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Polyethersulfone membrane biohybrid system using pig hepatocytes: Effect of diclofenac on cell biotransformation and synthetic functions

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Abstract

A biohybrid system constituted of primary pig hepatocytes and microporous synthetic membranes was used to investigate the diclofenac biotransformation and its long-term effect on hepatocyte specific functions. The maintenance of drug biotransformation functions and the effect of diclofenac continuous administration on the viability and metabolic functions of hepatocytes have been explored.

Pig hepatocytes were isolated from slaughterhouse organs and were cultured on polyethersulfone membranes and on collagen. The ability of cells to eliminate diclofenac and to synthesise metabolites was evaluated by an HPLC analysis. Urea and albumin productions were established as indicators of differentiated hepatocyte functions.

Pig hepatocytes eliminated diclofenac and produced two main metabolites: 4′-hydroxydiclofenac (4′-OHdic) and *N*,5-dihydroxydiclofenac (*N*,5-$(OH)_2$dic). Cells continuously exposed to subtoxic drug concentrations maintained their biotransformation functions until 10 days of culture. The rate of diclofenac elimination increased according to a saturation kinetics.

Diclofenac showed a cytotoxic effect at a concentration higher than 100 μM as confirmed by analysis of DNA fragmentation. The urea and albumin synthesis as well as the total protein production were also affected by diclofenac concentrations. An important concentration-dependent effect of diclofenac on protein synthesis was observed.

The results give information concerning the effect of diclofenac on specific functions of pig hepatocytes continuously exposed to the drug and on the long-term ability of cells to metabolise diclofenac in a membrane biohybrid system.
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1. Introduction

The development of biohybrid systems using cells and materials is important for the in vitro and in vivo reconstruction of organ/tissues. Information concerning cell response to the biomaterial in terms of maintenance of differentiated functions is required for the development of the biohybrid system.

As a good model of liver metabolism, isolated hepatocytes must ideally be able to undertake the full range of known in vivo synthetic, biotransformation and detoxification functions [1,2]. Thus, the isolation and maintenance of viable hepatocytes in vitro is desirable so that metabolic liver functions can be studied alternatively to animal experimentation in the establishment of new drugs or therapy that may cure patients. Hepatocytes are anchorage-dependent cells that need to adhere to the substratum for the maintenance of their cellular functions. Semipermeable membranes in fibre and flat configurations, owing to their functional characteristics, such as selective permeability, stability and cytocompatibility are among the most attractive biomaterials for cell culture in biohybrid systems [3–5]. Synthetic porous polyethersulfone (PES) membranes have been used in the biomedical field in applications, such as hemodialysis, filtration and ultrafiltration [6,7]. PES membranes could constitute a good mechanical and chemical support for cell adhesion because of their physico-chemical and morphological properties although

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few studies have been conducted to determine whether cells grow on PES.

We studied diclofenac biotransformation and its long-term effect on hepatocyte liver specific functions in a biohybrid system constituted of microporous PES membranes and primary pig isolated hepatocytes, which perform liver specific functions comparable to those of a human liver [8]. Diclofenac is a non-steroidal anti-inflammatory drug widely used in painful and inflammatory rheumatic and non-rheumatic conditions. Studies in animals and man showed that diclofenac undergoes an extensive hepatic biotransformation involving aromatic hydroxylations and conjugations [8–10]. The formation of reactive metabolites by drug oxidation seems to be related to diclofenac hepatotoxicity [11,12]. Previous short-term studies showed that diclofenac could induce cytotoxicity [13–15], apoptosis in hepatocytes [16,17] and kidney DNA fragmentation [18].

Most of the literature data were from experiments carried out during the first 24 h of culture [3–6] using collagen as a culture substrate. No study has been performed yet to identify the effects of diclofenac on specific functions of pig hepatocytes exposed continuously to the drug and on their in vitro long-term ability to metabolise diclofenac. Furthermore, information on the performance of a membrane biohybrid system in the study of the diclofenac effect on the hepatocyte metabolism has been achieved.

The present paper reports on diclofenac biotransformation and its effect on liver specific functions of pig liver cells cultured on PES semipermeable membranes. The morphological and physico-chemical properties of the membrane were investigated, the polymer surface characteristics being important in determining specific cell interactions [19]. The maintenance of drug biotransformation functions of isolated pig hepatocytes and the effect of continuous administration of diclofenac on cell viability (DNA fragmentation) and differentiated functions was explored. In particular the effect of diclofenac was investigated on urea and albumin production as well as on the synthesis of total protein by hepatocytes cultured on PES membranes and on collagen.

2. Materials and methods

2.1. Membranes for cell culture

Flat PES membranes with 0.1 μm pore size (PALL Corporation, Michigan, USA) were used for pig hepatocyte culture. The membrane morphology was characterised with respect to average pore size, porosity and thickness by scanning electron microscopy analysis (SEM). Water flux through the membrane was assessed by means of permeation measurements by using a flux tangential cell. Membranes were also characterised by attenuated total reflection (ATR) spectroscopy in order to identify the specific chemical groups of the polymer. The wettability of the membrane, which is an important parameter for cell adhesion, was characterised by means of water dynamic contact angle (DCA) measurements. The contact angle of water droplets was measured at room temperature with a CAM 200 contact angle meter (KSV Instruments Ltd., Helsinki, Finland). DCA measurements were performed under standardised conditions, which take into account various parameters (e.g., temperature, cleanliness of sample, drop volume). The instrument supported by video camera and software permits precise drop measurements and evolution in time. The DCA of the PES membrane as a function of time was measured. At least 30 measurements on different regions of each sample were averaged for each DCA value. Standard deviations are indicated as error bars.

The water sorption of the membrane surfaces during time was evaluated by following the decrease of drop volume in contact with the surface using the relation:

$$\text{surface water sorption}\,(\%) = \frac{\text{drop volume}_{(t=0)} - \text{drop volume}_{(t=i)}}{\text{drop volume}_{(t=0)}} \times 100.$$

2.2. Porcine hepatocyte isolation

Liver cells were isolated from slaughterhouse pigs weighing from 20 to 25 kg, which were slaughtered at the local slaughterhouse. The liver piece was removed from the animal and pre-perfused with 1 l of Ringer lactate at 4 °C. After transport to the laboratory in an ice-cold sterile Ringer lactate, the liver piece was perfused in single-pass at 80 ml/min 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} HEPES and 0.19 g l^{-1} 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) 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), 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 [20]. 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. Washing buffer (4 °C) is added to the filtered suspension to a final volume of 500 ml, followed by three washing procedures in the same buffer at 13.33 Hz (800 rpm) for 10 min. The centrifugation procedure, repeated three times, allowed liver cell preparation to be obtained, in which about 95–98% of the cells are hepatocytes because they are considerably larger than all other kinds [21]. The viability of the hepatocytes ranged between 90% and 95% and was assessed by trypan blue exclusion. On average, 3×10^9 cells were obtained from one isolation procedure.

