Cytotechnology (2008) 56:57–67
DOI 10.1007/s10616-007-9108-x

ORIGINAL RESEARCH

Separation of CHO cells using hydrocyclones

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Received: 3 April 2007 / Accepted: 20 October 2007 / Published online: 14 November 2007

Abstract Hydrocyclones are simple and robust separation devices with no moving parts. In the past few years, their use in animal cell separation has been proposed. In this work, the use of different hydrocyclone configurations for Chinese hamster ovary (CHO) cell separation was investigated following an experimental design. It was shown that cell separation efficiencies for cultures of the wild-type CHO.K1 cell line and of a recombinant CHO cell line producing granulocyte-macrophage colony stimulating factor (GM-CSF) were kept above 97%. Low viability losses were observed, as measured by trypan blue exclusion and by determination of intracellular lactate dehydrogenase (LDH) released to the culture medium. Mathematical models were proposed to predict the flow rate, flow ratio and separation efficiency as a function of hydrocyclone geometry and pressure drop. When cells were monitored for any induction of apoptosis upon passage through the hydrocyclones, no increase in apoptotic cell concentration was observed within 48 h of hydrocycloning. Thus, based on the high separation efficiencies, the robustness of the equipment, and the absence of apoptosis induction, hydrocyclones seem to be specially suited for use as cell retention devices in long-term perfusion runs.

Keywords Animal cells · Cell separation · CHO cells · Hydrocyclones · Perfusion · Retention device

Abbreviations

D_c	Hydrocyclone diameter (diameter of the cylindrical part)
D_i	Inlet diameter
D_o	Overflow diameter
D_u	Underflow diameter
E	Separation efficiency
HC	Hydrocyclone
L	Hydrocyclone total length
ℓ	Vortex finder length
Q	Feed flow rate
Q_u	Underflow flow rate
R_f	Flow ratio
X	Cell concentration
X_u	Underflow cell concentration
ΔP	Pressure drop
ΔV_u	Viability loss (cell viability in the feed minus viability in the underflow stream)
θ	Angle of the conical part

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Introduction

Animal cells are widely used for the production of biopharmaceuticals, monoclonal antibodies and vaccines. Continuous animal cell culture processes with cell retention, commonly known as perfusion processes, are characterized by constant fresh medium supply and removal of exhausted medium. Cell retention devices are employed for the separation of cells from spent medium. Thus, the combination of continuous nutrient feeding, metabolites removal and cell retention allows high-cell-density cultures (greater than 10^7 cells/mL) and high productivities (usually at least 10-fold higher than in batch cultures) to be obtained (Woodside et al. 1998; Castilho and Medronho 2002). However, the main concern in perfusion cultures is the technique employed to separate cells from the spent medium. Animal cell separation from the liquid medium is a difficult task because cells are sensitive to mechanical stress, have diameters in the range of 8–40 μm and densities of 1.05–1.14 g/cm^3, resulting in very low terminal settling velocities (Medronho 2003).

Adequate cell retention devices must present features such as high separation efficiency, long-term stable operation, no induction of death mechanisms and, preferentially, good resolution in separating viable from non-viable cells. However, the high protein titer in most cell culture media, associated to the small size and density of the cells, causes drawbacks in most of the conventional retention devices (Kretzmer 2002).

Different devices have been used to perform cell retention in bioreactors, but several limitations have been observed. Filtration-based techniques present problems of filter fouling after a relatively short period of operation (Kawahara et al. 1994; Van Reis and Zydney 2001). Centrifuges may suffer from cell adhesion and clogging, besides their high mechanical complexity and high cost (Jäger 1992; Tokashiki et al. 1990). Sedimentation-based apparatuses are susceptible to cell adhesion, and the high residence time needed to perform the separation exposes the cells to non-controlled environments (Searles et al. 1994; Voisard et al. 2003). Thus, special operation strategies like back-pulsing, to prevent cell build-up at the drain ports, and refrigeration systems, to induce cell aggregation and so enhance particle sedimentation, have to be employed (Lipscomb et al. 2004).

Hydrocyclones have been proposed in the recent years to promote animal cell separation in batch and perfusion processes (Lübberstedt et al. 2000a, b; Jockwer et al. 2001). These separation devices present several advantages for use in the biotechnology industry, such as their simplicity and reliability, as well as the absence of moving parts. Furthermore, for cell separation applications, hydrocyclones would require no maintenance, allowing long-term continuous operation of perfusion bioreactors (Castilho and Medronho 2002).

Previous works using three different commercially available hydrocyclones showed motivating results (Lübberstedt et al. 2000a, b). The best performance was obtained with a 10-mm Dorr-Oliver hydrocyclone, resulting in a separation efficiency of 81%. These experimental values agree with computational fluid dynamics (CFD) predictions that high levels of separation efficiencies for mammalian cells could be achieved with small-diameter hydrocyclones (Medronho et al. 2005). Since the preservation of high cellular viabilities is one main concern regarding animal cell retention devices, Lübberstedt et al. (2000a) also analyzed the influence of pressure drop on HeLa cell viability. It was shown that cell viability in the concentrated stream (underflow) remained constant for pressure drops varying in the range of 1–4 bar, while the diluted stream (overflow) presented viability losses for pressure drops above 3 bar. Although the shear stress levels inside hydrocyclones are relatively high, the residence times of the cells inside the equipment are very short (in the range of fractions of seconds), specially for those cells being separated in the underflow concentrated cell stream, which is the stream that is recycled to the bioreactor in a perfusion culture. Jockwer et al. (2001) studied hydrocyclones specially designed for animal cell separation and were able to successfully carry out a 23-day perfusion culture in a 5-L bioreactor with CHO (Chinese hamster ovary) cells.

