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Intracellular response of CHO cells to oxidative stress and its influence on metabolism and antibody production

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ABSTRACT

Chinese hamster ovary (CHO) cells are widely used for the manufacture of therapeutic mAbs with high productivity processes generating 5–10 g/L mAb. High productivity processes impose considerable strain on the intracellular metabolic and redox homeostasis resulting in increased oxidative stress. We investigated the mechanisms by which oxidative stress affects the productivity of a mAb producing CHO cell line by controlling bioreactors at dissolved oxygen (DO) set-points ranging from 20 to 175% of air saturation. The results showed that for the 20–75% DO conditions, despite similar growth and viability, there were significant differences in productivity, consumptions of oxygen and amino acids, intracellular redox, and mitochondrial function. Our results demonstrate that as the external dissolved oxygen increased, intracellular H_2O_2 increased, mitochondrial function diminished, flux into one-carbon metabolism pathway decreased, and cells diverted metabolic activity from mAb production. Intriguingly, although the reductase activities were similar irrespective of the DO concentration, the intracellular reduced-to-oxidized glutathione ratios were low for all conditions, which demonstrated that the intracellular antioxidant mechanisms had difficulties managing oxidative stress. Combined, these results indicate that mAb production may be limited by the cell's capacity to manage oxidative stress and thus provide a new attribute to leverage during upstream process development.

1. Introduction

Antibody therapeutics have achieved high success rates in the clinic, and this success has led to an increased focus on development processes to both increase the yield and improve product quality [1]. CHO cells are the most widely used cell types for the manufacture of therapeutic mAbs. Significant effort has been focused on developing high productivity processes that can achieve 5–10 g/L mAb [2]. The production of high quantities of mAbs imposes considerable strain on the metabolic and redox homeostasis of the CHO cells due to large energy demands associated with the synthesis, folding, quality control, and secretion of mAb [3]. A consequence of high energy demand is increased oxidative stress, which can result in elevated levels of reactive oxygen species (ROS). Sustained increased levels of ROS leads to irreversible oxidative damage to lipids, proteins, and DNA, ultimately leading to cell death [4]. Thus, we investigated the intracellular mechanisms by which oxidative stress and ROS effect the productivity of a CHO cell line producing a mAb.

Several *in vitro* models for the study of oxidative stress have been developed [5,6]. Most of these models are based on the bolus addition of a chemical oxidant that results in an acute but short-lived increase in oxidative stress [5]. Typically, when using these models, the cells are dosed with a chemical oxidant and the effects are measured a few hours later [6]. However, as cell culture processes used for production of therapeutic proteins typically run for 10-16 days, these models do not reflect the sustained and relatively low level of oxidative stress that cells may experience in a bioreactor. The model that most accurately reflects the production conditions involves the modulation of the DO set-point [5]. Cultures grown under conditions with elevated DO set-points experience increased cell culture redox potential and associated oxidative stress due to increased leakage of electrons from the mitochondrial electron transport chain; this results in superoxide production, which is immediately converted to H_2O_2 by enzymes in the mitochondria [7]. Increased production of H_2O_2 strains the intracellular redox homeostasis [8,9]. Although the effects of DO set-points on cell cul-

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Table 1
Comparison of corresponding DO set-points at 3L and 15,000L scales

3L DO Set Point	Equivalent DO at 15,000L
20%	13%
50%	33%
75%	50%
100%	66%
175%	116%

ture processes have been previously reported, [8,10] these reports have focused on cell growth and productivity, with little emphasis on the intracellular response. Therefore, we focused our investigations on understanding how altered oxidative stress affects the intracellular redox environment, ROS accumulation, nutrient metabolism, mitochondrial function, and specific productivity of a CHO cell line producing a mAb by running the related cell culture process at different DO set-points. In addition to providing a range of oxidative stress levels, a broad range of DO set-points was evaluated because DO can be affected by scale. Commercial scale bioreactors have increased hydrostatic and headspace pressure compared to 3L bioreactors. Thus, a DO set-point at 50% of air saturation has a significantly higher concentration of oxygen in a large-scale bioreactor compared to a small-scale bioreactor; refer to Table 1 for a comparison of DO set-points at 3L scale and their corresponding equivalent set-points in a 15,000L commercial scale bioreactor.

