CFD-aided optimization of a laboratory-scale centrifugation for a shear-sensitive insect cell line

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Abstract

A study has been conducted to assess cell damage during centrifugation and to optimize this operation for the insect cell line Se301. Experiments were carried out in a discontinuous centrifuge using Falcon tubes of different sizes as containers. The cells were easily recovered from the cell suspension and the time required for complete sedimentation was found to be independent of tube size or design. Cell damage was observed once the cells were sedimented, therefore this process depends on both the residence time of the cells in the pellet and on the acceleration applied. The sensitivity of the cells to this operation was higher than for other naked microalgae or for very sensitive red blood cells. Indeed, excessive treatment produced ~~irreversible~~ cell damage that reduced the productivity of subsequent cultures. CFD simulations were carried out in 2D and a good agreement was found between the CFD and experimental values in terms of the time required for full sedimentation. In addition, this technique allowed the calculation of shear stress, a key variable in the study of cell sensitivity to flow.

1. Introduction

Insect cells have been extensively used to express biologically active recombinant proteins (Kost et al., 2005) and are currently being investigated for the production of biopesticides (Beas-Catena et al., 2014). Such cells present several advantages compared to mammalian cells and are easier to handle as they often do not require the presence of CO_2. Insect cell expression systems produce proteins similar to those produced in mammalian cells. Moreover, insect cells can be adapted to suspension culture, although some cell lines are very shear sensitive, thus meaning that protective additives are needed (Beas-Catena et al., 2011).

In addition to insect cells, many other cell lines are particularly sensitive to agitation in suspension cultures (Lara et al., 2006). Indeed, although animal cells have traditionally been shown to be especially shear-sensitive, various insect cell lines (Tramper et al., 1986; Chalmers, 1996; Beas-Catena et al., 2011) or microalgae (Gallardo-Rodríguez et al., 2016) are difficult to grow in traditional culture systems. However, not all respond in the same way when subjected to high levels of shear or energy dissipation in a short period of time (Gallardo-Rodríguez et al., 2016). In addition to the culture stage, there are many steps in laboratory procedures and productive bioprocesses in which cells may experience energy dissipation rates, shear stresses or pressures high enough to cause cell damage (Mollet et al., 2004, Xu et al., 2015; García-Briones et al., 1994). For example, pipetting (Mollet et al., 2004) or centrifugation to recover cells (Xu et al., 2015; Westoby et al, 2010) can often damage them. Indeed, the more sensitive the cell line the more evident and marked the effects will be (Urbina et al., 2016).

Centrifugation is a common cell-harvesting technique in large- (Axelsson, 1999; Hutchinson et al, 2006) and small-scale bioprocesses (Hutchinson et al., 2006; Peterson et al., 2012). However, the prediction of the performance of the centrifugation process at large-scale is very difficult due to the fragile nature of the biological materials (Boychyn et al., 2001). On a laboratory scale, it is an essential operation for the maintenance, preservation, routine subcultivation and setting up of varied protocols (Perterson et al., 2012). Depending on the shear resistance of the cells, the same centrifugation treatment can be harmless or provoke cell damage or cell disruption (Hutchinson et al., 2006; Xu et al., 2015). Excessively intense centrifugation can also affect the protein of interest structure (Hutchinson et al., 2006; Neal et al., 2002). A linear relationship between cell disruption by centrifugation and energy dissipation in a pilot-scale disc-stack centrifuge has also been found (Westoby et al., 2010). There is currently a wide variety of centrifugation protocols (i.e. intensity and duration of treatment, equipment, etc.) in the literature, with very few criteria for choosing between them (Peterson et al., 2012). For example, Agathos (2007) recommends 100-200×g for 5 min for subculturing animal cells, whereas Lynn (2016) suggests 1000×g for 10 min for maintenance and storage. We also observed variability in the cell yields obtained with the insect cell line Se301 when subculture was performed after centrifugation applying standard literature methods. Thus, it seems imperative to optimize the centrifugation process specifically for the cells of interest (data not published).

Centrifugal stress is present in different bioprocess steps and, depending on its intensity, distribution into cell suspension and the exposure time of the cells, it may damage cells (Xu et al., 2015) or cause cell surface damage that is undetectable using standard viability tests (Peterson et al., 2012). The source of this variability may also depend on other factors, such as the height of suspensions or the geometry of

centrifugation containers, which are usually not reported but which could nevertheless modify the shear stress field experienced by the cells for a given *g* force. Computational Fluid Dynamics (CFD) may be useful for unifying centrifugation protocols since it has been shown to be useful for the characterization of centrifuges (Boychyn et al., 2004; Urbina et al., 2016). CFD allows shear rate fields or energy dissipation rates to be determined in flowing systems with living cells (Jain et al., 2005; Mollet et al., 2004; Gallardo-Rodríguez et al., 2016), which is essential for interpreting centrifugation-associated cell damage. CFD can also be used to reduce the experimental effort in selecting, designing or optimizing harvesting systems.

In this study, CFD-aided optimization of a discontinuous benchtop centrifugation process for the *Spodoptera exigua* Se301 insect cell line was addressed in terms of a novel variable named excess of treatment (*ET*), related to the height of suspension in tubes, *g* force and centrifugation time. Evaluation of the impact of centrifugation on subcultivation of cells was carried out using cells previously subjected to different *ET* values.