The present work investigates the use of different geometries of hydrocyclones that were specially designed for animal cell separation. The separation efficiency of CHO cells, as well as flow rates and cell viability were evaluated. Furthermore, mid-term tests for evaluating if any induction of apoptosis occurred were carried out with the parental CHO.K1 cell line and a recombinant one.

Materials and methods

Cell lines and cultivation conditions

The CHO.K1 (Chinese hamster ovary) cell line, obtained from DSMZ (German Collection for Microrganisms and Cell Cultures, Braunschweig, Germany), and a recombinant CHO cell line expressing human GM-CSF (granulocyte-macrophage colony stimulating factor), gently provided by Laboratorio de Cultivos Celulares from Universidad Nacional del Litoral (Santa Fe, Argentina), were used. Cells were cultivated in spinner flasks up to a volume of 1 L, in a mixture (1:1) of DMEM and Ham's F12 media, supplemented with 1% (CHO.K1) and 0.2% (CHO GM-CSF) fetal calf serum (FCS) (Cultilab, Campinas/SP, Brazil). The flasks were incubated at 37 °C and 5% CO_2 and stirred at 50 rpm.

Hydrocyclones

The hydrocyclones (HCs) tested in this work were specially designed to separate animal cells (Fig. 1). More details on their geometry can be obtained from Deckwer et al. (2005). Briefly, they have a double tangential inlet, a diameter (D_c) of 10.0 mm and a choice of two different underflow diameters (D_u) (2.0 and 3.0 mm) and three different overflow diameters (D_o) (1.0, 1.5 and 2.0 mm). Hence, six different geometries with varying underflow and overflow diameters were tested.

Experimental set-up

The cell separation tests were carried out using a 20-L stainless-steel tank containing cell suspension from a 1-L spinner culture diluted to 20 L with PBS (pH 7.2) containing 0.9% NaCl (Fig. 2). The concentration of the cell suspensions fed to the hydrocyclones was in the range of 6–7 $\times$ 10^4 cells/mL. In each experiment, the stainless-steel tank was pressurized with compressed air up to the required pressure, forcing the cell suspension to the hydrocyclone. Underflow and overflow outlets were open at atmospheric pressure, and the flow rate through these orifices was measured to determine the flow ratio (R_f). Samples (50 mL) were collected from each outlet stream to determine cell concentration and viability. Before cell counting, the samples were concentrated 10-fold to reduce the experimental error.

Further separation tests were carried out using hydrocyclone configurations 3020 (underflow diameter: 3.0 mm; overflow diameter: 2.0 mm) and 2010 (underflow diameter: 2.0 mm; overflow diameter: 1.0 mm) with the purpose of assessing the possible

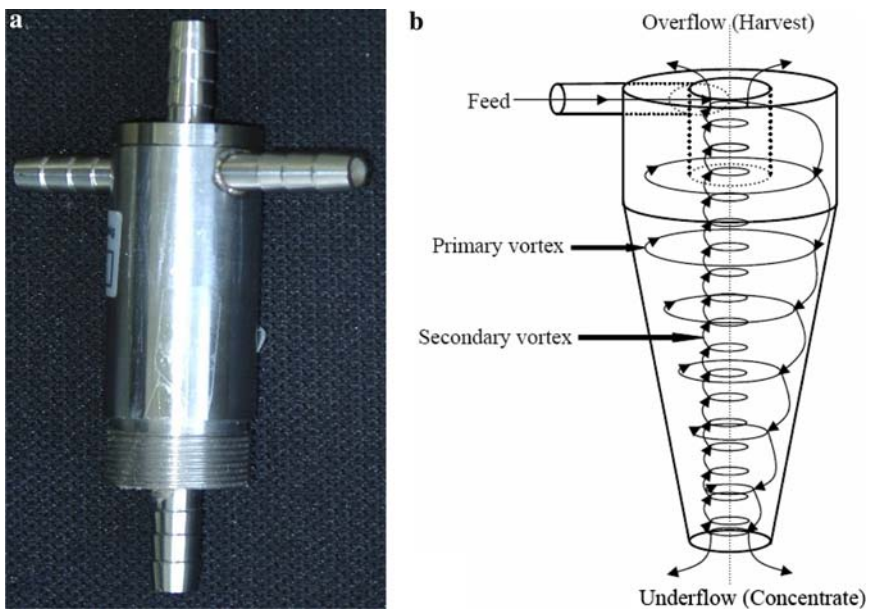

Fig. 1 (**a**) Photograph of the hydrocyclone specially designed for animal cell separation; (**b**) Schematic view of the fluid flow inside a hydrocyclone

