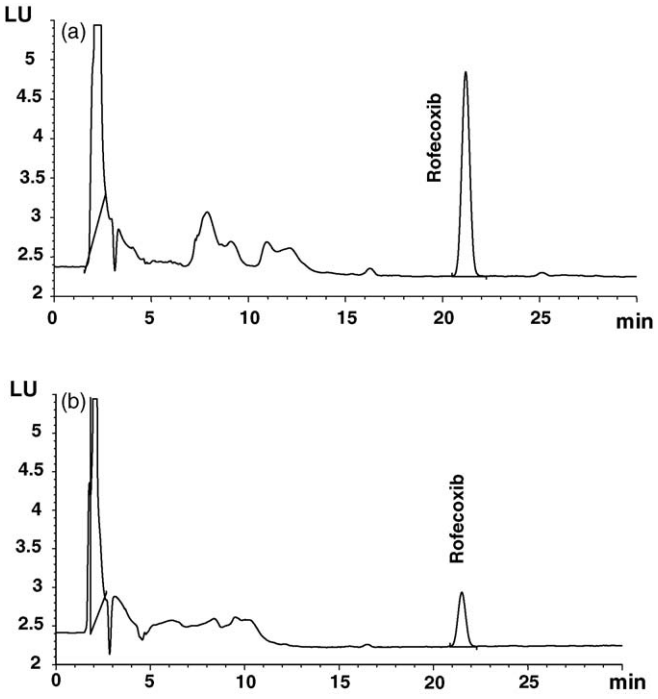

Fig. 9. HPLC chromatograms of rofecoxib elimination by pig hepatocytes cultured in the membrane bioreactor: (a) control medium from bioreactor without cells; (b) medium from bioreactor with cells.

cells including proteins involved in the cell adhesion and reorganisation process which occurs during the first day of culture.

The capacity of hepatocytes cultured in the membrane bioreactor to metabolise rofecoxib was also investigated. A peak with retention time of 21.5 min was assigned to rofecoxib, which was identified by injection of rofecoxib standard solution. A comparison of HPLC chromatograms at 21.5 min retention time corresponding to rofecoxib of the control medium (Fig. 9a), and the medium from bioreactor with cells (Fig. 9b) evidenced that the peak corresponding to rofecoxib, in the presence of cells, decreased about 50% with respect to the control at rofecoxib concentration of 100 nM. The rate at which the hepatocytes eliminated rofecoxib increased with increasing

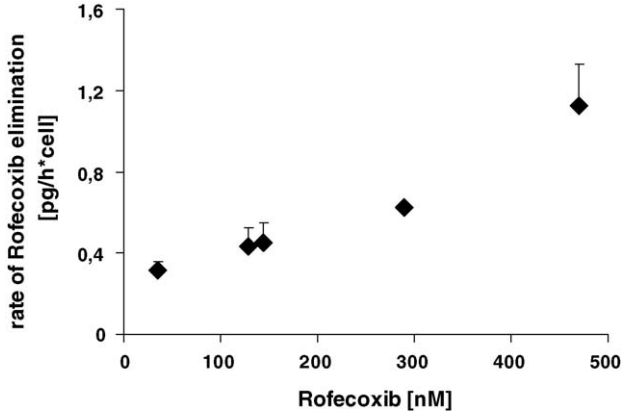

Fig. 10. Rate of rofecoxib elimination by pig hepatocytes cultured in the membrane bioreactor related to the investigated drug concentrations. The values are the mean of six experiments ± standard deviation.

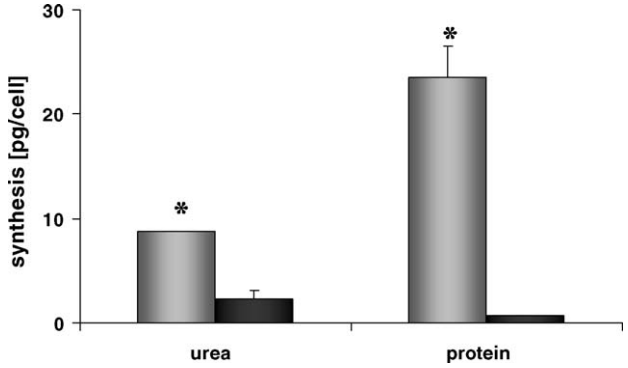

Fig. 11. Comparison of metabolic functions of pig hepatocytes at day 2 of culture in the membrane bioreactor (shade bar) and in the batch system on modified PES membrane (full bar) with rofecoxib 400 nM. The data represent the urea and proteins produced in 24 h of culture. The values are the mean of six experiments ± standard deviation. * Data statistically significant with respect to the batch ($p < 0.01$).

rofecoxib concentrations in the culture medium in the range 0–500 nM (Fig. 10).

A comparison of the batch and membrane bioreactor systems in terms of urea and total protein synthesis by pig hepatocytes at the 2nd day of culture at the concentration 400 nM of rofecoxib, is shown in Fig. 11. Liver specific function were expressed at very high levels in the bioreactor with high significant level ($p < 0.01$, Student's t-test). A substantial difference was measured also in terms of albumin synthesis. Hepatocytes cultured in the membrane bioreactor displayed an enhanced albumin synthesis (1.14 pg cell^{-1}) with respect to the batch system.

4. Discussion

The membrane bioreactor designed in this study permits to culture liver cells under controlled fluid dynamics conditions which make estimation of the metabolic rate unaffected by mass transport and easier and more reliable. The bioreactor was designed to permit the culture of hepatocytes adhered to the membrane and the fluid dynamics were modelled to resemble those of a CSTR. Therefore, the metabolite concentration at the cell compartment would equal that in the outlet stream and could be determining by assaying the effluent. In this case, the steady-state metabolite concentration difference between inlet and outlet streams to the bioreactor provides a direct estimation of the metabolic reaction rate at a given metabolite concentration.