2. Materials and methods

2.1. Cell Line, Culture Medium, Additives, and Maintenance

The insect cell line Se301, originally isolated from *Spodoptera exigua* (Hara et al., 1995), was used throughout this work and was kindly donated by the Department of Virology at Wageningen University (the Netherlands). Cells were grown in suspension culture in Ex-Cell 420 serum-free media (Sigma-Aldrich, Ref. 14420C) supplemented with 100 U L^{-1} penicillin-streptomycin (Sigma-Aldrich, Ref. P4333) and 0.125 $\mu g \cdot mL^{-1}$ amphotericin B (Sigma-Aldrich, Ref. A2942). The protective additives against shear

stress Polyvinyl Alcohol (PVA, Sigma- Aldrich, Ref. P8136) and Polyvinylpyrrolidone (PVP, Sigma-Aldrich, Ref. P2307), both at a concentration of 0.2% (w/v), and the disaggregant Dextran sulfate (DS, Sigma-Aldrich, Ref. 31404), at a concentration of 25 $\mu g \cdot mL^{-1}$, were used (Beas-Catena, 2013). The density and viscosity of the culture medium with additives was 1010 $g \cdot mL^{-1}$ and 1.26 Pa·s, respectively. Inoculum was grown in Erlenmeyer flasks of 250 mL (50 mL of culture) agitated at 75 rpm in an orbital shaker with an orbital diameter of 1.9 cm. Flasks were placed in an incubator at 27 ºC and humidification was achieved using a water tray on the bottom of the incubator. Cells were passaged every 4 days at a cell density of $5\text{-}6 \cdot 10^5$ $cell \cdot mL^{-1}$. Cells in the exponential phase were used in all assays.

The cell diameter was determined using the AnalySIS® software from images taken under the light microscope with a Pixelink camera (model no. PL-A662). The mean cell diameter was 18 µm.

Cell concentration and viability in all experiments were measured using a hemocytometer under a light microscope and the Trypan Blue dye-exclusion method.

2.2. Centrifugation assays

Suspensions at a cell concentration of 1.5×10^6 $cells \cdot mL^{-1}$ and a viability above 97%, obtained from the inoculum as described in section 2.1, were deposited in Falcon tubes and clarified in a benchtop centrifuge (model SIGMA 4-15C) using a rotor with a maximum radius of 18.2 cm. After every centrifugation, the supernatant was withdrawn and the cell pellet carefully resuspended in 5 mL of fresh medium. Cell concentration and viability were measured for the resulting suspension and the efficiency of the clarification process (η_c) was defined as follows,

$$\eta_c = \frac{N_p}{N_i} \quad (1)$$

where N_p and N_i are the number of total cells in the pellet and in suspension prior to treatment, respectively.

Two types of Falcon tubes (F15 and F50, with a maximum capacity of 15 and 50 mL respectively) were used. The characteristic dimensions of the tubes based on length (l), inner diameter (ϕ), cone height (h_c) and cone base (b_c) were as follows: (i) F15 (l = 118.5, ϕ = 14.6, h_c = 24.0, b_c = 2.0 mm); F50 (l = 114.4, ϕ = 27.0, h_c = 14.0, b_c = 4.6 mm). Experiments were designed by selectively combining the following factors: type of tube (F15 and F50), height of suspension in the tubes (h, 22 and 90 mm), g force (g_c, 15 levels ranging from 0 to 4000 ×g) and centrifugation time (t_c, 7 levels ranging from 0 to 45 min). A total of 68 trials were completed in duplicate. Experimental sedimentation times were determined from η_c versus time at η_c near 100% curves. Theoretical sedimentation times were estimated from Stoke's settling velocity equation given by

$$v_{stoke} = \frac{2}{9} g_c \frac{r_c}{\mu} (\rho_s - \rho_l) \quad (2)$$

where g_c is the acceleration applied, r_c the radius of the cell, μ the viscosity of the culture media, ρ_s and ρ_l the densities of the cell (1.025 g·mL^{-1}) and the culture media, respectively.

2.3. Sub-cultivation of previously centrifuged cells

To evaluate whether a given centrifugation treatment might have caused cell damage that was not detectable using viability measures, or if the damage observed by reduction of cell viability was reversible, previously centrifuged cells were sub-cultured. Briefly, cell pellets were resuspended in 4.5 mL of fresh medium and cultured for 7 days under the same environmental conditions as those for the inocula described above.

Initial cell concentrations were fixed near $5 \cdot 10^5$ cells·mL^{-1}. A control culture was carried out with non-centrifuged cells. All experiments were conducted in duplicate. Cell concentration and cellular viability were monitored at the beginning and end of the culture period (7 days).

Sub-cultured cells were obtained from different centrifugation treatments performed in F15 tubes, at an *h* of 22 mm, g_c varying from 0 to 4000 *g*, and t_c ranging from 0 to 16 min. Selection of treatments (n=17) provided a wide range of values for a novel, intensive variable, denominated excess of treatment (*ET*), defined as:

$$ET = g_C \cdot t_{pellet} \tag{3}$$

ET is similar to the g-second variable traditionally used in centrifugation (Urbina et al., 2016) but with the peculiarity that time of centrifugation (t_c) is replaced by the residence time of cells in the pellet (t_{pellet}), calculated as the difference between the treatment time (t_c) and the sedimentation time calculated from CFD simulations.

The cell concentration (*C*) and cellular viability were monitored and the relative cell yield (P_r) of each culture was determined as follows,

$$P_r = \frac{C_f - C_o}{C_{fC} - C_o} \tag{4}$$

where C_o is the initial viable cell concentration, C_f is the final viable cell concentration attained for a given *ET* and C_{fC} the final viable cell concentration observed in the control culture.

2.4 CFD simulations

Cell-sedimentation times and shear stress fields for each centrifugation treatment assayed were simulated using the CFD software Fluent® v16.2 (Ansys, Canonsburg, PA, USA). As the tube is symmetrical it was simulated in 2D using a structured grid

with an optimum size of 0.2 mm. A laminar model and a multiphase model with two Eulerian phases, in which the cells represent the granular phase, were also used. The properties of the phases can be found in section 2.1. The initial cell volume fraction in the suspension was 0.00458, as calculated from the cell diameter and the cell concentration in suspension. No energy balance was imposed, as isothermal conditions were assumed. Centrifuge acceleration was varied in the tube axis direction, as the tube rotates in a horizontal plane once the centrifuge reaches the selected speed. The reference for pressure was at the top of the suspension. Boundary conditions included non-slip conditions at the walls. The schemes used for spatial discretization were: for momentum, second order upwind; for gradient, Green-Gauss Node Based and Modified HRIC for Volume Fraction. The SIMPLE scheme with implicit formulation was chosen for pressure-velocity coupling. All simulations in the present study were performed in transient mode using a time step of 0.0005 s. The convergence criteria were checked at every time-step and residuals for all the variables were fixed at 10^{-5}. An HP Workstation Z840 with two Intel® Xeon E5-2670 v3 processors running at 2.3 GHz with 128.0 GB RAM and 3 TB ×2 hard disks was used for the simulations.