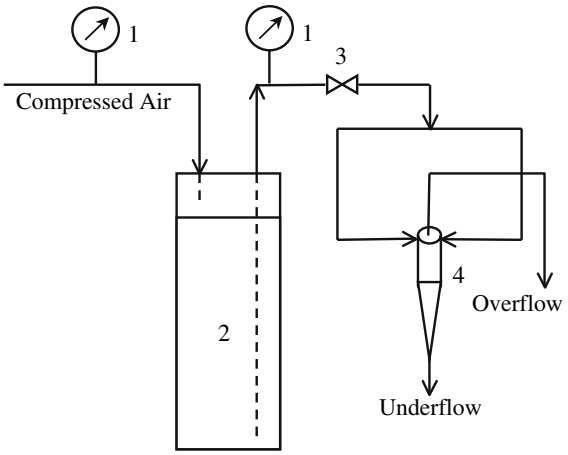

Fig. 2 Experimental set-up of the 20-L stainless-steel tank with two manometers (1), cell suspension (2), a valve (3) and the hydrocyclone (4)

mid-term effects of hydrocycloning on cell viability. High-viability cell suspensions (>98%), cultured in 500-mL spinner flasks, were fed under sterile conditions to the hydrocyclones using a low-pulse peristaltic pump (Watson Marlow, model 520 U with pumphead 505 L), at a pressure drop of 1 bar. After one passage through the hydrocyclone, the underflow stream (cell-concentrated stream) was collected in a sterile spinner flask, which was incubated for 48 h. Samples were collected for evaluation of LDH, apoptosis and cell growth in intervals of 0, 3, 6, 24 and 48 h after passage through the hydrocyclone.

The cell separation efficiency E was calculated as the fraction of cells (in number) recovered in the underflow (Eq. 1) (Castilho and Medronho 2008):

$$E = \frac{Q_u X_u}{QX} \quad (1)$$

where Q and Q_u are the flow rates and X and X_u are the cell concentrations, as cell number per unit volume, of the feed and concentrated streams, respectively.

The flow ratio R_f was evaluated using Eq. 2.

$$R_f = \frac{Q_u}{Q} \quad (2)$$

The cell number balances (comparison of the amount of cells leaving the hydrocyclone through the underflow and overflow streams with the amount of cells fed to the equipment) closed satisfactorily, with an average deviation of ±9.9%, which is approximately the same deviation associated to the cell counting technique in Neubauer chambers used for quantification of cell concentration in the different streams.

Experimental design

A statistical experimental design was employed to evaluate the effects of three variables (overflow diameter, D_o, underflow diameter, D_u, and pressure drop, ΔP). A fractional factorial experimental design (2^{3-1}), shown in Table 1, was used, and allowed obtaining mathematical models describing the effects of each variable and of the interactions among them on the responses (separation efficiency, flow rate and flow ratio). Four additional experiments were added to assess the experimental error using the central points of D_o and pressure drop. Statistical analysis of the results and mathematical modeling were accomplished with STATISTICA 6.0 (StatSoft, Tulsa, USA), using the non-linear parameter estimation module with least square objective function.

Cell quantification and viability assays

Cell quantification was carried out using a haemocytometer and an optical microscope. Dead cells were determined by the trypan blue exclusion method (Kuchler 2000). Total cells were quantified by counting total cell nuclei (Sanford et al. 1950).

Determination of cell size distribution

A particle size analyzer (Malvern Mastersizer) was used to determine cell size distribution of a suspension of CHO.K1 cells grown in 1% FCS.

Lactate dehydrogenase (LDH) activity determination

After centrifugation of samples at 250*g* for 4 min, lactate dehydrogenase activity in supernatants was determined at room temperature by following the conversion of NADH to NAD^+ by reduction of pyruvate to lactate, which is catalyzed by LDH. NADH concentration was measured at 340 nm

Table 1 Fractional factorial experimental design

Absolute and normalized (in parenthesis) values are shown for the tested variables: underflow diameter (Du), overflow diameter (Do) and pressure drop (ΔP)

Experiments	D_u	D_o	ΔP
1	0.20 cm (−1)	0.10 cm (−1)	1 bar (−1)
2	0.20 cm (−1)	0.20 cm (+1)	3 bar (+1)
3	0.30 cm (+1)	0.10 cm (−1)	1 bar (−1)
4	0.30 cm (+1)	0.20 cm (+1)	3 bar (+1)
5 and 6	0.30 cm (+1)	0.15 cm (0)	2 bar (0)
7 and 8	0.20 cm (−1)	0.15 cm (0)	2 bar (0)

(Racher et al. 1990). The reaction was initiated by the addition of 1 mL of supernatant to a mixture of 0.4 mL of 2.4 mM pyruvate and 0.025 mL of 6 mM NADH in 0.1 M phosphate buffer (pH 7.2). One activity unit (U) is defined as the amount of enzyme that catalyzes the consumption of 1 μmol of NADH per minute, under the assay conditions.