Our investigation of the impact of oxidative stress on CHO cell processes focused on the intracellular redox environment and mitochondrial function, both of which have been previously shown to influence specific productivity [11,12]. Intracellular redox was evaluated by quantifying the level of ROS and the ratio of reduced to oxidized glutathione. Mitochondrial function was evaluated by Seahorse flux analysis that provided information on the spare capacity of the mitochondria in response to oxidative stress as well as oxygen uptake rate that is related to ATP production. As an additional assessment of the mitochondrial state, flow cytometry was used to quantify the mitochondrial membrane potential. Finally, differences in metabolism were evaluated by analyses of amino acids, glucose, and lactate. The combined data provided a comprehensive evaluation of the effects of increased oxidative stress on CHO cell metabolism and the cells' capacity to produce therapeutic proteins.

2. Materials and methods

2.1. Bioreactor control

A stable GS-CHO cell line expressing an IgG_2 mAb was used for the described studies. A null-vector control cell line was used for comparison. The null-vector control cell line was transfected with the same vector used in the mAb-producing cell line except without the sequence coding for the mAb. The processes were run in 3-L glass bioreactors with a working volume of 1.5 L using in-house proprietary media and nutrient feeds as previously described [13]. All conditions were run in at least duplicates. The temperature, pH, dissolved oxygen, and agitation were monitored and controlled using SLC 5000 controller software (Rockwell Automation; Arlington, VA). All conditions used the same temperature, pH, and fixed agitation set-points for the duration of the runs. Dissolved oxygen was controlled using a fixed and low air flow rate initially with an increasing oxygen flow rate as needed through a drilled hole sparger and pH was controlled using sodium carbonate solution (at low end) added through the head plate and CO_2 gas (at high end) added through a drilled hole sparger. The extracellular redox potential was monitored using an InPro 3253i pH/oxidation reduction potential (ORP) probe (Mettler-Toledo; Columbus, OH). A Rapidpoint 400 Blood Gas Analyzer (Siemens; Malvern, PA) and BioFlex Analyzer (NOVA Biomedical; Waltham, MA) were used to measure the offline pH, gases (pCO_2, pO_2), and metabolites (glucose, lactate). Cell density and viability were measured using a Vi-CELL XR Analyzer (Beckman Coulter; Brea, CA). The mAb concentration was measured by protein A chromatography using an HPLC system (Agilent Technologies; Santa Clara, CA).

2.2. Reactive oxygen species analysis

The intracellular levels of H_2O_2 were determined according to a previously published procedure [14]. Briefly, cells were stained with 4 μM of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Invitrogen; Carlsbad, CA) and 25 nM TO-PRO-3 (Invitrogen) to detect intracellular H_2O_2 and exclude dead cells, respectively. Fifty micromolar N-ethyl-maleimide (NEM; Sigma-Aldrich; St. Louis, MO) was used as a positive control for H_2DCF-DA staining. A shake flask of cells used to inoculate 3-L bioreactors was maintained and passaged every 2-3 days. The shake flask cells were stained using the same method and run with samples from the 3-L bioreactors as a control. Cells were analyzed on an LSR II flow cytometer (Becton Dickinson; Franklin Lakes, NJ).

2.3. GSH/GSSG measurement

To measure reduced and oxidized glutathione content, cell culture samples were quenched, and the metabolites were extracted using methanol and ammonium bicarbonate buffer following a previously established procedure [15]. The extracted glutathione was analyzed using the GSH/GSSG Ratio Detection Assay Kit (Abcam; Cambridge, UK) following the manufacturer's protocol.

2.4. Reductase activity assay

The total reductase activity in each condition was measured from the cell lysate generated by freeze-thawing. Culture samples were stored frozen until the end of the run. All samples were analyzed at the same time using a previously established procedure [13]. Briefly, 10 μL of lysed cell culture was diluted into 190 μL of PBS (pH 7.4) containing 0.4 mM NADPH, 0.3 mM oxidized glutathione, and 3 mM 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB). Reductase activity was determined by monitoring the rate of increase in absorbance at 412 nm. Wells containing the dilution buffer described above without the cell culture were used to compensate for the background, and the activity was normalized based on the viable cell density (VCD) for each time point.

2.5. Amino acid analysis

Cell culture supernatant was centrifuged and filtered through a 10-kDa filter (Pall Life Sciences; Port Washington, NY). The filtrate was diluted using HPLC-grade water to an appropriate concentration. Amino Acid Standard (Waters; Milford, MA) was diluted to generate a standard curve. The diluted samples and standards were combined with the internal standard norvaline and AccQ Tag Derivation Kit reagents (Waters) in PCR plates. The samples were run on an Acquity UPLC system (Waters). The data were analyzed using the EMPOWER software (Waters).

2.6. Mitochondrial functional analysis

Mitochondrial function was evaluated using a Seahorse XF96 flux analyzer. The Seahorse instrument measures the oxygen uptake rates in the presence and absence of oligomycin, FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone), and rotenone/antimycin A, which enables the evaluation of basal