2.3. Liver cell culture

The isolated hepatocytes were seeded on PES membranes to give a surface concentration of 7×10^4 cells/cm^2 and were incubated in Williams medium (Biochrom) supplemented with 5% foetal bovine serum, prednisolone 0.76 μg ml^{-1}, glucagone 0.13 μg ml^{-1} (Sigma, Mainz, Germany), insulin 0.16 U ml^{-1}

(Sigma, Frankfurt, Germany), penicillin 200 U ml^{-1} and streptomycin 200 μg ml^{-1} (Biochrom). The culture medium was fully exchanged with fresh medium every 24 h. After 24 h from isolation the culture was continued under serum-free conditions. Hepatocytes were incubated in Williams E medium. Collagen was used as the reference substratum. Type I lyophilised collagen from rat tail (Roche Diagnostics, Mannheim, Germany) was dissolved with sterile acetic acid to a final concentration of 2 mg/ml; pH was adjusted to 7.4 with 10× concentrate Dulbecco's modified Eagle medium diluted 1:10 with the collagen solution. This 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.

The metabolic rates of the investigated hepatocyte reactions were assessed at every change of the culture medium by incubating the cells and controls with diclofenac sodium salt (Sigma) to final concentrations of 100, 300 and 700 μM. Cells were continuously exposed to diclofenac for up to 10 days of culture for each concentration. Every 24 h fresh medium containing diclofenac was replaced.

The effect of diclofenac on liver cell metabolism was evaluated in terms of albumin production, urea synthesis and secretion of total proteins. The viability and proliferation of cells cultured in the presence of diclofenac were determined by DNA analysis and the mitotic index. The effect of diclofenac on cells was evaluated by qualitative and quantitative analysis of DNA fragmentation.

2.4. HPLC analysis of diclofenac and metabolites

The HPLC method was used to analyse diclofenac and metabolites [11]. The samples from the culture medium were precipitated with acetonitrile and centrifuged for 10 min at 8000 × *g*. After dilution with 20 mM phosphate buffer pH 7.4 to reach 25% (v/v) acetonitrile samples were HPLC analysed using C-8 Lichocart 5 μm, 250 mm × 4.6 mm column with precolumn. The mobile phase (75% triethanolamine, 0.02% in 20 mM phosphate buffer pH 7.4, 25% acetonitrile) was delivered at 0.7 ml/min and the effluents were monitored at 282 nm. The column temperature was 55 °C. Ibuprofen was used as the internal standard and diclofenac as the external standard. The chromatographic retention of diclofenac and its metabolites was found by measuring the time between the injection point and maximum of the detector response for the correspondent compound. This parameter is usually called "retention time".

Diclofenac biotransformation was followed by its elimination and the formation of the metabolites 4′-hydroxydiclofenac (4′-OHdic) and *N*,5-dihydroxydiclofenac (*N*,5-$(OH)_2$dic). The diclofenac metabolites were identified by HPLC analysis of chemically synthesised probes (Sigma) and by comparison with literature data.

2.5. DNA fragmentation

The DNA fragmentation of cells exposed to diclofenac was evaluated by means of qualitative and quantitative assays. A fluorometric method by Patel et al. [22] was used to observe the nuclei isolated from hepatocytes by fluorescent microscopy. The isolation of nuclei was performed by the incubation of the hepatocytes in 1 ml of a hypotonic solution containing 0.01 M Hepes, 0.25 mM spermine and 0.5 mM spermidine for 5 min at room temperature. Next, 100 μl of lysis solution (containing 30 ml l^{-1} glacial acetic acid and 50 g l^{-1} ethylhexadecyldimethylammonium bromide) was added and the cells were gently agitated for 15 min at room temperature. The nuclei were then pelleted by centrifugation at 1000 × *g* for 5 min and fixed by resuspension in 1 ml of fixative (1% formaldehyde, 20 mM Hepes in Hanks buffered saline solution, pH 7.0) at 4 °C for 15 min. The nuclei were pelleted, washed twice and resuspended in phosphate-buffered saline solution, as is reported in Patel's method [22]. Terminal deoxynucleotidyl transferase (TDT) was used to enzymatically label 3′-OH DNA ends with fluorescein-12-dideoxyuridine triphosphate (fluorescein-12-ddUTP) in the assay using fluorophore end-labelling. Fluorescein-12-ddUTP end-labelling of DNA in intact nuclei was performed by suspending the nuclei obtained from 10^7 cells in 50 μl of reaction mixture, which consisted of five units of TDT and 10 μM fluorescein-12-ddUTP (Perkin-Elmer, USA), the TDT reaction buffer (Sigma). The samples were then incubated for 35 min at 37 °C. The reaction was terminated by adding 500 μl of stop solution (30 mM sodium chloride, 30 mM sodium citrate) and washed twice with PBS for 5 min at room temperature to remove unbound fluorescent nucleotide. Nuclei were resuspended in phosphate-buffered saline containing 1 μg/ml of 4′,6-diamino-2-phenylindole (DAPI) (Sigma). The samples were observed by fluorescence microscopy. Green (fluorescein-12-ddUTP) and blue (DAPI) fluorescence of individual nuclei was analysed and measured.

The quantitative determination of DNA fragmentation was based on the method of Ray et al. [23]. Colour reaction was based on Burton's method [24]. To measure hepatocyte DNA fragmentation by spectrophotometer, the hepatocytes were lysed by lyses buffer (5 mM Tris–HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.00). The cells were centrifuged at 27,000 × *g* for 20 min to separate intact chromatin in the pellet from fragmented DNA in the supernatant. The pellets were resuspended in 0.5N perchloric acid and the supernatants were treated with concentrated perchloric acid to reach a final concentration of 0.5N. All the samples were heated at 90 °C for 15 min and centrifuged at 1500 × *g* for 10 min and then the resulting supernatants were treated with diphenylamine for 16–20 h at room temperature to develop colour. DNA standards were also prepared and treated with diphenylamine. Absorbance for standards and samples was measured at λ, 600 nm. DNA fragmentation was calculated as:

$$\text{DNA fragmentation (\%)} = \frac{\text{fragmented DNA}}{\text{fragmented DNA} + \text{intact DNA}} \times 100.$$

2.6. Gel electrophoresis of proteins

Proteins from a medium of hepatocyte cultures treated with diclofenac 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), which permitted band molecular weights (MW) 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 protein MWs. The MW of the proteins contained in the culture medium was calculated from the logarithm curve fitting, which relates the standard MWs to the relative mobility by using calibration Kit proteins (HMW and LMW) (Amersham Biosciences). The amounts of proteins identified in the gels were calculated from the quantity calibration curve, which relates the band volume in terms of image intensity to protein quantity by using standard proteins.