Determination of apoptosis and necrosis

Normal, apoptotic and necrotic cells were determined according to visible aberrations of the chromatin using an epifluorescence microscope (Nikon, TS100F). Two fluorescent dyes that bind to DNA (acridine orange and ethidium bromide, both from Fluka) were used. 4 μL of a solution containing 100 μg/mL of each of both dyes was added to 100 μL culture suspension (5×10^5–5×10^6 cells/mL). The samples were examined using a 40× objective with epi-illumination and a combined filter-set for fluorescein (Mercille and Massie 1994). Five different cell physiological states were identified: viable non-apoptotic (VNA), viable apoptotic (VA), non-viable apoptotic (NVA), necrotic (NEC) and chromatin-free (CF).

Results and discussion

Influence of pressure drop and outlet diameters on hydrocyclone performance

CHO.K1 cell suspensions, previously grown in DMEM/F12 medium containing 1% FCS and presenting cell viability above 95%, were fed from a pressurized 20-L steel tank to the six different configurations of the specially designed hydrocyclones, at pressures of 1, 2 and 3 bar. The experiments followed a fractional factorial design. The hydrocyclone geometries 2015 ($D_u = 2.0$ mm and $D_o = 1.5$ mm) and 3015 ($D_u = 3.0$ and $D_o = 1.5$) were analyzed in duplicate to evaluate the experimental error. The results are shown in Table 2 in terms of feed flow rate (Q), flow ratio (R_f), total separation efficiency (E) and cell viability loss in the underflow stream (ΔV_u = viability in the feed minus viability in the underflow).

High cell separation efficiencies, varying from 97.9% to 99.9%, were obtained for 5 out of 6 hydrocyclone configurations. The geometry 3010 ($D_u = 3.0$ mm and $D_o = 1.0$ mm) presented a flow ratio of 100%, diverting the total feed flow to the underflow, indicating that this configuration is inappropriate to perform any kind of cell separation. Loss of viability was within the range of 2.9–9.1% for all configurations with exception of HC 2020, which presented a viability loss of 14.4%.

Hydrocyclone separation efficiencies for CHO.K1 cells shown in Table 2 are higher than the efficiencies obtained with other devices described in the literature. Wen et al. (2000) employed a two-step sequential sedimentation device for the retention of an IgG-producing hybridoma, achieving a separation efficiency of 88%. In CHO-cell perfusion cultures with spin-filters, the efficiencies attained were in the range of 75–95% (Iding et al. 2000), as compared to a minimum efficiency of 97.9% found for hydrocyclones in the present work. Furthermore, unlike hydrocyclones, fouling and clogging of spin-filter meshes is considered a major problem, which may cause premature interruption of the culture process (Esclade et al. 1991; Deo et al. 1996; Voisard et al. 2003). Furthermore, when compared to other hydrocyclone geometries, the efficiencies found in the present work are higher than the best efficiency (81%) obtained for HeLa cells by Lübberstedt et al. (2000b). These authors used commercial hydrocyclones designed for conventional (non-biological)

Table 2 Separation of CHO.K1 cells grown in DMEM/F12 medium (1% FCS)

Hydrocyclone	D_u (cm)	D_o (cm)	ΔP (bar)	Q (L min^{-1})	R_f (%)	ΔV_u (%)	E (%)
2010	0.20	0.10	1	1.13	93.9	5.8	99.6
2015	0.20	0.15	2	1.60 ± 0.01	77.4 ± 0.1	9.1 ± 0.8	99.1 ± 0.2
2020	0.20	0.20	3	2.12	60.4	14.4	97.9
3010	0.30	0.10	3	2.46	100.0	–	–
3015	0.30	0.15	2	2.01 ± 0.02	97.3 ± 0.1	5.7 ± 1.9	99.9 ± 0.0
3020	0.30	0.20	1	1.44	87.8	2.9	99.1

Feed flow rate (Q), flow ratio (Rf), underflow viability loss (ΔVu) and separation efficiency (E) for different geometries and pressure drops (ΔP)

solid-liquid separations (a 7-mm Bradley, a 10-mm Mozley and a 10-mm Dorr-Oliver hydrocyclone), which have geometric proportions that are different from those of the hydrocyclones used in this work. These HCs were specially designed for the separation of animal cells (Deckwer et al. 2005) based on the knowledge of how geometric proportions of hydrocyclones affect separation efficiency (Table 3). A comparison of some geometric proportions of the hydrocyclone used in this work (Deckwer et al. 2005) with those of the HCs used by Lübberstedt et al. (2000a, b) is given in Table 4. According to Tables 3 and 4, a low D_o/D_c range and a high D_u/D_c range were chosen for the specially designed hydrocyclones with the aim of maximizing their separation efficiency.

The viability losses found in the present work were slightly higher than those obtained with commercial Dorr-Oliver hydrocyclones for HeLa cells (Lübberstedt et al. 2000a), but lower than those obtained by Jockwer et al. (2001) for the separation of CHO cells cultured in serum-free medium, using the same type of hydrocyclone as in this work. Just in the case of HC 2020, viability loss was identical (14.4%) in this study and in that by Jockwer et al. (2001). These results suggest that there may be a difference between HeLa and CHO cells regarding sensitivity to shear generated inside hydrocyclones. Furthermore, comparing the present results with those obtained for CHO cells grown in serum-free medium (Jockwer et al. 2001), the data also suggest that the presence of seric proteins could be attenuating the effects of the hydrodynamic stress on cells maintained in serum-supplemented medium, as previously observed by Van Der Pol et al. (1990).

