

AGITATION EFFECTS ON MICROCARRIER AND SUSPENSION CHO CELLS

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SUMMARY

The growth properties of attachment-dependent and attachment-independent CHO cells cultured in spinner-flask bioreactors were compared to investigate cell damage from rapid agitation. Damage was attributed to bulk-fluid turbulence for attachment-dependent CHO cells and bubble breakup associated with vortex formation for attachment-independent CHO cells. Radiolabeling demonstrated that cell damage included intrinsic changes in DNA synthesis.

INTRODUCTION

In vitro cultures of animal cells are hosts for an increasing assortment of biological products. Efficient production of these biologicals requires an understanding of the influence that the culturing environment has on cell growth and product yield. To this end, this work investigated the effects of agitation on microcarrier and suspension cultures of Chinese Hamster Ovary (CHO) cells grown in spinner flasks. The rationale for selecting this cell type is three fold: they are extremely robust (Gottesman, 1985), are adaptable from attachment-dependent to attachment-independent growth (Stanley *et al.*, 1975), and are widely used to produce recombinant proteins (Manos, 1988). As such, this research should be applicable to a number of production processes.

Agitation, shear and other hydrodynamic phenomena have been found to alter cell function, including metabolism (Chittur *et al.*, 1988; Diamond *et al.*, 1990) and growth (Croughan and Wang, 1989; Papoutsakis, 1991). To date, research on these hydrodynamic effects has been performed primarily with either attachment-dependent or attachment-independent cells, but comparisons of the two cell types have been very limited (Kretzmer *et al.*, 1989). The majority of comparative studies have not investigated hydrodynamic effects; rather, they have focused on the relationship between cell function and cell attachment (Farmer *et al.*, 1983).

The research described below expands and enriches previous work on hydrodynamic effects by presenting a model system to study both attachment-dependent and attachment-independent cells with the same cell line and in the same reactor environment. In the following, a comparison of growth properties of microcarrier and suspension CHO cultures in rapidly agitated spinner flasks provides insight into fluid-mixing characteristics which induce cell damage. Moreover, DNA synthesis was investigated to determine whether apparent changes in cell growth rate reflect intrinsic changes in DNA synthesis.

MATERIALS AND METHODS

Cultivation. The CHO subclone, Pro⁻5 (CRL 1781, American Type Culture Collection, Rockville, MD, USA), was developed by Stanley *et al.* (1975) and can be adapted from attachment-dependent to attachment-independent growth. Prior to adaptation, these cells were cultivated at 37 °C, 5% CO₂ and 95% relative humidity in a 1:1 mixture of F-12/DME medium at pH 7.4 containing 3150 mg/L glucose and supplemented with 0.042 mg/L linoleic acid (L 1012, Sigma Chemical Co., St. Louis, MO, USA), 2.0 mM L-glutamine (G 7513, Sigma), 10.0 ml/L penicillin/streptomycin/neomycin (PSN) solution (P 9032, Sigma) and 10.0 ml/L HB CHO protein solution (T401, Hana Biologics Inc., Alameda, CA, USA). This medium could not

support attachment-independent growth; instead, a 1.5:1:1 mixture of Joklik (410-2300EB, Gibco Laboratories, Grand Island, NY, USA)/F-12 (N 6760, Sigma)/DME (D 5030, Sigma) medium at pH 7.1 that was supplemented with 5% (v/v) defined fetal bovine serum (A-1111-L, HyClone Laboratories, Logan, UT, USA), 6 mM L-glutamine, 11.5 ml/L MEM non-essential amino acid solution (M 7145, Sigma) and 10.0 ml/L PSN solution was employed for this purpose. Procedures for cell maintenance are described by Freshney (1987).

For agitation experiments, both attachment-dependent and attachment-independent CHO cells were cultivated in 100 ml spinner flasks (1965-00100, Bellco Glass Inc., Vineland, NJ, USA). The attachment-dependent CHO cells were grown as a microcarrier culture on Cytodex-3 beads (C 3275, Sigma) at a density of 5 g beads/L and seeding of 5 viable cells/bead. Previous research found that this bead density and seeding favored cell proliferation (Croughan *et al.*, 1988). The microcarrier culture was prepared according to the protocol provided by Pharmacia (1981) and agitated using a low-profile magnetic stirrer (7761-06005, Bellco). Experimental conditions for both the microcarrier and suspension cultures are given in the figure legends.

