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Maximizing mouse embryonic stem cell production in a stirred tank reactor by controlling dissolved oxygen concentration and continuous perfusion operation

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ABSTRACT

One of the key challenges in stem cell bioprocessing is the large-scale cultivation of stem cells in order to meet the demanding meaningful cell numbers needed for biomedical applications, especially for clinical settings. Mouse embryonic stem cells [1], used as a model system herein, were cultivated on microcarriers in a fully controlled stirred tank reactor (STR) [2]. The impact of varying the concentration of dissolved oxygen (at 5%, 10%, 20% and 30% DO) and operating under a continuous perfusion mode on cell growth and pluripotency maintenance was investigated. In addition, in order to further optimize the feeding strategy of the STR operating under continuous perfusion toward maximal cell production, the influence of different medium residences times (12 h, 24 h, 32 h, 48 h and 96 h) was evaluated. Overall, the maximal cell concentration of 7.9–9.2 $\times$ 10^6 cells/mL were attained after 11 days, with no passaging required, under a DO of 10–20% in the continuous perfused bioreactor with cell retention and medium residences times of 32–48 h. Importantly, mESC expanded under these conditions, retained the expression of pluripotency markers (Oct4, Nanog and Ssea-1), as well as their differentiation potential into cells of the three embryonic germ layers.

The STR-based cultivation platform optimized herein represents a major contribution toward the development of large-volume production systems of differentiated cell derivatives for a wide range of biomedical applications.

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1. Introduction

Stem cells have emerged as the starting cell source of choice to produce cells and tissues to potentially treat immunological, degenerative and genetic diseases, as well as to serve as a platform for drug screening and toxicological studies. For the successful implementation of stem cell technology, in particular using embryonic stem cells (ESC), a high number of these cells and their derivatives are necessary for cellular therapies and other biomedical uses. Indeed, practiced and experimental cell-therapy protocols require 10^7–10^9 cells per patient [3].

Several studies aiming at the scaling-up of ESC expansion/differentiation from the traditional static to stirred cultured systems have been reported (reviewed in [4]). The grounded knowledge accumulated over the last century on the design and operation of the stirred tank reactor (STR) in the pharmaceutical industry, the versatility of this reactor type and its relative simplicity [5] have supported the preference by this bioreactor configuration in large-scale cultivation of mammalian cells either as suspension cells or using microcarrier technology.

In order to establish a reliable, cost-effective and controlled bioprocess featuring automation and standardization, the operating culture parameters should be rationally optimized toward the maximization of ESC yield, while maintaining the original features of the cell product, namely pluripotency. Importantly, considering large-scale ESC cultivation in a microcarrier-based STR platform [6], special attention should be given to agitation conditions (*e.g.* impeller type and rotation speed) which should: (i) maintain the cell-containing microcarriers in suspension, (ii) assure the homogeneity of the culture medium, and (iii) together with aeration maintain the optimal oxygen mass transfer rate (k_La) in the culture [5,7]. In fact, aeration is also a very important parameter since it provides oxygen and CO_2 (for pH control) to the culture system.

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In particular for stem cell cultivation, oxygen is known to have an important impact not only on cell growth and metabolism, but also on cell phenotype. Thus O_2 content is a very important parameter to be closely monitored and controlled when designing new strategies for stem cell expansion and/or controlled differentiation in bioreactors. In equilibrium with air, the dissolved oxygen (DO) concentration is just about 0.2 mM at 37 °C and therefore oxygen in the medium can be quickly depleted unless it is constantly replenished. Indeed, oxygen supply is often the limiting factor in animal cell cultures, especially at high cell densities. Hence, the oxygen transfer rate in the bioreactor must be sufficient to meet the oxygen consumption requirements to sustain optimum cell growth, with no toxic effects (*i.e.* production of reactive oxygen species (ROS)). Although agitation and aeration are essential for the success of STR cultures, both of them are associated with hydrodynamic damage, *e.g.* 'shear stress'. According to Cherry and Papoutsakis [8], there are three potential damage mechanisms caused by agitation in a microcarrier-based cell culture: collision among cell-covered microcarriers; collision with parts of the reactor (especially the impeller); and interaction with turbulent eddies [8]. Additionally, the direct sparging of air into high protein-containing medium causes foaming, which can result in the agglomeration and flotation of cell-containing microcarriers on the surface of the foam. The use of surfactant (*e.g.* Pluronic F68) and the addition of antifoam (*e.g.* Antifoam C) agents have been successfully implemented in the biopharmaceutical industry to minimize the negative impact of shear and foaming in animal cultures [9,10].

Although in a STR pH, DO and temperature are typically monitored and controlled, nutrient and metabolite concentrations change, sometimes greatly over time, resulting in decreased cell growth. Therefore, the design and optimization of feeding strategies that allow higher cell densities in a cost-effective way is an important goal. For instance, fed-batch is the culture mode of choice in the pharmaceutical sector due to the high product titers (*e.g.* antibodies) and high cell densities attained, as well as its simplicity, among other features. In our previous studies of ESC cultivation in microcarrier-based stirred systems [6,11], the culture medium exchange scheme consisted of a manual protocol of medium withdrawal/replenishment performed everyday (once or twice a day) upon stopping the agitation to allow the cell-containing microcarriers to settle. An alternative strategy when the target product is the cell is to operate the STR under continuous perfusion with cell retention. In this operation mode, operator errors are minimized as no extensive handling for feeding is needed, also minimizing the contamination risks; in addition, as nutrients and growth factors are continuously replenished and metabolites diluted, time-varying conditions are minimized [5,12,13]. Several strategies can be applied to retain the cells within the STR including using filters or spin-filters and sedimentation [14].