Suspension cultures of CHO.K1 cells naturally present aggregates that can be easily visualized. Using a Malvern particle size analyzer, the size distribution of CHO.K1 cells in the feed was determined (Fig. 3), and it was possible to identify two distinct populations with mean diameters of 12 μm, representing isolated cells, and 110 μm, indicating aggregates. Oxygen and nutrient diffusion inside these clumps is limited, creating inadequate conditions for the innermost cells, resulting in non-viable cells that cannot be detected by the trypan blue technique, which in turn leads to over-estimated viability values. However, due to the high shear rates inside the hydrocyclone, cell clump disaggregation could be an important factor leading to a decrease in cell culture viability that is rather apparent, since only individual cells or small clumps (3–6 cells) had been previously visualized and counted in the haemocytometer by the trypan blue technique. Some authors (Mercille et al. 1994; Castilho et al. 2002) have shown that DNAse treatment of CHO cell cultures can disaggregate cell clumps, significantly increasing the countable concentration of non-viable cells.

Table 3 Effects of increases in the values of geometric and operational variables on the capacity (feed flow rate) and separation efficiency (Eq. 1) of hydrocyclones (+: increase, −: decrease). Adapted from Matta and Medronho (2000)

	D_c	D_i	D_o	D_u	L	ℓ	θ	ΔP
Capacity	+ + +	+ +	+ +	+	+	−	−	+ +
Efficiency	− −	− −	− −	+ +	+	−	−	+ +

Table 4 Comparison of some geometrical proportions of the Bradley hydrocyclone family (Bradley and Pulling 1959), two commercial hydrocyclone designs (Richard Mozley Ltd., and FLSmidth Dorr-Oliver Eimco) and the hydrocyclone used in this work (Deckwer et al. 2005)

Geometric proportion	Bradley	Mozley	Dorr-Oliver	This work
D_o/D_c	0.20	0.32	0.25	0.10–0.20
D_u/D_c	0.07	0.22	0.23	0.20–0.30

Therefore, one important factor that may have influenced the increase in non-viable cell concentration in the underflow stream is the disaggregation of clumps. Visually, it was possible to observe that the number and size of cell clumps collected in the hydrocyclone underflow stream were lower than in the feed before hydrocycloning.

The results shown in Table 2 were also used to determine empiric mathematical models to describe separation efficiency (E), flow ratio (R_f), and flow rate (Q) as a function of the geometric variables (D_u and D_o) and the pressure drop (ΔP).

Equation 3 shows the model for separation efficiency (E), which presented a correlation coefficient (R) of 0.988. This correlation has the form of a typical polynomial equation, as commonly used in experimental design analyses, and only includes the terms that presented statistical significance.

$$E = 90.29D_o^2 + 112.34D_u^2 - 3.56\Delta P^2 - 374.34D_oD_u + 66.43D_o\Delta P \quad (3)$$

where D_o and D_u are in centimetres, ΔP in bar and Q in L min^{-1}.

The models for flow ratio (Eq. 4) and flow rate (Eq. 5) presented correlation coefficients of 0.977 and 0.961, respectively. The structure of these equations was based on classical hydrocyclone models (Plitt 1976; Coelho and Medronho 2000).

$$R_f = 0.79D_o^{-0.53}D_u^{0.65} \quad (4)$$

$$Q = 3.42D_u^{0.75}\Delta P^{0.62} \quad (5)$$

where D_o and D_u are in centimetres, ΔP in bar and Q in L min^{-1}.

Figure 4 compares the predicted values with the experimental data, considering a confidence level of 98%, for the efficiency, flow ratio and flow rate. These three models show that although the efficiencies were high in all cases, the flow rate and flow ratio varied considerably, resulting in important differences in overflow flow rate, for example. Thus, these models allow predicting the effects of changes in D_o, D_u and ΔP within the tested ranges on variables that are of great relevance in establishing e.g. a perfusion process, such as harvest flow rate.

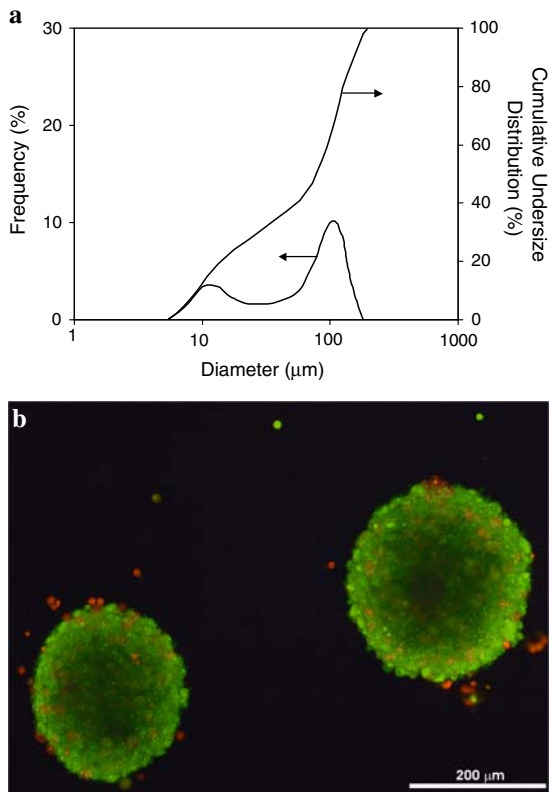

Fig. 3 (**a**) Size distribution of CHO.K1 cells cultured in spinner flasks in DMEM/F12 medium supplemented with 1% FCS. (**b**) Cell clumps stained with acridine orange and ethidium bromide observed under a fluorescence microscope