2.7. Biochemical assays

Samples of the culture medium were collected from cell cultures and controls 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) by spectrophotometer analysis. The urea concentration was assayed by the enzymatic urease method (Sentinel, Milan, Italy).

For DNA analysis, the cells were detached from the membrane by the addition of 0.05% collagenase in PBS followed by incubation at 37 °C for 30 min. The cells were centrifuged for 10 min at 41.66 Hz (2500 rpm). The cell pellet was washed twice with 10 ml PBS and resuspended in 1 ml of nuclear lyses buffer (10 mM Tris–HCl, pH 8.0, 400 mM NaCl, 2 mM EDTA, pH 8.0, 1 mg/ml proteinase K). After overnight incubation at 39 °C, the samples were precipitated in saturated NaCl solution (6 M) and centrifuged at 41.66 Hz (2500 rpm) for 15 min at 4 °C. DNA was then extracted after the addition of isopropanol at ratio 1:1. The precipitated DNA was washed with EtOH 70% (v/v) and resuspended in TE buffer (0.01 M Tris–HCl, pH 8.0 and 0.001 M EDTA, pH 8.0). The DNA was measured spectrophotometrically using diphenylamine [25].

The mitotic index is calculated as the fraction of cells in mitosis, determined by counting mitoses in stained cultures as a proportion of the whole population. According to Feulgen-Rossenbeck's method, DNA was hydrolysed with 6N hydrochloric acid for 1 h and stained with Schiff's reagent (1 h) at room temperature to produce magenta-stained nuclei. The mitoses were counted by fluorescence microscopy.

Albumin secretion was measured in the samples by the immunometric method (ELISA) [26] with the modification that antibodies against pig albumin and pig albumin were used (Sigma). ELISAs were done on cells of six different isolations. Chromatographically purified porcine albumin and monoclonal antibody for porcine albumin was used from Bethyl Laboratories Inc., USA. Ninety-six well plates were coated with 50 μg/ml in coating buffer and left overnight at 4 °C. After washing the plates four times with a solution of 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) was added for 7 min. The reaction was stopped with 100 μl of 8N H_2SO_4. Absorbance was measured at 450 nm using a Multiskan Ex plate reader (Thermo Lab Systems).

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

3. Results

From ATR spectroscopy analysis the typical chemical groups of PES membranes were identified (Fig. 1). Large bands at 835–871 cm^{-1} corresponding to benzene 1,4-disubstituted were observed in the PES membrane spectrum. The characteristic bands for ethers groups at 1100–1238 cm^{-1} and for sulfonate groups between 1200 and 1400 were also detected.

The PES membrane surface appears to have pores with a mean diameter of 0.1 μm regularly distributed on the surface (Fig. 2). The symmetric membrane has 72.5 ± 0.19% porosity and a thickness of 190 ± 15 μm.

The hydraulic permeance was calculated from the filtration measurements at different transmembrane pressures and assumes a linear correlation between water flux and the convective driving force. The observed steady-state hydraulic permeance of the membrane, calculated as the slope of the water flux versus transmembrane pressure straight line, was 4.087×10^{-2} l/m^2 h Pa (5.45 l/m^2 h mmHg), with a *R*-squared value of 0.99.

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. Furthermore, the hydraulic permeance provided transport information specific for the use of membrane in biohybrid device.

Advancing and receding contact angle measurements were performed on PES membranes at standardised conditions, the first contact angle at $t = 0$ was automatically measured when the droplet contacted the membrane surface. This value depends on membrane physico-chemical properties. For this purpose, these data should be taken into account in the early contact of membrane with cells and medium. Time related contact angle (Fig. 3a) showed an advancing and receding contact angle of PES membranes and collagen. The advancing contact angle remains more or less at the same values while the receding decreased in time. PES membranes displayed advancing and receding values, respectively, of 30° and 13°. The receding contact angle was measured in time up to complete absorbance of the water droplet on the surface which occurred after 1.6 s. Advancing of about 86° and receding of about 59° was measured on collagen. Most

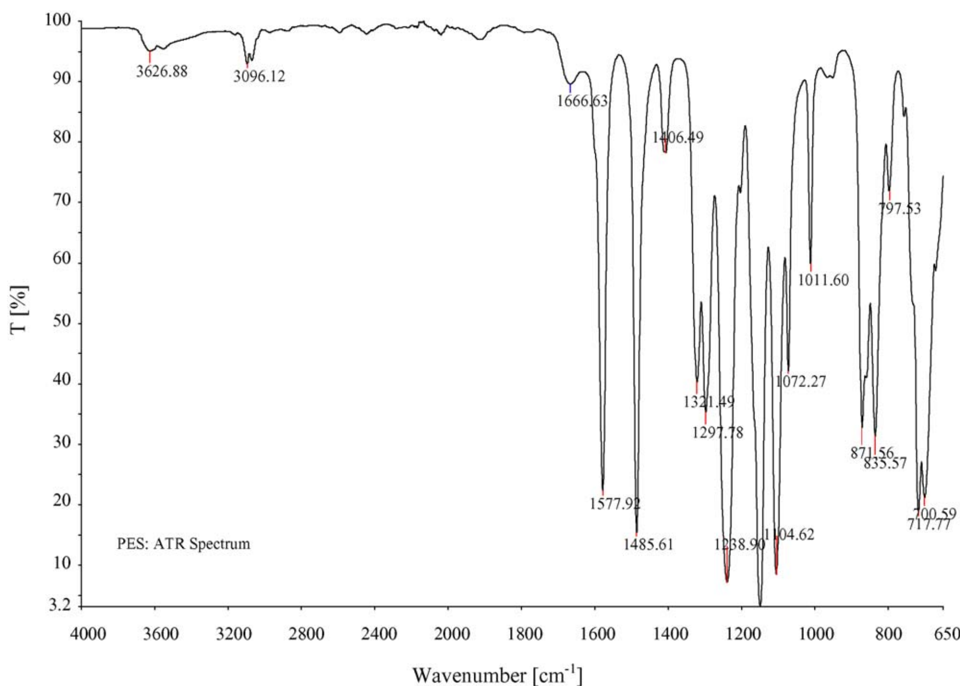

Fig. 1. ATR spectrum of the PES membrane.

registered changes in receding contact angles were noticed during the first seconds of measurements. This trend is confirmed by the surface water sorption, as illustrated in Fig. 3b. PES proved to be the surface that adsorbed the largest percentage of water and the collagen the surface with low water sorption. The DCA and water sorption measurements demonstrated the high wettability of the membrane surface, which was important for cell adhesion and the consequent reorganisation over the substrate.