Assays. Trypan blue exclusion was employed to measure cell viability (Freshney, 1987). Cell concentration was determined with a Coulter Multisizer (Coulter Electronics Inc., OPA Locka, FL, USA) for suspension cultures (Kunas and Papoutsakis, 1990) and by counting released nuclei for microcarrier cultures (Pharmacia, 1981). DNA synthesis was quantified with radiolabeling by measuring the incorporation of ^3H -thymidine into the genome of viable CHO cells. Radiolabeling was performed on samples of suspension cells from spinner flask cultures. Each sample contained 5×10^4 viable cells and was processed immediately to prevent adaptation to a non-agitated environment. To maximize radiolabel uptake, conditioned medium containing unlabeled thymidine was removed and replaced with 200 μl serum-free medium. The sample was then incubated for 30 min at 37 $^{\circ}\text{C}$ with 2 μCi ^3H -thymidine (24042, ICN, Irvine, CA, USA). After the incubation, radioactive medium was removed, and cells were rinsed with 1.0 ml PBS to wash off unincorporated label. Cells were resuspended in 200 μl NETS (100 mM NaCl, 1 mM EDTA and 10 mM Tris at pH 7.4, and 0.5% (w/v) SDS) and 700 μl H_2O , and lysed by vigorous pipetting. Genomic DNA was precipitated by adding 100 μl of 100% (w/v) trichloroacetic acid (TCA) and storing the sample overnight at -20 $^{\circ}\text{C}$. Precipitated DNA was collected on the Millipore 1225 sampling manifold (XX2702550, Millipore Corp., Bedford, MA, USA). Filtered samples were washed first with 10% (w/v) TCA and then with 95% (v/v) EtOH to promote drying and prevent aqueous quenching of the scintillation count. The dry filter was transferred to a 6 ml polyethylene scintillation vial (986644, Wheaton, Millville, NJ, USA) containing 5 ml of Formula 989 cocktail (NEF989, Dupont Co., Wilmington, DE, USA) and incubated for 12 hr to allow the cocktail to completely penetrate the filter. Tritium decay was counted for 1 min per sample on a scintillation counter (Tri-Carb 1900CA, Packard Instrument Co., Meriden, CT, USA).

RESULTS AND DISCUSSION

Cell Growth. Figure 1 shows the growth properties of microcarrier cultures of CHO Pro⁻5 agitated at 60, 100 and 150 rpm. In this set of experiments, all cultures were inoculated at 60 rpm where agitation effects were negligible. After the cultures had entered into exponential phase at 20 hr post inoculation, the impeller speed in the Bellco spinner flasks was increased. (Raising the impeller speed while a culture is still in lag phase produces false positive results.) At 60 rpm, the CHO cells had a doubling time of 26.1 hr. The doubling time increased by a factor of 1.25 at 100 rpm to 32.5 hr and by a factor of 1.46 at 150 rpm to 38.1 hr. As shown in Figure 1a, the exponential phase of cultures grown at 150 rpm was reduced from greater than 60 hr to under 30 hr followed by a rapid reduction in cell concentration as cells were dislodged from the Cytodex microcarrier beads.

Since CHO Pro⁻5 is adaptable to suspension growth, we examined whether the cells that were dislodged from the Cytodex beads remained viable and proliferated in suspension. The results are given in Figures 1b and c. Initially the inoculum contained 7.06×10^4 viable cells/ml at 80.6% viability. During the 20 hr post inoculation, cell concentration and viability were reduced by a factor of 10 and 4, respectively, as cells attached to the Cytodex beads. For cultures at 60 and 100 rpm, the ratio of viable suspension to Cytodex-bound cells never exceeded 0.06 for the remainder of the experiment; however, the ratio rose to 0.69 for the 150 rpm culture at 165 hr post inoculation. The F-12/DME medium that was used in this experiment does not support proliferation of suspension cells, only cell maintenance. In our

microcarrier experiments, the viability of the suspension cells was always less than 28% after inoculation, and was frequently less than 15%.