Mid-term effects of hydrocycloning on cell viability and apoptosis induction

In order to evaluate any mid-term effects of hydrocycloning on the cells, fluorescence microscopy with acridine orange and ethidium bromide was used as a sensitive technique to determine cell viability and to assess any possible induction of programmed cell death (apoptosis). Two hydrocyclone configurations (HC 3020 and HC 2010) were tested at 1 bar using a peristaltic pump, in order to mimic the set-up in a

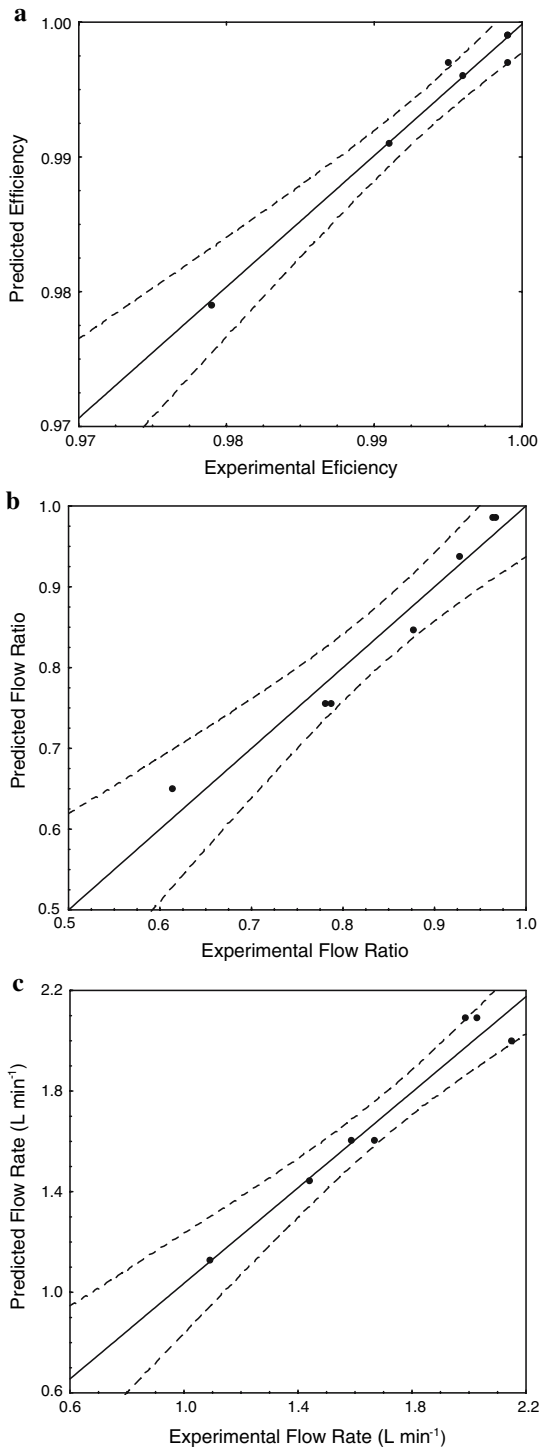

Fig. 4 Separation of CHO.K1 cells. Experimental data versus data predicted by the models for: (**a**) separation efficiency; (**b**) flow ratio; (**c**) flow rate. Dotted lines show a confidence level of 98%

Table 5 CHO.K1 culture conditions before ("Culture") and after (0, 3, 6, 24, 48 h) hydrocycloning (HC 3020) at a pressure drop of 1 bar

HC 3020 CHO.K1	Concentration (10^5 cells mL^{-1})	Viability (%)	LDH (U L^{-1})
Culture	8.0	99	71.5 ± 6.9
0 h	7.7 ± 0.1	97	95.9 ± 6.1
3 h	8.7 ± 0.0	93	110.4 ± 11.5
6 h	9.2 ± 0.8	93	116.1 ± 18.4
24 h	8.1 ± 0.5	96	132.0 ± 12.4
48 h	11.7 ± 0.4	99	126 ± 17.9
Overflow	0.8 ± 0.0	82	83.4 ± 3.3

perfusion bioreactor. The CHO.K1 cell line was used in tests carried out with HC 3020 (Table 5) and in a control test, in which cell suspension was pumped through the test system without the hydrocyclone. This control test gave evidence that the pumping system had no significant influence on the cells (data not shown). Due to the good results observed in these tests, recombinant CHO cells expressing GM-CSF were also tested for separation efficiency, viability and apoptosis with HC 3020 and HC 2010 (Tables 6 and 7, respectively).