Isolated pig hepatocytes adhered on the membrane surfaces and formed three-dimensional aggregates. Cells were cultured on membranes and on collagen in the presence of different diclofenac concentrations 100–700 μM for 10 days. Hepatocytes eliminated diclofenac and produced two main metabolites. A peak with retention time of 4.05 min was assigned to 4′-OHdic, while a peak with retention time of 2–3 min was

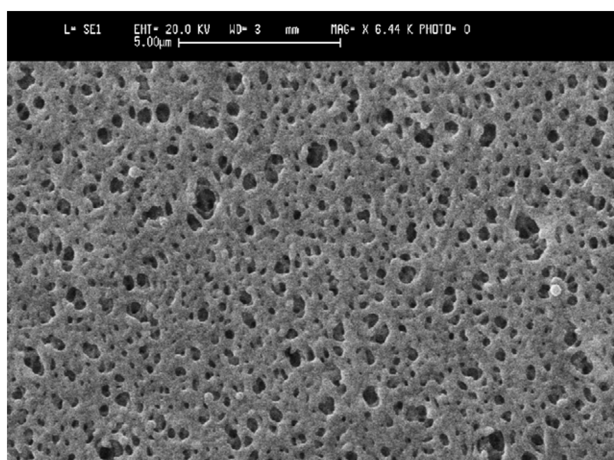

Fig. 2. SEM image of the PES membrane surface.

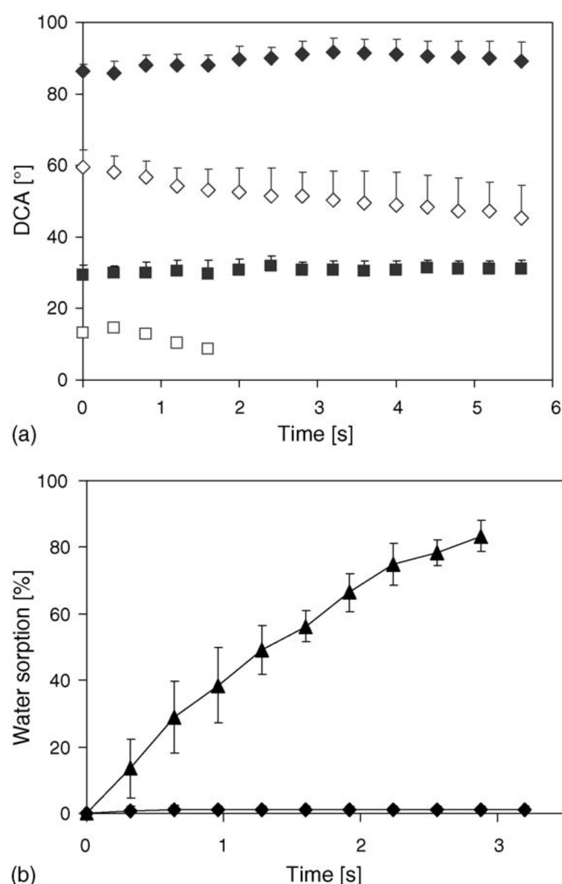

Fig. 3. Physico-chemical properties of PES membrane and collagen surfaces: (a) advancing (full symbol) and receding (empty symbol) contact angle of PES membrane (■) and collagen (♦) surfaces. (b) Time-related water sorption: (▲) PES membrane; (♦) collagen. The values are the mean of 30 measurements per sample ± standard deviation.

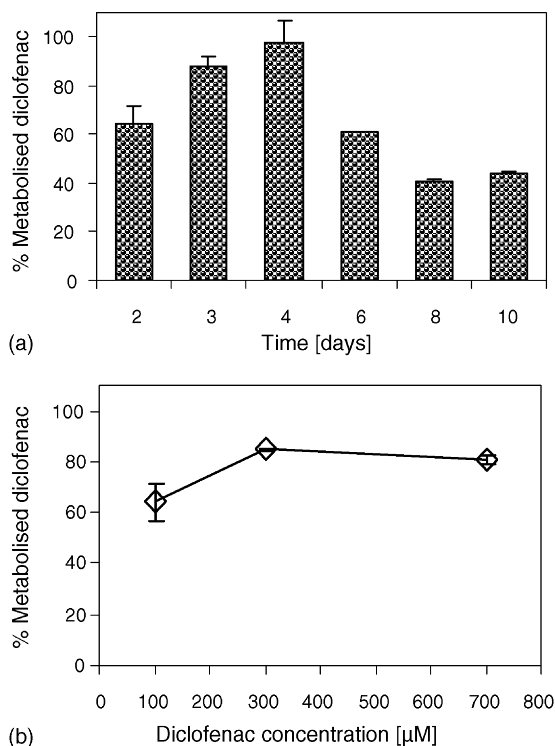

Fig. 4. (a) Time course of diclofenac biotransformation of pig isolated hepatocytes cultured with drug concentration of 100 μM; (b) rate of diclofenac elimination by pig isolated hepatocytes on day 2 of culture on PES membranes related to diclofenac concentrations. The values are the mean of eight experiments ± standard deviation. *Significantly different data.

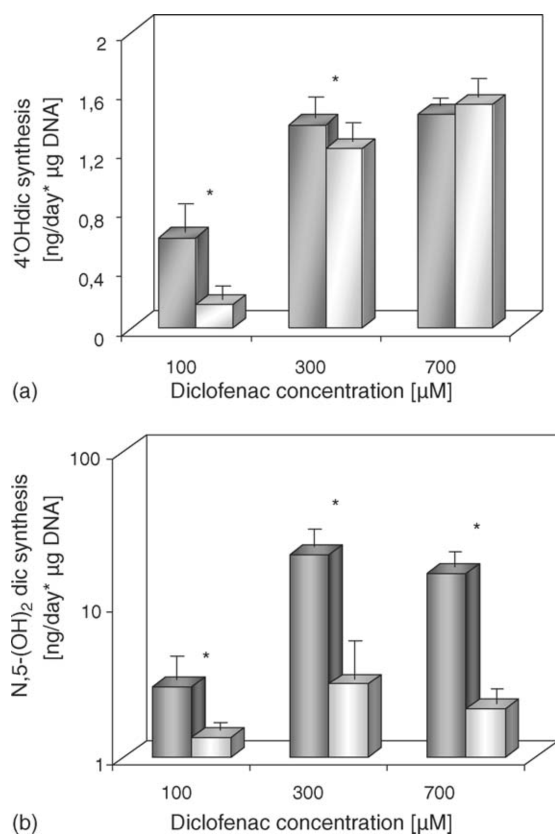

Fig. 5. Formation of diclofenac metabolites by pig hepatocytes on day 2 of culture on PES membranes (full bar) and on collagen (shade bar) related to drug concentrations: (a) 4′-OHdic; (b) *N*,5-$(OH)_2$dic. The reported values are the mean of eight experiments ± standard deviation. *Significantly different data.

assigned to *N*,5-$(OH)_2$dic. These metabolites were identified on the basis of their retention times and by comparison with chemically synthesised probes. Cells incubated with 100 μM diclofenac eliminated 64.2% of administered drug on day 2 (Fig. 4a). The biotransformation increased during the culture time and reached high values on day 4. A decrease of cell ability to eliminate diclofenac was observed only during the last days of culture.