3. Results and discussion

3.1. Determination of cell sedimentability

Figure 1 shows the effect of g_c and t_c on the efficiency of clarification (η_c). As can be seen, cells were effectively separated by centrifugation. Moreover, the shortest t_c (i.e. 1 min) required a minimum g_c value of 200 ×g to settle all the cells at the bottom of the tube. In contrast, the lowest g_c value of 10×g needed 16 min of centrifugation for complete cell separation. A relatively short and soft treatment could be established at the g_c and t_c values of 40-50×g and 8 min, respectively, although a higher h value would evidently entail an increase in t_c. Values of g_c reported for standard protocols ranged

from 800 to 2000×g (Murhammer, 2016; King et al., 2016; Lynn, 2016) and are much higher than those needed for complete separation in this study. As such, centrifugation may have been excessive in many literature reports. Indeed, cells may be damaged or lysed as result of this excess treatment (Xu et al. 2015). CFD can help prevent this scenario by predicting separation times for a given cell line, culture medium and acceleration, without needing to perform experiments.

Table I compares the effective sedimentation times obtained by CFD simulation with the experimental ones presented herein for different combinations of tube type, h, g_c and t_c. The results for assays 1 to 4 in Table I are also represented in Fig. 1.

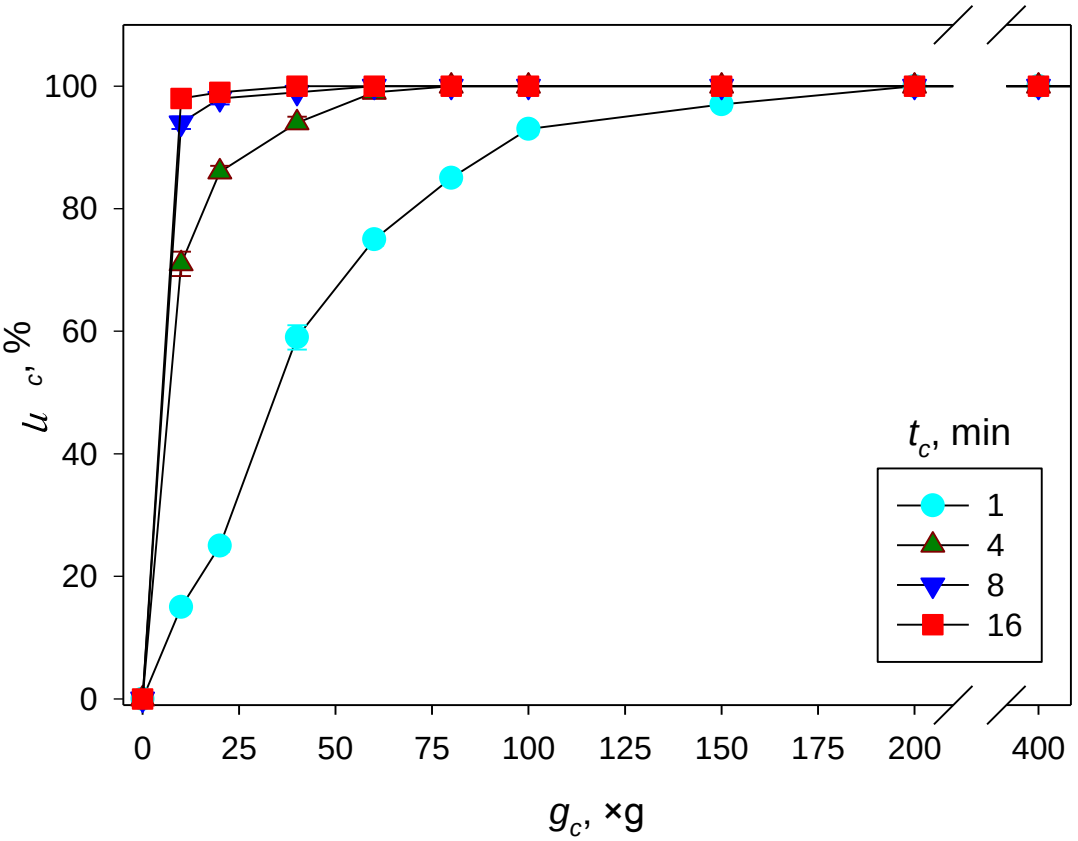

Figure 1. Influence of the acceleration (g_c) and centrifugation time (t_c) on the clarification efficiency (η_c). Type of tube: F15; height of suspension (h): 22 mm. Data points are averages, and vertical bars are difference between duplicate samples. When the bar is absent, the bar is smaller than the point. ~~Data points are averages, and vertical bars are standard deviations (SDs) for duplicate samples. When the SD bar is absent, the SD is smaller than the point.~~

As can be seen from Table I, the theoretical sedimentation times obtained from

the Stoke's equation are slightly shorter than the simulated and experimental values. This result was expected, since Stoke's equation does not take into account possible cell-cell or cell-tube interactions, which may hinder the settling of cells. However, this equation can nevertheless be used to obtain an approximate value for the minimum duration of the treatment. Simulated CFD sedimentation times were also lower than their experimental counterparts but closer to the theoretical ones. Any discrepancies were due to a lack of accuracy in the graphical determination of sedimentation times. For example, from the data represented in Fig. 1, it is predicted for assay n 2 in Table I (i.e., a g_c of 20×g and h of 22 mm) a 100% η_c between 8 and 16 min, in other words around 12 min. Therefore, the CFD results were similar to the experimental ones, thus demonstrating CFD to be a useful tool for predicting precise sedimentation times for cell suspensions as a function of g_c.

Table 1. Theoretical sedimentation time estimated from the Stoke's settling velocity (v_{stoke}), sedimentation time from CFD simulations and experimental sedimentation time for various combinations of tube size (15 mL (F15) and 50 mL (F50)), height of suspension in the tubes (h), g-force (g_c) and centrifugation time (t_c).