The separation of both cell lines with hydrocyclones was accomplished with high separation efficiencies (>97%) and low viability losses (<7%), maintaining cell culture viability above 92%. Previous studies have shown that some cell lines are susceptible to hydrodynamic stress, presenting a significant decrease in cell viability when subject to stress levels above a threshold limit (Born et al. 1992). However, due to the low viability drops observed in this work, the viability of CHO cells separated by hydrocyclones was kept above 92%, which is higher than the cell viability obtained for most cell retention devices reported by Voisard et al. (2003). In this review article, a compilation of 21 works using different cell retention devices showed that in only two cases cell viability was kept at values higher than the minimum viability (92%) obtained in the present work. In those two works cited by Voisard et al. (2003), the cell viability was 95%.

LDH activity released to the supernatant of the CHO.K1 culture (Fig. 5a) presented an increase up to 24 h after hydrocycloning, becoming stable within 48 h. For the recombinant cell line, rather stable

Table 6 CHO-GMCSF culture conditions before ("Culture") and after (0, 3, 6, 24, 48 h) hydrocycloning (HC 3020) at a pressure drop of 1 bar

HC 3020 CHO-GMCSF	Concentration (10^5 cells mL^{-1})	Viability (%)	LDH (U L^{-1})
Culture	16.2 ± 2.0	99	23.0 ± 1.7
0 h	19.1 ± 0.1	92	77.9 ± 5.4
3 h	20.8 ± 1.3	94	76.7 ± 4.6
6 h	17.1 ± 1.7	93	85.6 ± 7.5
24 h	18.2 ± 0.3	95	94.9 ± 6.6
48 h	22.1 ± 0.1	98	54.9 ± 10.7
Overflow	3.6 ± 0.3	84	45.6 ± 6.7

Table 7 CHO-GMCSF culture conditions before ("Culture") and after (0, 3, 6, 24, 48 h) hydrocycloning (HC 2010) at a pressure drop of 1 bar

HC 2010 CHO-GMCSF	Concentration (10^5 cells mL^{-1})	Viability (%)	LDH (U L^{-1})
Culture	11.4 ± 0.3	98	21.4 ± 1.8
0 h	10.9 ± 0.1	97	49.0 ± 2.9
3 h	13.3 ± 0.6	97	49.8 ± 6.2
6 h	12.2 ± 0.2	95	48.0 ± 3.2
24 h	15.4 ± 0.7	99	57.1 ± 6.5
48 h	18.8 ± 0.5	99	52.9 ± 6.4
Overflow	2.9 ± 0.3	95	25.3 ± 2.1

values were observed between 0 and 24 h, with a tendency to decrease within 48 h (Fig. 5b and c). In all cases, the LDH values were in agreement with the trypan blue viability data, with increases in LDH activity released to the supernantant correlating to decreases in cell viability. These increases in LDH activity could also be related to the disaggregation of cell clumps, as discussed previously. Due to the break up of clumps upon hydrocycloning, dead cells become freely suspended in the medium, increasing the release of LDH to the supernatant.

Samples collected from the overflow stream showed a lower viability and, thus, a higher relative amount of dead cells when compared to the underflow stream (samples designated "0 h" in Tables 5–7). These data give evidence that the hydrocyclones showed a preferential retention of viable cells, separating non-viable cells preferentially in the overflow stream, which in a perfusion run would be the harvest stream. Acoustic filters also presented

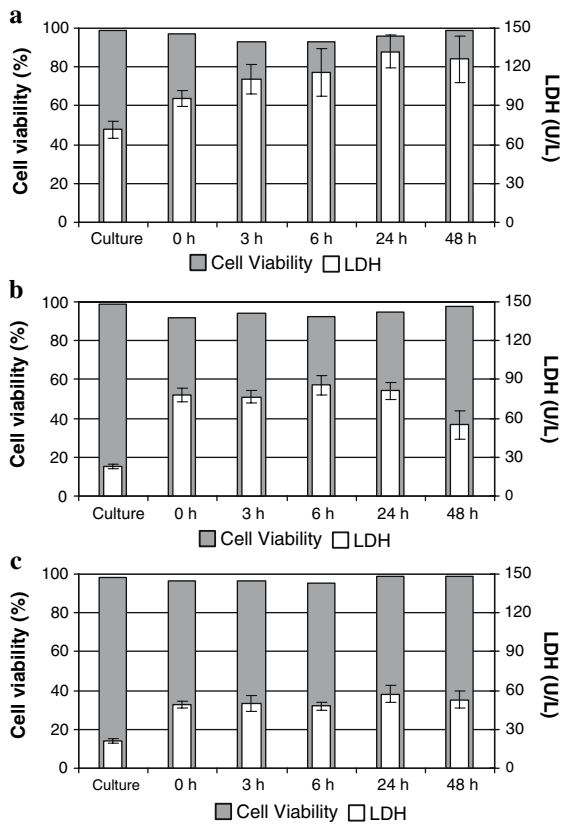

Fig. 5 Cell viability and LDH activity in the supernatant before ("Culture") and after (0, 3, 6, 24 and 48 h) hydrocycloning at a pressure drop of 1 bar: (**a**) CHO.K1 cells, HC 3020; (**b**) CHO-GMCSF cells, HC 3020; (**c**) CHO-GMCSF cells, HC 2010