Fig. 4b shows the relationship between the percentage of metabolised diclofenac by hepatocytes on membrane and the drug concentration. Increasing the diclofenac concentration in the culture medium from 100 to 300 μM the percentage of biotransformed drug increased from 64% to 85%. There were no further significant changes of diclofenac elimination when the drug concentration was increased from 300 to 700 μM. Both 4′-OHdic and *N*,5-$(OH)_2$dic metabolites appeared in the culture medium as shown in Fig. 5a and b. The formation rates of both the metabolites increased when cells were incubated with 300 μM diclofenac with respect to the concentration of 100 μM approaching to saturation values at 700 μM. The production rate of 4′-OHdic by hepatocytes cultured on PES membranes increased from 0.15 to 1.2 ng/day μg DNA when the concentration of diclofenac increased from 100 to 300 μM (Fig. 5a). The same trend was observed for *N*,5-$(OH)_2$dic metabolite (Fig. 5b). Both metabolites were produced by hepatocytes cultured on PES membranes at higher rates with respect to those measured on collagen.

Diclofenac affected the viability and proliferation of cells and showed a cytotoxic effect dependent on its concentration in the culture medium. The amount of DNA determined as a parameter of cell viability decreased at high diclofenac concentration. Similar behaviour was observed in both culture systems: collagen and PES membranes. Diclofenac affected also the ability of liver cells to proliferate: the mitotic index was reduced significantly when cells were cultured in the presence of 700 μM diclofenac on both PES membrane and on collagen.

Fig. 6 shows changes in DNA integrity of cell nuclei after exposure to diclofenac (300 μM). Fluorophore end-labelling was performed on nuclei from both cells exposed to diclofenac and control. Following fluorophore end-labelling (green), nuclei were counter-stained with DAPI (blue) to determine the total DNA content of the nuclei. Fluorescent images evidenced that nuclei of cells treated with diclofenac appeared to be coloured green and blue in comparison with the control, which was blue. Fragmented DNA was end-labelled with fluorescein-ddUTP (green). Because only one labelled dideoxynucleotide can be added per 3′-OH end of DNA, the fluorescence intensity is directly proportional to the number of DNA strand breaks. We observed the same DNA damage on both substrates used for cell culture: membranes and collagen after exposure to diclofenac. Quantitative changes in integrity of the genomic DNA are reported in Fig. 7. An increase of DNA fragmentation was measured in diclofenac-treated cell

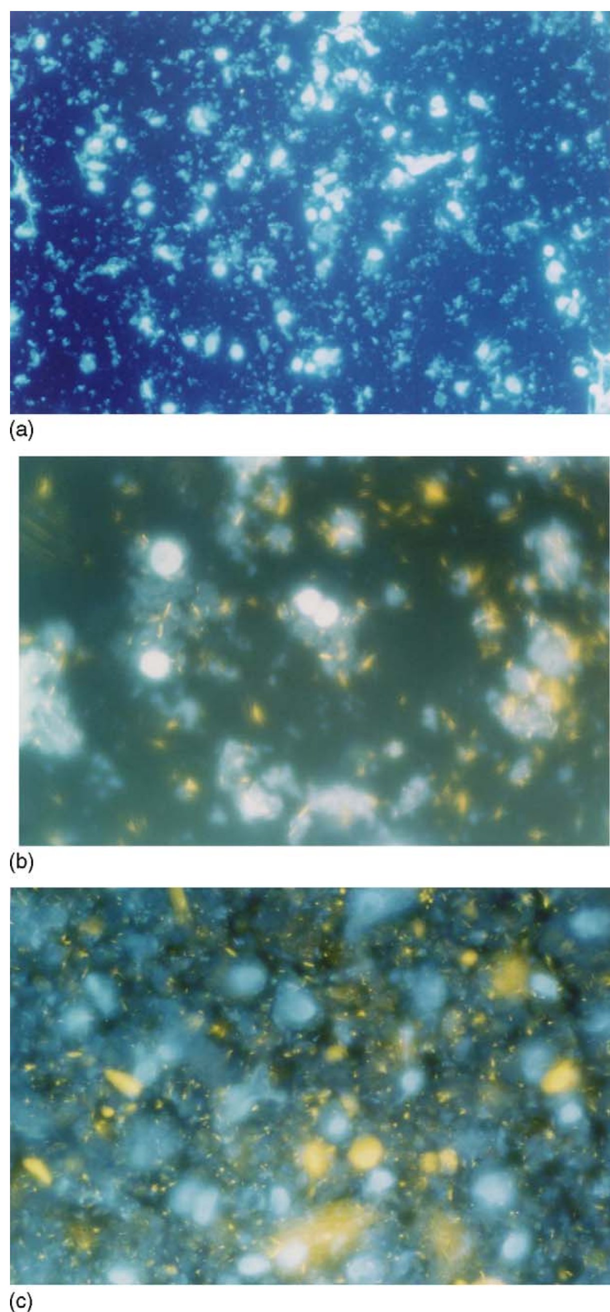

Fig. 6. Fluorescence photomicroscopy of hepatocyte nuclei following fluorescein-12-ddUTP end-labelling and staining with DAPI. Fluorescein fluorescence in nuclei from cells (a) incubated in the absence of diclofenac (control); (b) cultured on collagen in the presence of diclofenac 300 μM; (c) cultured on PES membrane in the presence of diclofenac 300 μM.

nuclei with 100 and 300 μM concentrations with respect to the control.

Identification of the proteins released by cells treated with diclofenac was evaluated by gel-electrophoresis (Fig. 8). We identified four bands in the medium collected from both hepatocytes cultured on collagen and on PES membranes. The band with 66 kDa MW corresponding to albumin was very marked on both PES and collagen treated with diclofenac. A decrease of band intensity was observed at a drug concentration of 300 μM. Other bands with MW of 80 and 130 kDa were obtained. A further small band revealed the presence of proteins with MW of about 690 kDa. The samples collected on the third day of culture present three bands corresponding to proteins with MW of 66, 80 and 690 kDa. The band intensity was different on the basis of the culture substrates: only the band corresponding to albumin was marked on collagen, while the other bands also appeared intensive on PES (Fig. 8c). The quantitative analysis of the proteins identified by gel electrophoresis confirmed that the hepatocytes to a large extent secreted proteins with 66 kDa MW and to low extent proteins with higher MWs (Fig. 9). Diclofenac concentration affected the secretion of low MW proteins. The secretion of proteins with 66 kDa MW was expressed at higher levels on PES membranes. Increasing the concentration of diclofenac from 100 to 300 μM strongly decreased the secretion of protein corresponding to MW of albumin.