Nº Exp.	Tube, mL	g_c, ×g	h, mm	v_{Stoke}, m·s^{-1}	Sedimentation time, min		
					Theoretical	Simulated	Experimental
1	F15	1	22	$2.10 \cdot 10^{-6}$	187	201.8	216
2	F15	20	22	$4.20 \cdot 10^{-5}$	9.3	12	16
3	F15	40	22	$8.41 \cdot 10^{-5}$	4.7	5.1	8
4	F15	60	22	$1.26 \cdot 10^{-4}$	3.1	3.3	4.0
5	F15	40	90	$8.41 \cdot 10^{-5}$	19.1	18.8	19.0
6	F50	40	90	$8.41 \cdot 10^{-5}$	19.1	19.0	19.0

A wide variety of centrifuge tubes of different sizes and shapes (e.g. flat-, round- or conical-bottomed) designed for use in any biotechnology application are available. As such, the impact of container size and geometry on recovery of the biomass was evaluated by performing two experiments in the F15 and F50 tubes with the same h (90 mm) and g_c (40 ×g; assays 5 and 6 in Table 1, respectively). The evolution of the clarification efficiency with time for both tubes was virtually identical, thus indicating no effect of geometry or volume on cell recovery. The measured data agreed

significantly well with the CFD model predictions.

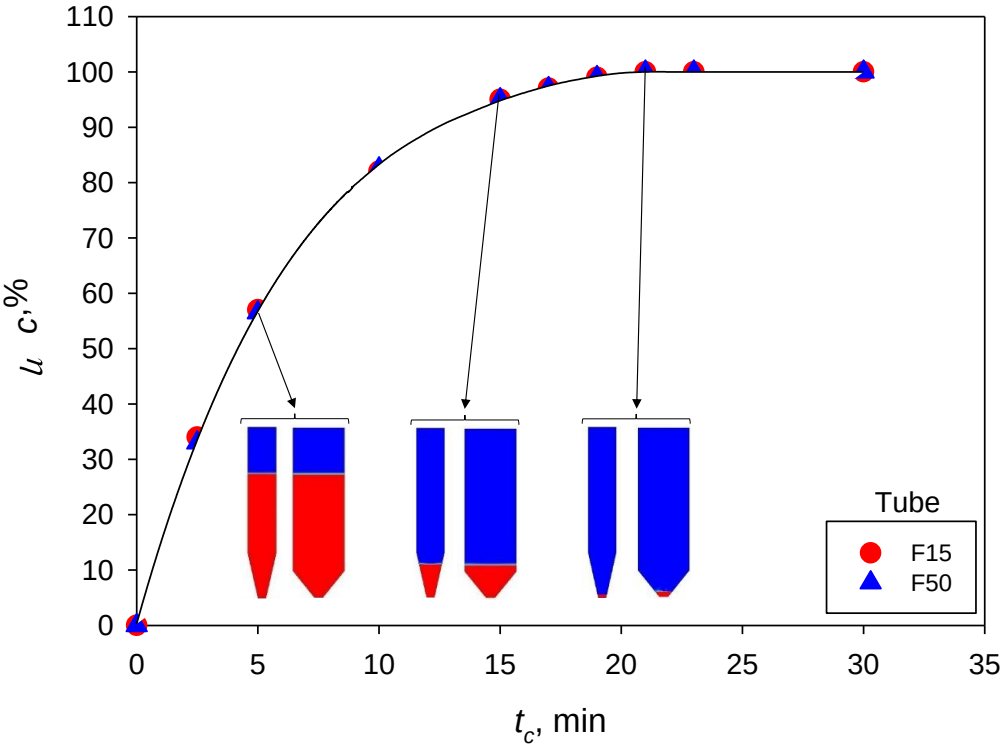

Figure 2. Separation efficiency (η_c) as a function of centrifugation time (t_c) for two types of tubes (F15 and F50 represent Falcon tubes with capacities of 15 and 50 mL, respectively). The inset figures represent the state of the suspensions in both tubes as predicted by CFD: the blue area corresponds to the region of clarified liquid (i.e. cell-free zone) and the red zone to the region of cell suspension. The initial height of the suspension in the tubes (h) was 90 mm and g_c was 40 ×g. Data points are averages, and vertical bars are difference between duplicate samples. When the bar is absent, the bar is smaller than the point.

The inset graphs in Fig. 2 show the progression of the suspension interface (i.e., boundary between the cell-free upper zone and cell suspension zone). As expected, once the sedimentation time is achieved (around 19 min) the pellet geometry coincides with the conical shape of the tubes.

In order to verify that the sedimentation time is independent of the conical-bottomed geometry of the tube, additional CFD simulations were performed changing the aspect ratio of the Falcon tube. Fig. 3 shows the progression of the suspension interface height for four different aspect ratios. As can be seen, the interface moves forward at the same velocity in all four configurations. These results make CFD

simulations a solid and useful tool for predicting the behavior of any single-cell suspensions under discontinuous centrifugation and for accurately predicting settling times without the need for experimentation.