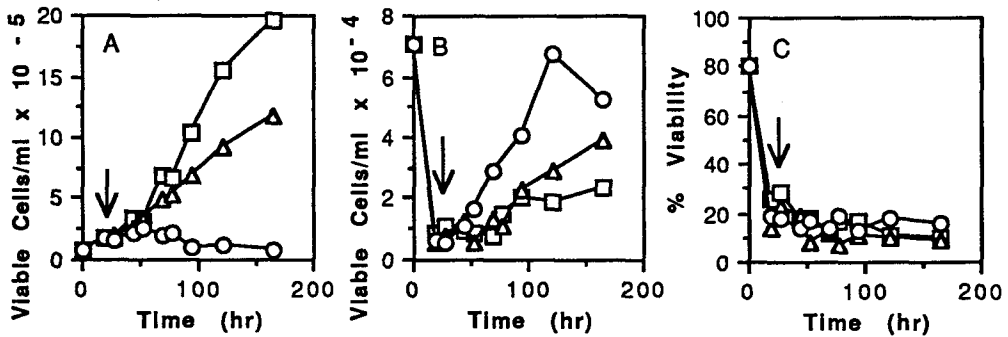


Figure 1. Effects of agitation on microcarrier cultures of attachment-dependent CHO Pro⁻5 bound to Cytodex-3 beads. Cells were cultivated in 100 ml Bellco spinner flasks each containing 5 g beads/L in 100 ml of F-12/DME medium and were maintained at 37 °C and 5% CO₂. Spinner flasks were inoculated with 7.1×10^4 viable cells/ml at a seeding of 5 viable cells/bead. Cultures were stirred at 60 rpm for 20 hr after which time the impeller speed was increased to 100 rpm (Δ), to 150 rpm (\circ), or maintained at 60 rpm (\square). The change in impeller speed is designated by an arrow. Each data point represents the average performance of two spinner cultures. Plotted are the concentration of Cytodex-bound cells (A), concentration of viable cells suspended in solution (B) and viability of the suspended cells (C).

For suspension CHO cultures, agitation effects were apparent at 340 rpm which was substantially greater than in microcarrier cultures. The suspension and microcarrier cultures were grown in the same spinner flasks except that the former contained a 2.5 x 5 cm plastic paddle from Bellco attached to the impeller to enhance fluid turbulence, resulting in cell damage at lower rpm's. (Spinner flasks had a tendency to vibrate and wobble above 400 rpm.) Figures 2a and b show growth and viability curves for suspension cultures at 100, 270, 340 and 400 rpm. All cultures were initially grown at 100 rpm. The impeller speed in the spinner flasks was increased 20 hr (100 - 340 rpm) or 26 hr (400 rpm) after inoculation. At 270 rpm and below, agitation effects were negligible, and cultures had a doubling time of 19.2 ± 1.2 hr. At 340 rpm and above, cell growth was inhibited. The viable cell concentration remained constant at $1.7 \pm 0.2 \times 10^5$ cells/ml for the 340 rpm culture and diminished from 2.1×10^5 cells/ml at 26 hr post inoculation to 3.2×10^4 cells/ml at 44 hr post inoculation for the 400 rpm culture. After this time, the viable cell concentration of the 400 rpm culture remained constant; however, cell viability rose sharply from 23% to 65%. This could reflect cell adaptation to rapid agitation and/or preferential lysis of dead cells over viable cells. Cell adaptation is further discussed in the section on DNA synthesis below.

In the literature, cell damage from bulk-liquid turbulence has been correlated to the ratio of the Kolmogorov-scale eddy size to bead diameter for microcarrier cultures (Cherry and Papoutsakis, 1986; Croughan *et al.*, 1987) and cell diameter for suspension cultures (Kunas and Papoutsakis, 1990; McQueen *et al.*, 1987). Damage initiates as the ratio approaches unity and intensifies at lower values. It has been proposed that eddies of the same size or smaller than cell particles (microcarrier beads or individual cells) cause high shear stresses on the cell surface, interparticle collisions and reactor-particle collisions (Cherry and Papoutsakis, 1986). For larger eddies, shearing and collisions are minimized as cell particles move in eddy streamlines.

Table I lists the values for the Kolmogorov-scale eddy size, η , for all experiments described in this work. Our estimates of η were based on a kinematic viscosity, ν , equivalent to that of water at 20 °C ($0.01 \text{ cm}^2 \text{ s}^{-1}$) and an energy dissipation rate per unit mass, ϵ , of $14 \text{ cm}^2 \text{ s}^{-3}$ at 60 rpm to $4,500 \text{ cm}^2 \text{ s}^{-3}$ at 400 rpm according to the following expressions given by Kolmogorov's theory (Cherry and Papoutsakis, 1986):

$$\eta = \left(\frac{\nu^3}{\varepsilon}\right)^{1/4} \quad \text{where} \quad \varepsilon = \frac{N_p n^3 d_i^5}{V}$$