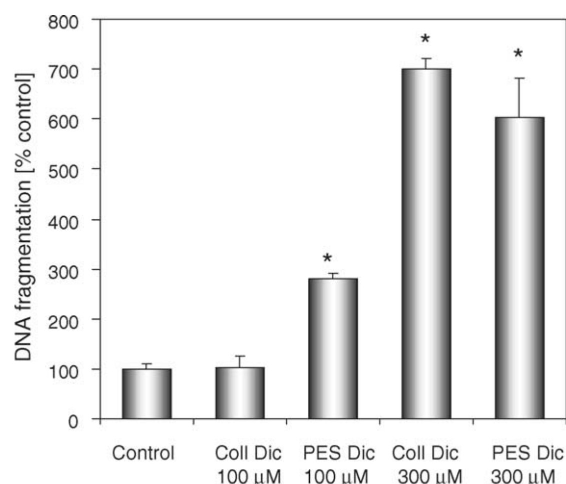

Fig. 7. DNA fragmentation measured in cell culture treated with different diclofenac concentration. Diclofenac induced marked fragmentation of DNA at 300 μM on PES membranes and on collagen. *The values are significantly different from control ($p < 0.05$).

The ability of hepatocytes to perform liver specific functions was investigated with respect to urea and albumin productions at different concentrations of diclofenac. As reported in Fig. 10a, diclofenac-treated hepatocytes (100 μM) on PES membrane exhibited high rates of urea synthesis during the first days of culture, thereafter their ability to synthesise urea decreased from 42.3 μg/day μg DNA measured at day 4 to 5.6 μg/day μg DNA at day 10. On the contrary, the untreated cells maintained their metabolic activity at higher values over the culture time. Quantitative changes in the ability of cells to synthesise urea during exposure to increasing doses of diclofenac are shown in Fig. 10b. An increase of diclofenac concentration in the culture medium from 100 to 300 μM resulted in an increase of urea synthesis rates in membrane and in collagen culture systems. No significant differences were measured between untreated cells and cells incubated with 100 μM of diclofenac. However, hepatocytes cultured on PES membrane expressed liver functions at higher levels with respect to collagen. The diclofenac concentration of 700 μM proved to be toxic.

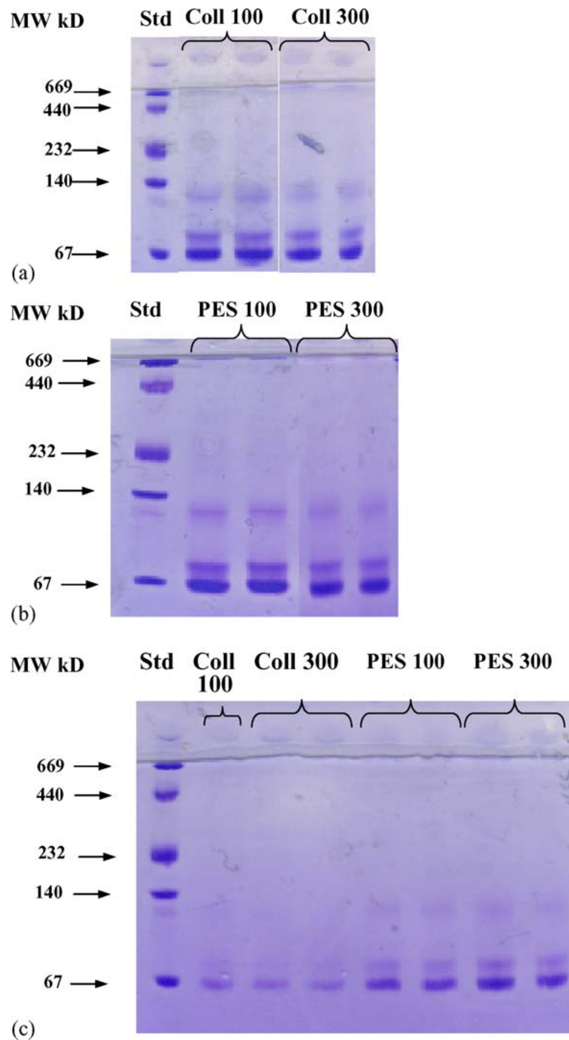

Fig. 8. Native-PAGE with PhastGel gradient 8–25% of standard proteins (std) and medium samples collected by pig hepatocytes cultured (a) on collagen (COLL) with diclofenac at 100 μM (COLL 100) and 300 μM (COLL 300) on day 2 of culture; (b) on PES membrane in the presence of diclofenac at 100 μM (PES 100) and 300 μM (PES 300) on day 2 of culture. (c) Samples of hepatocytes on collagen incubated with diclofenac at 100 μM (COLL 100) and 300 μM (COLL 300) and on PES membranes incubated with diclofenac at 100 μM (PES 100) and 300 μM (PES 300).

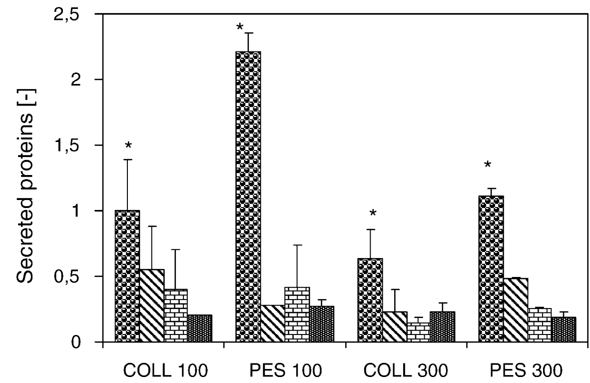

Fig. 9. Analysis of secreted proteins in culture medium by cells on PES membrane incubated with diclofenac at 100 μM (PES 100) and 300 μM (PES 300) and on collagen incubated with diclofenac at 100 μM (COLL 100) and 300 μM (COLL 300): (▩) proteins with 66 kDa MW; (▧) proteins with 80 kDa MW; (▤) proteins with 130 kDa MW; (■) proteins with 690 kDa MW. Data were normalised with respect to the protein with 66 kDa MW secreted by cells on collagen incubated with diclofenac (100 μM). *Statistically significant with respect to other proteins ($p < 0.05$).

In agreement with urea synthesis data, hepatocytes incubated with 100 μM diclofenac synthesised proteins with rates that peaked on day 4 (Fig. 11a). Thereafter the protein synthesis decreased at values which were similar to those measured in the first days and did not change significantly until 10 days of culture. Untreated cells over culture time exhibited average higher rates of protein synthesis with respect to those diclofenac-treated except for day 4. Diclofenac concentration however caused an important effect on the synthesis of total proteins, as is shown in Fig. 11b. Increasing the diclofenac concentration decreased the amount of proteins secreted by cells on both PES membrane and collagen. The exploration of the liver specific functions with respect to albumin synthesis confirmed the results of urea synthesis and total protein production. The ability of diclofenac-treated hepatocytes (100 μM) to synthesise albumin was maintained at values of about 10 ng/day μg DNA during the first days thereafter decreased at values of 0.4 ng/day μg DNA whereas the untreated cells over the culture time maintained their ability to synthesise albumin at values of about 9 ng/day μg DNA (Fig. 12).