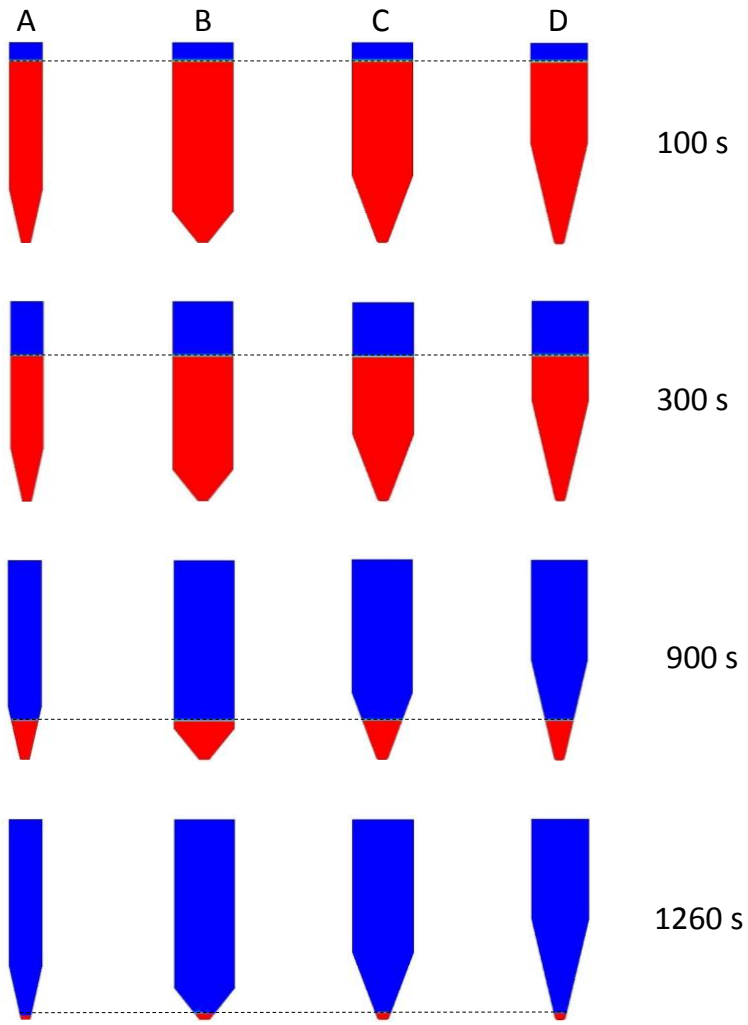

Fig. 3. CFD simulation of the progression of the suspension interface height for different aspect ratios of Falcon tubes: **(A)** 15 mL commercial Falcon tube (F15); **(B)** 50 mL commercial Falcon tube (F50); **(C)** fictitious 50 mL Falcon tube with an enlarged cone of 30 mm, and (D) fictitious 50 mL Falcon tube with an enlarged cone of 45 mm. h = 90 mm; g_c = 40 ×g. Dashed horizontal lines indicate the interphase between the two zones.

3.2. Effect of applied acceleration on cell viability

The origin of the cell damage observed in the centrifugation process could be related to the relative cell-fluid movement, the velocity gradients that cells encounter on their way to the bottom of the recipient or the compressive forces to which they are

submitted once in the pellet. In this regard, Xu et al. (2015) carried out a detailed study of the potential source of cellular damage during centrifugation using the microalga *Dunaliella salina*. These authors determined the values of hydrodynamic stress due to turbulence, viscous drag (F_d), the increase in cell "weight" due to the centrifugal force, hydrostatic pressure due to the liquid head (P_h) and pressure due to the "weight" of cells in the pellet of recovered cell material. They concluded that, for a fixed centrifugation time, all forces were negligible compared to the hydrostatic pressure, which was mainly responsible for the damage to *D. salina* cells.

CFD simulations are very useful for calculating flow-related magnitudes such as shear stress (τ) or energy dissipation rates. In this work it was possible to precisely determine the shear stress levels from the CFD-simulated shear stress field. Figure 4 displays the contour graph of shear-stress in the conical zone of the tube at a value of 4000 ×g.

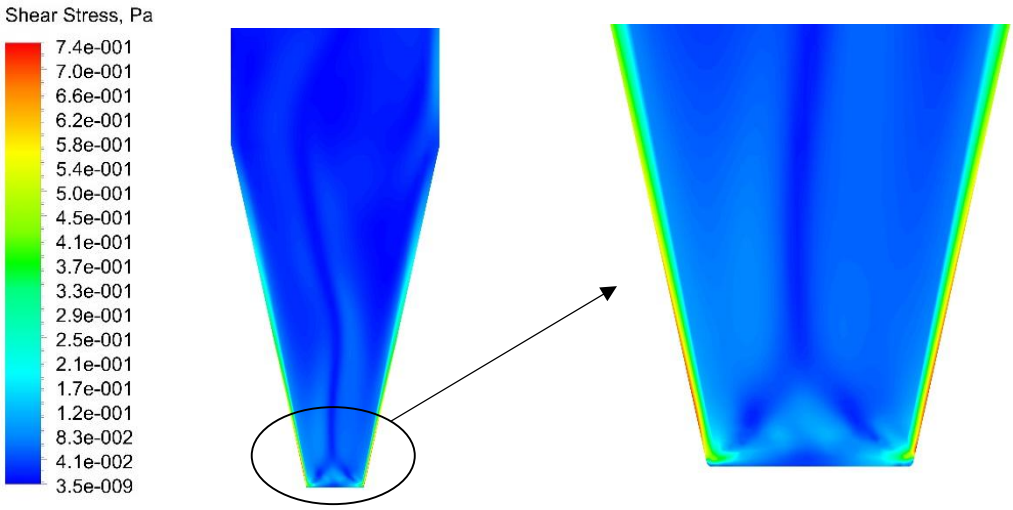

Figure 4. Contours of Shear Stress in the cone of the centrifuge tube from CFD simulations at g_c = 4000 ×g and h = 90 mm.

As expected, the highest values of τ ($7.4 \cdot 10^{-1}$ Pa) were found at the wall of the lower part of the cone. An average breaking shear stress value of 233±25 Pa was determined for the same insect cell line used herein (Se301) employing a microfluidic

flow-contraction device (Gallardo-Rodríguez et al., 2016). Consequently, it can be definitively inferred that any cell damage observed is not due to velocity gradients that the cells encounter on their way to the pellet.

Moreover, as no cell damage was observed in cells that did not settle, P_h does not cause cell damage in the cells' journey to the bottom of the container either. This is also expected because the intracellular fluid (which is mainly water) can be considered to be incompressible and P_h provokes a radial compressive force.

Peterson et al. (2012) also observed that, once a cell has been deposited, it is squeezed against the tube walls by the action of total compressive force. The authors defined what they termed the "compaction parameter", which depends on the mass and volume of the pellet but not on the acceleration or the time the cells remain in the pellet. Using this parameter they were able determine the volume of the pellet damaged in the centrifugation.