In these equations, n is the impeller rotational speed in units of s^{-1} rather than rpm; d_i is the impeller diameter of 4.5 cm for microcarrier cultures and 5.0 cm for suspension cultures; N_p is the dimensionless power number; and V is a characteristic fluid volume in units of cm^3 and is traditionally taken as the reactor volume or $(d_i)^3$. The latter gives better correlations of η to cell damage (Kunas and Papoutsakis, 1990) and, thus, was used here. (The difference between the two volumes was less than 10% in our spinner flasks.) Values for N_p are shown in Table I and were obtained from plots of N_p vs. Re, impeller Reynolds number, for unbaffled stirred tanks mixed with a two-blade paddle (Nagata, 1975). These plots are a function of d_i/D and b/d_i where D is the tank diameter and b is the paddle width. For our system, D was 6 cm and b was 1 cm for microcarrier cultures and 2.5 cm for suspension cultures. To obtain N_p for the four-blade paddle used in the suspension cultures, b was doubled as described by Nagata. In these plots, the Reynolds number was defined with impeller diameter as the characteristic distance.

$$Re = \frac{d_i^2 n}{\nu}$$

All values for Re in Table I exceed 1,000, confirming that fluid flow in our spinner flasks was turbulent.

	Microcarrier Cultures			Suspension Cultures			
Calculated Parameter	60 rpm	100 rpm	150 rpm	100 rpm	270 rpm	340 rpm	400 rpm
Re	2,000	3,400	5,100	4,200	11,000	14,000	17,000
N_p	0.70	0.60	0.50	0.95	0.70	0.65	0.60
ε ($cm^2 s^{-3}$)	14	57	160	110	1,600	3,000	4,500
η (μm)	160	120	90	100	50	45	40

Table I. Eddy size and related parameters for microcarrier and suspension cultures.

As demonstrated in Table I, the correlation between eddy size and cell damage holds for our microcarrier cultures. Inhibition of cell growth became apparent and intensified as η became increasingly less than the average diameter of Cytodex-3 beads, 175 μm (Pharmacia, 1981). At first glance, the data in Table I for suspension cultures contradict the correlation; cell growth was inhibited at 340 rpm and completely arrested at 400 rpm with eddy sizes that were significantly larger than the average cell diameter of 12 μm . This paradox can be resolved by considering a recent study of CRL-8018 hybridoma cells which found that two phenomena participate in cell damage: bulk-liquid turbulence and vortex formation with associated gas entrainment and bubble breakup (Kunas and Papoutsakis, 1990). In the absence of vortex formation, hybridoma cultures survived and flourished at high impeller speeds up to 600 to 700 rpm. At higher speeds, eddy size (nearly 13 μm at 800 rpm when V is taken as $(d_i)^3$) approached cell diameter, and cell damage ensued. With a vortex, however, growth became inhibited at much lower speeds, 200 rpm. Hydrodynamic forces that accompany gas bubbles bursting at the gas/liquid interface of a vortex are the source of cell damage (Chalmers and Bavarian, 1991; Kunas and Papoutsakis, 1990). In our own experiments, vortices were present in suspension but not microcarrier cultures. As such, the dominant factor in cell damage was bulk-fluid turbulence for the microcarrier cultures and vortex formation for the suspension cultures.

Research in our group indicates that the greater sensitivity of our microcarrier CHO cultures to rapid agitation relative to our suspension CHO cultures was not due to a change in

media composition. Microcarrier cultures of bovine embryonic kidney (BEK) cells in a MEME serum-supplemented medium grown under the same conditions as in this work closely approximated our microcarrier CHO cultures in their response to agitation (Lakhotia and Papoutsakis, 1992). The analogous response of these cell systems suggests the relationship of cell particle to eddy size and vortex formation, and not medium composition, are the dominant factors in determining agitation effects on our CHO cultures.