Modified PES membranes have properties that fulfil many of the requirements of a biomaterial. In addition to exhibiting high surface wettability, which favours interactions with the cells [13,31,32] this membrane has a porous surface that offers cells more anchorage points for their adhesion.

This study demonstrated the feasibility of the membrane bioreactor in maintaining functional integrity of pig hepatocytes cultured in a similar in vivo microenvironment, therefore it allowed to study the effect of drugs on hepatocyte metabolism in a controlled system.

Rofecoxib was used as a model drug to investigate the capacity of the bioreactor to perform drug biotransformation functions. Connected to perfusion system, membrane bioreactor allows the continuous feeding of nutrients and metabolites and the simultaneous catabolite removal together with specific cell products. In this system, cells were exposed at constant concentrations of oxygen, nutrients and metabolites thus sustaining cell viability and functions in the time.

Pig hepatocytes, the most similar to human hepatocytes for metabolic functions, were cultured in adhesion on modified PES membrane that represents a good surface favouring the anchorage-dependent hepatocyte property. In batch, pig hepatocytes were also cultured on collagen gel as control being the natural substratum for liver cells.

Pig hepatocytes cultured on membranes without and with Rofecoxib at concentration of 400 nM synthesised urea and proteins at higher levels with respect to those on collagen gel. The capacity of the membranes to provide an adequate mechanical and chemical support for cell culture, influencing cell adhesion, viability and metabolic rates of isolated hepatocytes was also confirmed in previous studies [12–14]. The liver specific functions of pig hepatocytes in the bioreactor were maintained at different concentration of rofecoxib: cells exhibited the highest metabolic rates at drug concentration of 300–400 nM as shown in Figs. 6–8.

Hepatocytes cultured in the membrane bioreactor were able to biotransform rofecoxib at a rate that increased with increasing drug concentration in the range between 100 and 500 nM, achieving no saturation values (Fig. 10). In literature, the estimated kinetic parameters for rofecoxib metabolism using appropriate liver subcellular fraction and redox cofactor indicated saturation rates at higher concentration than those used in our study. In particular, for the glucuronidation of 5-hydroxyrofecoxib was estimated a value of $K_M = 44.2$ μM [28], and for NADPH-dependent reduction of rofecoxib to the isomeric 3,4-dihydrohydroxy acid (DHHA) metabolites were estimated K_M values of 90, 214 and 164 μM for *erythro*-DHHA, *threo*-DHHA and *erythro*- + *threo*-DHHA, respectively [27]. Many studies in vitro on human and animal tumour cell line demonstrated that these concentrations have an apoptotic effect by changing gene expression favouring the cell cycle arrest [16]. In order to preserve pig hepatocytes cultured in the bioreactor and to estimate the metabolic rates without any possible interference by high concentration of rofecoxib, we evaluated the rofecoxib elimination in the range 0–500 nM thus avoiding apoptotic effect.

Rofecoxib is metabolised by CYP1A2 and CYP3A4 isoenzymes of P450 cytochrome [27]. These enzymes are among the most sensitive and fragile found in hepatocytes, responding quickly to unfavourable culture conditions with loss of activities. The enhanced biotransformation ability of pig hepatocytes cultured in the membrane bioreactor is also an indication of the high-energy status of cells and of high efficiency of the membrane bioreactor.

A comparison of the metabolic functions of cells cultured in batch system with those cultured in the membrane bioreactor evidenced as liver specific functions were expressed at high levels in the bioreactor system.

Cells in batch system resulted to be metabolically less active than the corresponding cells in the bioreactor. This is not completely unexpected because in the batch system cells are exposed to change of metabolite concentration in the time and cell products together including catabolites are accumulated. For liver cells which are highly perfused in vivo, such conditions are susceptible to oxygen and nutrient limitations with consequent reduction of cell viability and functionality. Furthermore, in the batch system, it is difficult to obtain reliable information on cell metabolism because concentration changes of a given metabolite measured cannot be related completely to cell metabolism: in fact, other factors, as the development of concentration gradients in culture medium resulting from the diffusive resistances to mass transport or of poor mixing, affect reaction rates.

On the contrary, in the bioreactor oxygenated fresh medium containing nutrients and metabolites is continuously supplied to the cells and catabolites and spent medium are continuously removed from them, as it occurs in vivo. These results sustain the applicability of the bioreactor as model system that permits to culture cells in a well-defined fluid dynamics microenvironment overcoming the limitations of batch culture systems. Furthermore, the estimated metabolic reaction rates are reliable and related to the effective concentration of the substrate because the metabolite concentration at the cell site is easily measured and controlled. Overall the results demonstrated that the developed membrane bioreactor improves the process oxygenation helps in end-products removal and optimise the distribution of fluid molecules inside the cell environment improving cell maintenance.

5. Conclusions

We evaluated the performance of cells cultured in a membrane bioreactor that was developed to assess the drug biotransformation by isolated hepatocytes. This study demonstrated that hepatocytes cultured in the membrane bioreactor exhibited liver specific functions at higher levels than those of cells cultured in batch systems. In particular, rofecoxib elimination by cells increased with increasing drug concentration in the range 0–500 nM. The rates of urea and albumin synthesis and the maintenance of cytochrome P450 demonstrated the good performance of the membrane bioreactor for in vitro studies of cell drug metabolism.

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