The duration of the centrifugation can be selected arbitrarily as the same separation can be obtained from treatments of different duration (see Fig. 1). If the time of this operation is not accurately adjusted, the cells stay in the pellet for longer than required to settle the whole cell population. As a consequence, the pressure on the pelleted cells can act for long periods of time. As the time in the pellet has been previously seen to possibly influence cell damage (Maybury et al., 2000), the time cells can remain in the pellet without suffering damage should be determined. In addition to physical damage, high residence times in the pellet could be deleterious to the cells as a result of deprivation of oxygen or other nutrients (Häggstrom, L, 2003) even though this happens at lower P_h values than those that provoke cellular rupture. As such, the time that the entire cell population remained in the pellet (t_{pellet}) was defined (section 2.3). If there is no available CFD simulations or experimental sedimentation times, a reasonable

approach may be to calculate them from the Stoke's equation (equation 2) by dividing the suspension height by the settling velocity. The time in the pellet, thus, is a magnitude that can easily be calculated from sedimentation times. The cell viability relative to the control for all the assays carried out in F15 tubes at an *h* of 22 mm (n = 68) is plotted against t_{pellet} in Figure 5.

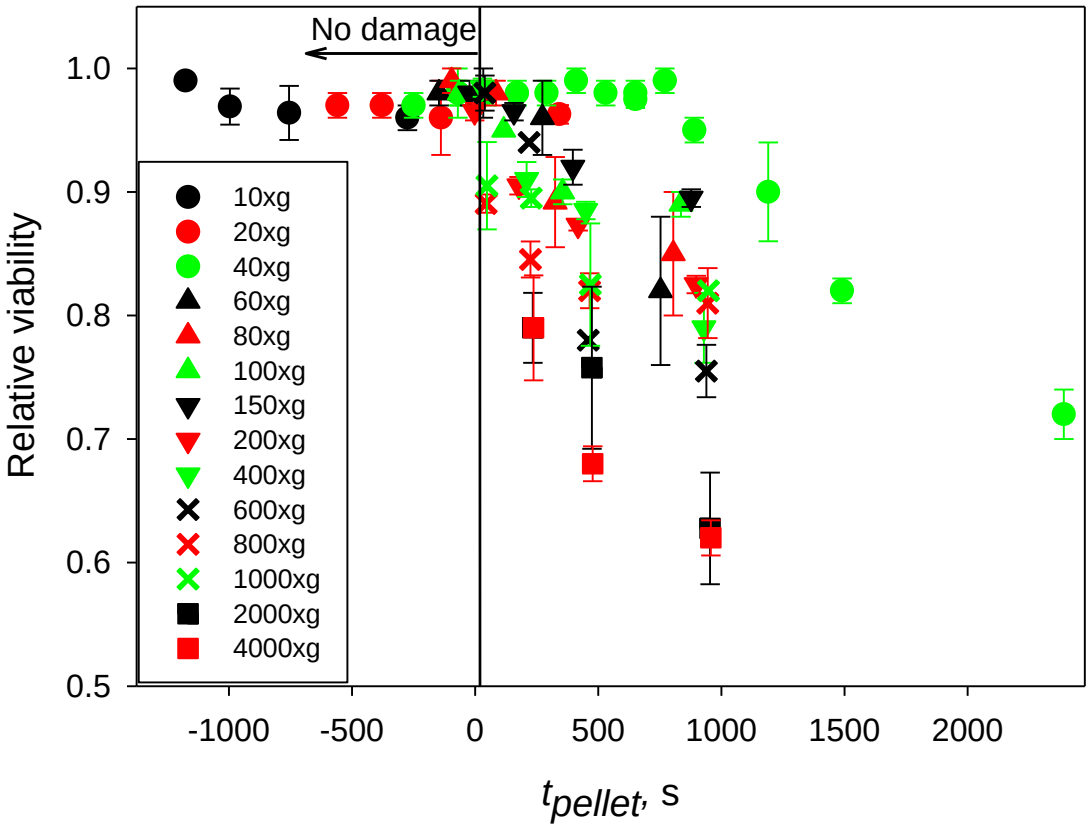

Fig. 5. Influence of the time that the cell population remains in the pellet (t_{pellet}) on the relative cell viability for assays performed in the F15 Tube with a suspension height (*h*) of 22 mm. The vertical line indicates the value of time in pellet at which the cells start to be damaged. Data points are averages, and vertical bars are difference between duplicate samples. When bar is absent, the difference is smaller than the point.

The negative values of time in pellet in Figure 5 correspond to experiments in which not all the cells were sedimented. The vertical line indicates the value of t_{pellet} at which cell damage starts to be observed at some accelerations. At t_{pellet} bellow that value no cell damage is observed irrespective of the acceleration applied. Hutchinson et al. (2006) also observed no cell breakage at laboratory level on their centrifugation experiments.

As flow-related damage is fast acting (Hutchinson et al., 2006; Gallardo Rodríguez et al., 2016), the reduction in cell viability observed must be associated with processes that appear once the whole cell population has settled. Probably being the cells that sediment first the ones that first suffer cell damage. Although the sedimentation time at g_c values higher than 400×g was below the minimum t_c assayed (1 min), damage appears at positive values of time in pellet. In light of this, it can be inferred that cells are not damaged in their journey to the bottom of the container. It can also be observed that, irrespective of the acceleration used, the cells can stay in the pellet for about 45 s without being damaged. A special behavior is observed at 40×g. This condition was chosen to carry out experiments with a duration of up to 45 minutes because it is a soft treatment and the sedimentation time is relatively short. Under these conditions the cells can stay in the pellet for about 15 min (900 s) without any measurable damage. The decrease in cell viability at longer times could be related to oxygen depletion in the pellet rather than to mechanical cell damage. Oxygen depletion triggers a cascade of cell responses that start in the cell membrane, changing its mechanical resistance and, if prolonged, result in cell death (Barvitenko et al., 2015). Any other higher acceleration causes damage to the cells in the pellet if they remain therein for longer than 45 s. The most acute damage is seen for more intense treatments, with a maximum at 4000×g. This would indicate that such damage is mechanical rather than biological in nature.