Cultispher S microcarriers offer several advantages over other microcarrier types including effective harvesting, shear protection and biodegradability [15]. In fact, the ability to completely harvest cells from microcarriers is an important aspect of microcarrier-based cultures since the cells are the product itself. As the gelatin matrix from *Cultispher S* microcarriers completely dissolves in the presence of an enzymatic agent, there is no need for separating the cells from the beads. In addition, these microcarriers are FDA approved and recent reports have showed their application in *in vivo* dermis regeneration in humans [16,17]. Kallos and co-workers also demonstrated that the biodegradable nature of *Cultispher S* offers great potential for tissue engineering bone and cartilage as it provides a suitable environment for expansion and differentiation of ESCs and degrades upon transplantation [15].

Here we investigate the impact of varying the concentration of dissolved oxygen (5%, 10%, 20% and 30% DO) and adopting a continuous perfusion operation mode (*versus* common medium exchange) in our previously established microcarrier-based STR platform [6] toward the maximization of mouse Embryonic Stem Cells (mESC) production, as a model of pluripotent stem cells, in a controlled, reproducible and cost-effective bioprocess.

2. Materials and methods

2.1. Model ESC line

The 46C mESC line [19] was established in the laboratory of Professor Austin Smith at the Wellcome Trust Centre for Stem Cell Research, University of Cambridge, England, UK. 46C mESCs were kept cryopreserved in a vapor/liquid-phases nitrogen tank until further use.

2.2. mESC expansion under static conditions

Upon thawing, 46C mESCs were expanded on gelatinized (1%, v/v gelatin solution in water, prepared from a 2% gelatin solution, Sigma) tissue culture plates/flasks for at least 2 passages using Knockout Dulbecco's modified Eagle's medium (KDMEM) (GibcoBRL) containing 15% (v/v) knockout serum replacement (KSR) (GibcoBRL), 1% (v/v) glutamine 200 mM (GibcoBRL), 1% (v/v) penicillin (50 U/mL)/streptomycin (50 μg/mL) (GibcoBRL), 1% (v/v) non-essential Amino acids 100× (Sigma), 0.1% (v/v) 2-mercaptoethanol 0.1 mM (Sigma) supplemented with 0.1% (v/v) human leukemia inhibitory factor (hLIF) (produced *in house* by 293-HEK EBNA cell line). This expansion medium will be referred as SF (serum-free) medium thereafter. Cells were cultured at 37 °C under a 5% CO_2 humidified atmosphere. At each passage, cell number and viability were determined using the Trypan Blue (GibcoBRL) exclusion method in a hemocytometer under an optical microscope (Leica Microsystems).

2.3. mESC expansion under stirred conditions

Bioreactor experiments were carried out in a 1.3 L modular benchtop mechanically stirred bioreactor (New Brunswick Bioflo 110) equipped with a 3-blade pitched stainless-steel impeller, with dissolved oxygen (DO), pH and temperature (T) probes and with a ring-shaped air sparger. Before inoculation of the bioreactor, 5×10^4 cells/mL and 1 mg/mL of *Cultispher S* microcarriers (±60 cells/bead) were incubated at 37 °C in 1/6 of the final medium volume in a 250 mL spinner flask (Bellco) for a 24-h period with intermittent stirring (15 min of stirring at 30–40 rpm, followed by 60 min statically). Then, 1/6 of fresh pre-warmed (37 °C) SF medium was gently added, and cell suspension was transferred to the bioreactor, which contained already 500 mL of SF medium. Based on previous results [6], the operational parameters were set to a pH of 7.2, a temperature of 37 °C and an agitation rate of 60 rpm. DO concentrations of 5%, 10%, 20% and 30% were evaluated, which corresponds to approximately 1%, 2%, 4% and 6% atmospheric O_2, respectively, according to the Henry's law. All controlled parameters were kept constant throughout time in culture with the exception of the sparging rate that was increased from 100 cubic centimeter per minute (cm^3/min) to 200 cm^3/min upon day 6 for DO concentrations of 5%, 10% and 20%. For DO concentration of 30%, the sparging rate was increased from 200 cm^3/min to 300 cm^3/min upon day 4. The dimensions of the stirred tank bioreactor are summarized in Table 1. The culture pH was maintained with the addition of CO_2 gas. The DO was monitored by a polarographic oxygen electrode previously calibrated by sparging SF medium with nitrogen (DO = 0%) and compressed air (DO = 100%). Aeration was achieved through gentle sparging from the base of the bioreactor with a mixture of N_2, Air and CO_2 gas bubbles and temperature was kept at 37 °C by an electric heating jacket. The feeding was

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