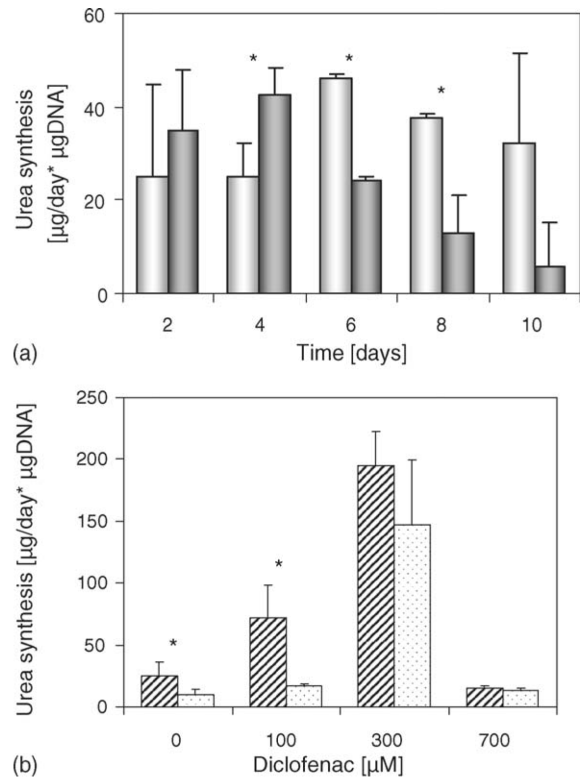

Fig. 10. (a) Time course of urea synthesis of untreated (shade bar) and diclofenac-treated (100 μM) (full bar) pig hepatocytes on PES membrane. *Statistically significant with respect to the corresponding values in control culture. (b) Effect of diclofenac concentration on the urea synthesis of hepatocytes at day 2 of culture on PES membranes (ruled bar) and on collagen (dotted bar). The reported values are the mean of eight experiments ± standard deviation. *Significantly different data ($p < 0.05$).

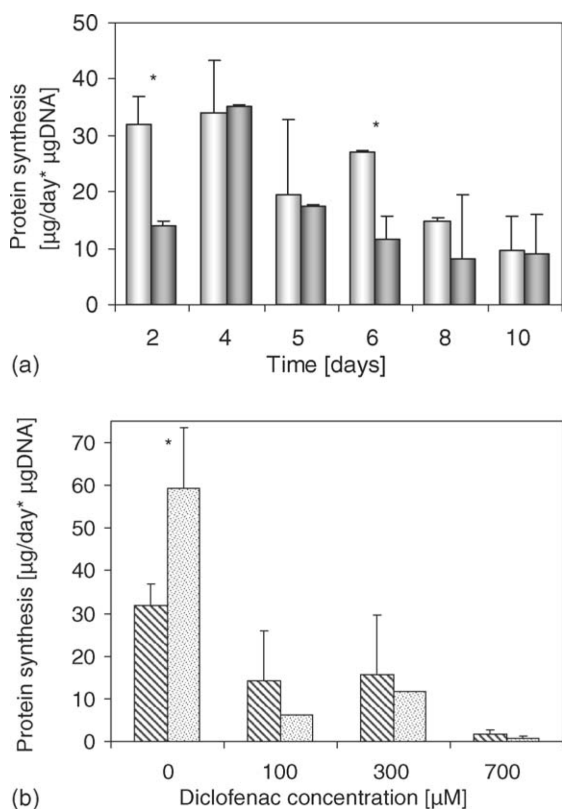

Fig. 11. (a) Time course of protein synthesis of untreated (shade bar) and diclofenac-treated (100 μM) (full bar) pig hepatocytes on PES membrane exposed to diclofenac. (b) Effect of diclofenac concentration on the protein synthesis of hepatocytes at day 2 of culture on PES membranes (ruled bar) and on collagen (dotted bar). The reported values are the mean of eight experiments ± standard deviation. *Significantly different data ($p < 0.05$).

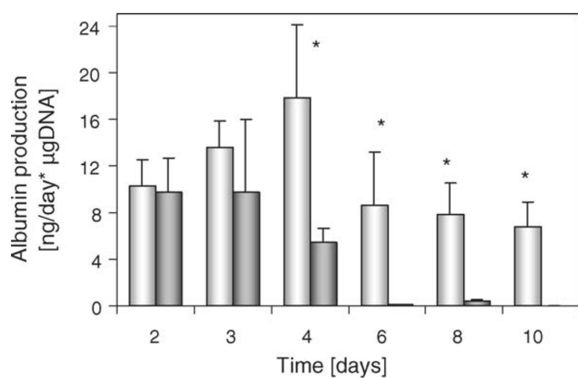

Fig. 12. Time course of albumin synthesis of untreated (shade bar) and diclofenac-treated (100 μM) (full bar) pig hepatocytes cultured on PES membrane. The reported values are the mean of eight experiments ± standard deviation. *Statistically significant with respect to the corresponding values in control culture.

4. Discussion

The in vitro biohybrid system, which employs isolated hepatocytes cultured on synthetic polymeric membranes, could be a potent tool to study hepatic cellular metabolism and the effect of pharmaceutical substances as well as the mediators of different liver metabolic response. Furthermore, relevant information concerning the specific response of pig hepatocytes to PES membranes may be obtained and addressed to the reconstruction of a system that comprehensively reproduces the human liver function. Although collagen is the most common substrate to culture hepatocytes in batch systems, in this report we used PES membranes with the long-term goal of developing a membrane hepatic bioreactor that reproduces the liver functions in vitro. Membranes in a biohybrid device allow the transport of nutrients and metabolites to the cells while providing transport pathways for catabolites and metabolic products from the cells to the medium or blood. In the case of anchorage-dependent cells, they offer a high surface area available for cell adhesion and culture. The PES membranes described here have a homogeneous porous morphology with interconnecting pores and a rough surface. These properties together with hydraulic permeance are not only important for the attachment of cells but also as routes for the delivery of metabolites towards the cell mass and the selective removal of secreted products in a dynamic system, such as the membrane bioreactor.

The dynamic contact angles of PES membrane and collagen evidenced the different physico-chemical properties of these substrates (Fig. 3) being the advancing and receding angle, respectively, a measure of the apolar and polar aspect of the surfaces. 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 [4,27,28] this membrane has a porous surface that offers cells more anchorage points for their adhesion. PES membranes proved to be good material for the culture of isolated hepatocytes. Cells cultured on membranes exhibited metabolic functions at high levels as well as those cultured on collagen, which is the natural substrate of hepatocytes.