As different evolutions can be seen from Figure 5, it was decided to determine the influence of the time in pellet, the acceleration applied to the centrifugation process and the replica on cell viability by means of an ANOVA analysis of the experiments shown in that figure. The results indicated that the replica (p-value > 0.05) had no statistically significant influence on the variance of cell viability. However, both t_{pellet}

(p-value < 0.05) and acceleration (p-value < 0.05) had an effect, with the latter showing the highest influence (F-value of 28). Taking these results into account and in order to be able to compare different time-acceleration combinations, we defined an intensive variable denominated Excess of treatment (ET), as described by equation (3) in section 2.3.

Fig. 6 plots the results of the relative cell viability in Fig. 5 with respect to the corresponding calculated ET values. For clarity, since no cellular damage was observed at negative values of t_{pellet} (see Fig. 5), only positive values were plotted in Fig. 6.

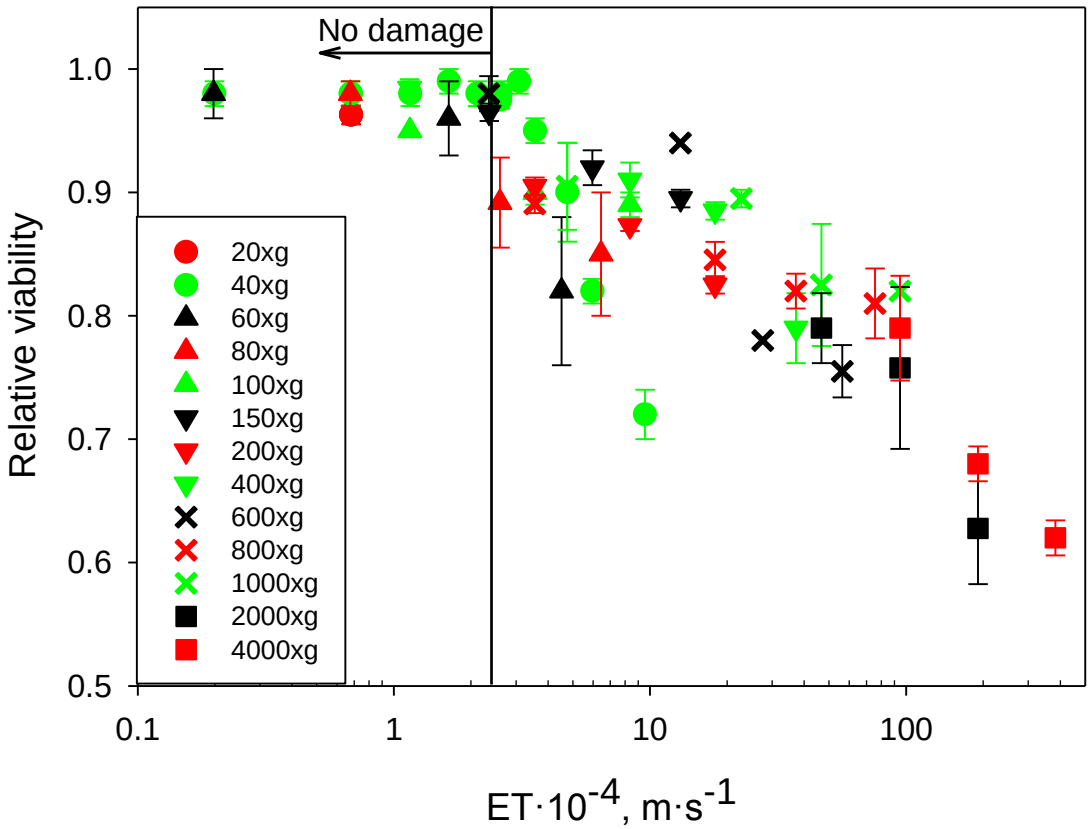

Figure 6. Influence of the excess of treatment (ET) on relative cell viability for assays performed in the F15 Tube with a suspension height (*h*) of 22 mm. The vertical line indicate the value of ET at which the cells start to be damaged. Data points are averages, and vertical bars are difference between duplicate samples. When bar is absent, the difference is smaller than the point.

As can be seen for all combinations, even for 40×g, there is a zone (up to $2.5 \cdot 10^4$ $m \cdot s^{-1}$) in which viability is not affected and there is no cell damage. From this value onwards there is a steep and sustained decrease of this variable for all conditions, to a

minimum value of 0.6 at close to ET = $400 \cdot 10^4$ $m \cdot s^{-1}$, corresponding to 4000×g and 16 min. This behavior, although at different ET values, is extendable to any cell line, therefore experiments of this type should be performed with the cell line of interest if viable cells are to be recovered by centrifugation. According to the results in Figure 6, at a suspension height of 22 mm, this cell line should not be centrifuged at accelerations higher than 600×g, since the cells sediment in less than 20 seconds. Therefore, in a treatment as short as 1 min the cells remain in the pellet for more than 40 seconds (ET above the threshold value of $2.5 \cdot 10^4$ $m \cdot s^{-1}$) and suffer damage. The results shown are independent of the suspension height, as is ET.

Centrifugation is a sufficiently intense treatment to cause damage to cells as robust as plant cells, microalgae and bacteria (Xu et al., 2015; Urbina et al., 2016; Peterson et al., 2012). The results in Figure 6 demonstrate that the Se301 cell line is very sensitive to centrifugation, therefore special attention should be paid to this operation if a healthy cell population is to be recovered. This cell line has also been shown to be especially sensitive to suspension culture, much more so than other insect cell lines (Beas-Catena et al., 2013). For example, the experiments of Xu et al. (2015) with the microalgae *D. salina*, which does not have a cell wall, indicate that it may withstand an ET about two orders of magnitude (> $2.5 \cdot 10^6$ $m \cdot s^{-1}$, ie, 5000×g for 10 min) higher than our cell line. In addition, even cells as sensitive as red blood cells have been shown to be more resistant than Se301 cells since a decrease in cellular viability of only 4% was observed in treatments of 10 min at up to 1500×g (Urbina et al., 2016). However, the authors observed an increase in sub-lethal damage with centrifugation intensity.