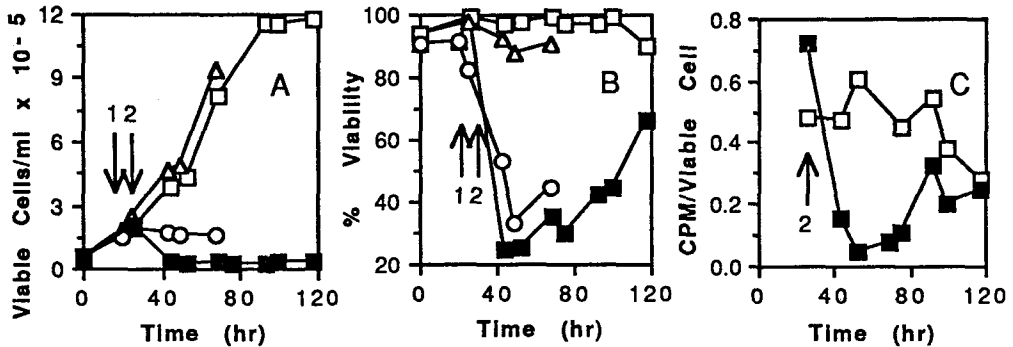


Figure 2. Agitation effects on suspension cultures of attachment-independent CHO Pro⁻⁵. Cultivation conditions were the same as in the microcarrier experiments with the following exceptions: cell were grown in Joklik/F-12/DME medium; each spinner flask was equipped with a 2.5 x 5 cm Bellco paddle on the impeller shaft; and spinners were inoculated with $5.6 \pm 0.5 \times 10^4$ viable cells/ml at 100 rpm (\square). The impeller speed was raised 20 hr (1st arrow) post inoculation for cultures grown at 270 rpm (Δ) and 340 rpm (\circ), and 26 hr (2nd arrow) post inoculation for cultures grown at 400 rpm (\blacksquare). Plotted are the viable cell concentration (A), percent viability (B) and uptake of ³H-thymidine into CHO suspension cultures as measured by counts per min (cpm) per viable cell (C). In order to observe adaptation with ³H-thymidine, culturing at 100 and 400 rpm was extended from 70 to 120 hr. For Figure 2c, radiolabeling was performed on the 100 rpm and 400 rpm cultures shown in Figures 2a and b according to the protocol given in the Materials and Methods section. Data points represent the average cpm's from four separate samples.

DNA Synthesis. Damage to cell culture can result in cell death and/or intrinsic changes in cellular processes. The first of these was verified with viability assays (Figure 2b). For the second, we chose to focus on cell replication and, in particular, DNA synthesis by measuring the uptake of ³H-thymidine into genomic DNA. Results from the radiolabeling experiments are shown in Figure 2c. Suspension cultures grown at 100 and 400 rpm were radiolabeled as a function of time. At 100 rpm, thymidine uptake per viable cell and, thus, DNA synthesis was constant within experimental error during exponential phase from 25 to 90 hr and decreased after 90 hr as the cells entered stationary phase. In contrast, the 400 rpm culture initially experienced a greater than 7 fold reduction in thymidine uptake during the first 25 hr and subsequently regained part of this loss as the CHO cells adapted to rapid agitation. The recovery in thymidine uptake is verified by a corresponding increase in viability. These results demonstrate that agitation altered DNA synthesis in CHO cells and that these cells have the capacity to adapt to adverse conditions.

Stress-induced changes in DNA synthesis have been previously reported. Sublytic, viscometric shear stress reduced DNA synthesis in T lymphocytes activated with mitogenic lectins (Chittur *et al.*, 1988). This change in synthesis corresponded and probably resulted from a decrease in interleukin-2 produced by the lymphocytes. Lakhotia *et al.* have recently reported an activation of DNA synthesis in CHO suspension cultures when agitated at 235 - 250 rpm in a Setric Genie 2 L bioreactor (1992). At these moderate impeller speeds, viability was reduced by 45 - 55%, and the rate of DNA synthesis in surviving S-phase cells was enhanced by 40 - 60%, rather than inhibited as observed in our work. This difference may, in part, be attributed to the cell population examined in each study: our data was based on the total number of viable cells, while Lakhotia *et al.* based their measurements on the S-phase viable cells. It is also possible that agitation can both activate DNA synthesis at moderate impeller speeds and be inhibitory at higher speeds such as those investigated in this work.

This duality has been noted by others, primarily with respect to biochemical effectors. For example, low calcium concentrations in media inhibit multiplication and favor the secretory cell phenotype in human tracheobronchial epithelial cells, while higher concentrations stimulate multiplication and inhibit the secretory phenotype (Chopra *et al.*, 1990).

The ability of DNA synthesis to adapt to environmental stress has been observed in cultures of root tip cells from *Vicia faba* seedlings with attached cotyledons (Yee and Rost, 1982). After the cells were stressed by adding 20% polyethylene glycol (PEG) to the culture medium, DNA synthesis was lower than in control cultures for the first 40 to 56 hr. As in Figure 2c, reduced DNA synthesis was followed by a period of adaptation during which synthesis approached the level of the control.

Conclusions. This research has demonstrated the usefulness of our CHO cell system in investigating changes in cell function resulting from adverse bioprocessing conditions and in elucidating the mechanisms which underlie these changes. Our knowledge of these phenomena is at its infancy, and additional information is required. The CHO model is a low-cost and efficient cell system with which to obtain this information. As our knowledge develops, so will the productivity and applications of *in vitro*, animal-cell culture.

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