According to our contact angle measurements, collagen used in this study proved to be less wettable than PES membranes; this is due to the high percentage of hydrophobic amino acids moieties present in the collagen molecule. The measured hysteresis of the contact angle could be affected by the surface rearrangement and reorganisation of chemical groups on the surface as well as the surface roughness; since collagen exhibits a smooth surface, the hysteresis value of dynamic contact angle is probably due to a heterogeneous distribution of polar and apolar domains on its surface.

Pig hepatocytes similarly to human cells, metabolised diclofenac through the formation of 4′-OHdic, which is the major oxidative product by CYP2C9 found in liver microsomes [29,30]. In addition, in the culture medium of hepatocytes, the presence of N,5-$(OH)_2$dic was detected.

Differently from other studies carried out in literature, the diclofenac metabolism was studied in pig hepatocytes long-term cultured on PES membrane. The cells are continuously exposed to different drug concentrations. Furthermore, the effect of diclofenac on cell viability and liver specific functions, such as albumin synthesis and urea synthesis, in long-term culture is reported here. Results of this study demonstrated the breakdown of the integrity of hepatocyte genomic DNA and the involvement of cell deaths.

The ability of cells to biotransform diclofenac varied according to the dose used. Hepatocytes eliminated diclofenac according to saturation kinetics (Fig. 4b). The formation of 4′-OHdic and N,5-$(OH)_2$dic increased when the concentration of diclofenac increased from 100 to 300 μM but at 700 μM no further significant increase in biotransformation rate was observed (Fig. 5a and b). This suggests that diclofenac at a high dose could saturate the enzyme systems of cytochrome P450C that are responsible for the biotransformation root.

Hepatocytes continuously exposed to drug concentrations of 100 μM maintained their biotransformation functions until 10 days of culture (Fig. 4a). Diclofenac elimination and metabolite formation were maintained at high levels during the first days and decreased on the last days of culture. The remarkable decrease in the time of cell biotransformation activity that was observed at high drug concentrations (Fig. 4b) could be due to drug metabolism. In fact, literature studies have shown that diclofenac is biotransformed to unstable epoxide metabolites that could destroy the cytochrome P450 [11,15,31]. In our study this mechanism could explain the decrease in the time of metabolic rates at high drug concentrations.

The diclofenac at concentration of 300 and 700 μM decreased the viability proliferation activity of hepatocytes cultured on PES and on collagen. This finding suggests a cytotoxic effect of diclofenac, which would inhibit the proliferation of cells inducing DNA damage. Cell growth occurs as cells pass through interphase and mitosis to complete the cell cycle. Generally, liver cells lose the capacity to divide as they mature or divide only rarely. In this case, by quantifying properties of cell division in cultures exposed to increasing diclofenac concentrations, the observed differences in cell growth were in response to the amount of the drug present in the culture medium. A qualitative and quantitative DNA analysis of diclofenac-treated hepatocytes demonstrated the DNA damage due to exposure to the drug. Diclofenac induced a genomic DNA fragmentation as is shown by the fluorescence images of cell nuclei enzymatically labelled with fluorophore dideoxynucleotide and by quantitative analysis (Figs. 6 and 7). DNA fragmentation is considered as one of the later steps in the apoptotic program, which involves the degradation of DNA into 300 and/or 50 kb fragments by endogenous endonuclease activity [22]. The fragmentation of kidney genomic DNA was observed also by Hickey et al. as a result of the endonuclease action, which is activated by diclofenac as probable consequence of oxidative damage or factor imbalance [18]. Recent evidence also suggested that the oxidative stress at the mitocondrial level is in the root of apoptosis induction [16]. However, the toxicity could also be related to drug metabolism, in particular with the formation of N,5-$(OH)_2$dic and the 5′-OHdic, which cause decrease in NADPH concentration, which could be at the root of the hepatocyte toxicity mechanism [12]. In the culture medium the presence of this metabolite was detected, which could be responsible for the cytotoxic effect.

The analysis of the proteins contained in the medium collected by culture samples confirmed the presence of some proteins with MW similar to those plasmatic proteins synthesised by hepatocytes. The molecular masses of the detected proteins corresponded to the MWs indicated by the standards. On the first day of culture, we found proteins with the same MW in both collagen and PES membranes and also at different diclofenac concentrations (Fig. 8a and b). The PES membranes stimulated the cells to produce a large amount of proteins with respect to collagen. In particular the presence of proteins with MWs ranging from 66 to 80 kDa was very pronounced in all samples. We observed also a slight mark of proteins at high MW, which could be involved in the adhesion and reorganisation of the cells on the substrata. The decrease of the protein amount with MW 66 kDa secreted by the cells in the samples exposed to diclofenac 300 μM is also supported by the decrease of albumin production in diclofenac-treated cells. A hypothesis to explain this effect is the possible formation of diclofenac–protein adducts induced by diclofenac with a concentration-dependent effect (Fig. 9). The formation of diclofenac-protein adducts between 60 and 100 kDa was observed by some authors in bile samples of rats treated with diclofenac [32]. However, further investigations are needed to clarify this point.

Also the differentiated functions of liver cells were affected by diclofenac in different way. In fact, hepatocytes exposed to a drug concentration of 100 μM expressed urea synthesis and albumin production at high levels during the first days of culture, thereafter a decrease of their metabolic functions was observed (Figs. 10–12), whereas untreated cells maintained their differentiated functions over the culture period. The continuous exposition of cells to the drug could affect their functions. Although the concentration of 100 μM is considered "subcytotoxic" other authors observed inhibition in the rat of liver synthetic functions at subcytotoxic diclofenac concentrations, which are not essential for cell survival, but that require a substantial amount of ATP [33]. An interesting point was the different concentration-dependent effect of diclofenac on protein production with respect to urea synthesis. The diclofenac at concentrations of up to 300 μM seems to stimulate cellular catabolism producing an increase of urea synthesis. The decrease of this metabolic activity at high concentration is due to a cytotoxic effect of the drug, in agreement with DNA fragmentation data.

5. Conclusions

Overall the results of this study demonstrated the biotranformation of diclofenac and its effect on cell viability and functions by using a biohybrid system constituted of isolated pig hepatocytes and PES membrane. The effect of drug concentration on liver specific functions of hepatocytes, such as urea and albumin productions as well as the synthesis of proteins, which was unknown, was evaluated. The cytotoxic effect due to DNA fragmentation induced by diclofenac was also evaluated. Pig isolated hepatocytes on PES membrane and on collagen maintained their diclofenac biotransformation functions for about 10 days of culture although during the last days a decrease of activity was observed. On the basis of the evaluation of biotransformation and liver specific functions expressed by pig hepatocytes, PES membrane proved to be a good substrate for in vitro liver cell culture.

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