3.3. Effect of centrifugation on subcultivation

The above discussion highlights the need to establish a cell line dependent centrifugation protocol suitable for subcultivation purposes in order to prevent ~~sub-lethal (cell damage that does not appreciably affect culture performance) or lethal~~ damage that may reduce the productivity of a given bioprocess (Lara et al., 2006; Urbina et al., 2016). In this sense, cells centrifuged at different *ET* values within the same range used in Fig. 5 (i.e., from 2000 to $4 \cdot 10^6$ ms^{-1}), were subcultured as described in section 2.3. Fig. 7, which shows that the effect of *ET* on the relative cell productivity measured in subcultures, follows an analogous pattern to Fig. 6, with a safe zone up to about $6 \cdot 10^4$ $m \cdot s^{-1}$ of ET in which culture productivities were not affected. Comparing Figs. 6 and 7, there apparently is a certain ~~reversible~~ cell damage ~~(sub-lethal cell damage)~~ produced between 2.5 and $6 \cdot 10^4$ $m \cdot s^{-1}$ that does not appreciably affect cell growth. Similarly to Fig. 6, a steep descent is also observed after that threshold.

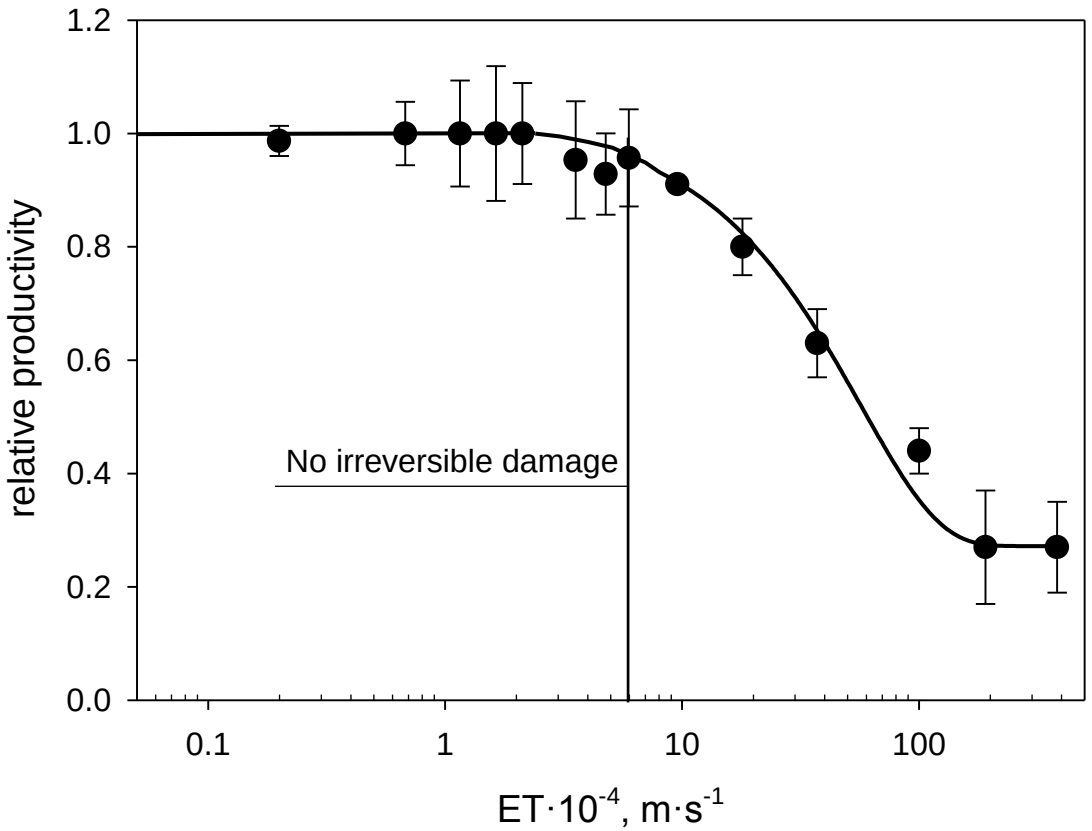

Figure 7. Influence of the excess of treatment (ET) on culture productivity relative to a control culture with no prior centrifugation. Centrifugation performed in the F15

Tube with a suspension height (*h*) of 22 mm. Culture assays performed in shake flasks with a culture volume of 5 mL. The vertical line indicates the ET value at which the cells cannot recover from the damage suffered (lethal cell damage). Data points are averages, and vertical bars are difference between duplicate samples. When bar is absent, the difference is smaller than the point.

Therefore, there was a small range ~~of sub-lethal~~ damage that did not affect culture productivity. However, as this interval of ET for this cell line is very narrow, it is recommended to use cell viability measurements results to safely subculture centrifuged cells.

4. Conclusions

The Se301 insect cell line can be easily sedimented by centrifugation, with a soft treatment (4-8 min at 40-80×g) being necessary to settle 100% of cells. Other treatments with higher times or accelerations are excessive and may cause cell damage.

Cell damage was observed in the centrifugation operation, with a combination of residence time in the pellet and acceleration applied being responsible for this damage. Centrifugation-related cell damage was adequately predicted using an intensive variable defined as Excess of Treatment (ET), which is the product of time in pellet and acceleration. This variable is easy to calculate as the time in pellet can be easily obtained from CFD calculations, from centrifugation experiments or, less precisely, from the Stoke's sedimentation velocity. ET is also of general use for any cell line and can be obtained from simple experiments similar to those performed in this work. ET values greater than $6 \cdot 10^4$ $m \cdot s^{-1}$ indicate irreparable damage to the cells, thereby greatly reducing the productivity of subsequent cultures, although values greater than $2.5 \cdot 10^4$ $m \cdot s^{-1}$ start to affect cell viability ~~(sub-lethal damage)~~. Viability reduction between both values does not appreciably affect culture productivity. For safety reasons, this fact should be checked with the cell line of interest and, as a general recommendation,

centrifugation intensity for subculture should be performed bellow the ET value that reduces cell viability.

CFD simulations of the experiments performed were especially useful for determining the values of cell damaging variables such as shear stress and for determining the time of sedimentation. This technique can be used for virtually any cell suspension and centrifuge and reliably reduces the experimental work required to calculate settle times. In light of the results obtained, it is important to carry out studies of this type in those cases in which viable and healthy cells are to be recovered since the application of an inadequate protocol can damage these cells irreversibly.

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