preferential separation of viable hybridoma cells, and this was credited to the decrease of the mean diameter of dead cells (Batt et al. 1990; Lipscomb et al. 2004). However, it is probable in the present case that not only the alterations in size, but also the changes in density have influenced cell separation. Whereas apoptotic cells usually have a decreased size, it is known that necrotic cells are prone to experience an increase in size due to cell swelling after membrane breakdown (Al-Rubeai 1998; Buja et al. 1993). However, due to loss of intracellular components, the density of necrotic cells becomes lower than that of living cells. In both cases (decrease in diameter or in cell density), the terminal settling velocity of the cells decreases, favoring the separation of dead cells in the overflow stream. A similar behaviour has been observed in inclined settlers and Centritech centrifuges (Batt et al. 1990; Takagi et al. 2000; Lipscomb

et al. 2004), which are equipment that also separate particles based on their terminal velocity.

The cells were also monitored within the first 48 h after passage through the hydrocyclone by fluorescence microscopy to evaluate any possible induction of apoptosis. According to the literature, exposure to high levels of hydrodynamic stress can induce cell death mechanisms such as apoptosis and necrosis, depending on its intensity and frequency (Al-Rubeai et al. 1995a, b). Analysis of cell samples taken along 48 h, upon labelling with ethidium bromide (BE) and acridine orange (AO), showed insignificant levels of apoptotic cells (Fig. 6). Thus, the present results indicate that the shear stress inside the hydrocyclones, combined to the short residence times of cells inside the equipment, does not induce apoptotic mechanisms. Considering that the apoptosis assay using fluorescence microscopy with BE/AO is significantly more sensitive than the trypan blue method, these results indicate that hydrocyclones can most probably be used as separation devices in long-term runs, e.g. in perfusion processes.

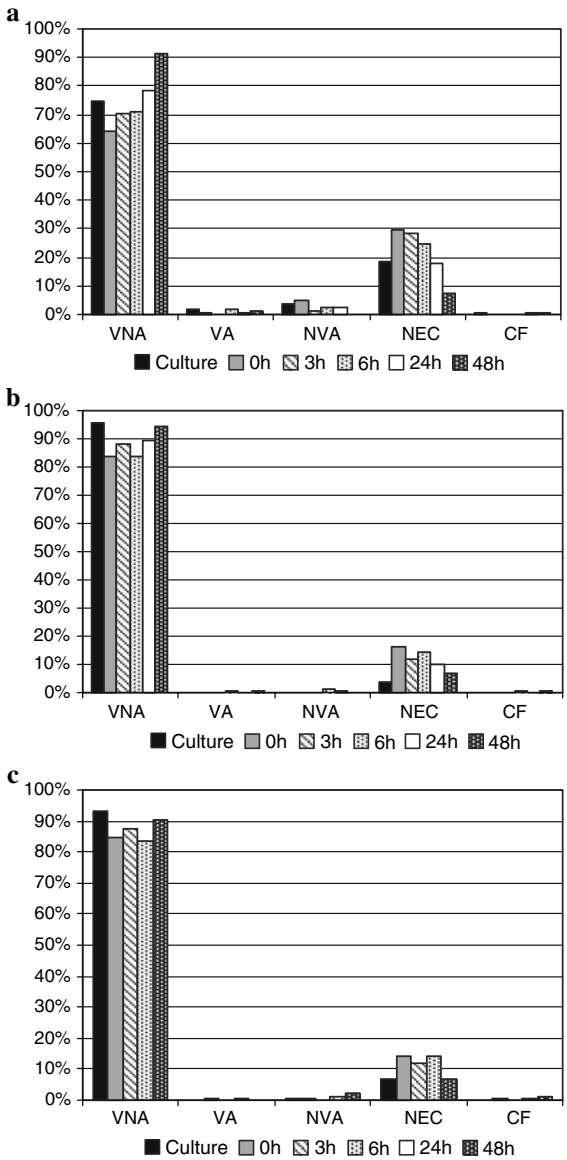

Fig. 6 Monitoring of cells before ("Culture") and after hydrocycloning (0, 3, 6, 24 and 48 h): (**a**) CHO.K1 cells, HC 3020; (**b**) CHO-GMCSF, HC 3020; (**c**) CHO-GMCSF, HC 2010. VNA: viable non-apoptotic cells; VA: viable apoptotic cells; NVA: non-viable apoptotic cells; NEC: necrotic cells; and CF: chromatin-free cells

Conclusions

In the present work, the separation of CHO cells with hydrocyclones was evaluated. The separation efficiencies attained in all cases were high (>97%), and low drops in cell viability were observed. Tests performed with two CHO cell lines (wild-type CHO.K1 and recombinant CHO-GMCSF) have suggested that apoptotic cell death mechanisms are not activated upon passage through the hydrocyclones tested. Furthermore, the results suggest the same behaviour for both cell lines, indicating that the expression of a heterologous protein has not affected cell sensitivity to the hydrodynamic stress generated inside the hydrocyclones. Due to the high separation efficiency, the absence of apoptosis induction and the lack of moving parts, it can be postulated that hydrocyclones are suitable devices for cell retention in perfusion bioreactors.

Acknowledgements The authors wish to thank the Brazilian funding agencies CNPq, FAPERJ and FINEP for financial support.

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