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Control of redox potential in hybridoma cultures: effects on MAb production, metabolism, and apoptosis

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Abstract Culture redox potential (CRP) has proven to be a valuable monitoring tool in several areas of biotechnology; however, it has been scarcely used in animal cell culture. In this work, a proportional feedback control was employed, for the first time, to maintain the CRP at different constant values in hybridoma batch cultures for production of a monoclonal antibody (MAb). Reducing and oxidant conditions, in the range of −130 and +70 mV, were maintained in 1-l bioreactors through automatic control of the inlet gas composition. Cultures at constant DOT, in the range of 3 and 300 %, were used for comparison. The effect of constant CRP on cell concentration, MAb production, metabolism of glucose, glutamine, thiols, oxygen consumption, and programmed cell death, was evaluated. Reducing conditions resulted in the highest viable cell and MAb concentrations and thiols production, whereas specific glucose and glutamine consumption rates remained at the lowest values. In such conditions, programmed cell death, particularly apoptosis, occurred only after nutrient exhaustion. The optimum specific MAb production rate occurred at intermediate CRP levels. Oxidant conditions resulted in a detrimental effect in all culture parameters, increasing the specific glucose, glutamine, and oxygen consumption rates and inducing the apoptotic process, which was detected as early as 24 h even when glutamine and glucose were present at non-limiting concentrations. In most cases, such results were similar to those obtained in control cultures at constant DOT.

Keywords Culture redox potential · Hybridomas · Monoclonal antibody · Apoptosis · DOT control

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Introduction

The increasing demand of monoclonal antibodies (MAb) for diagnostics and therapeutic applications has been met during the last three decades through continuous improvements in their concentration and productivity. Such improvements have resulted from combined engineering and molecular approaches, yet novel control and culture strategies are still needed. A useful parameter can be the culture redox potential (CRP), although it has received only limited attention in the cell culture area. CRP has been used for process monitoring and control in microbial fermentations, yielding relevant physiologic and metabolic information of microbial cells, as well as being a useful tool for optimizing microbial processes [1, 4, 23, 35]. CRP has also been used in animal cell culture [39], although to a much lesser extent than in microbial bioprocesses. The importance of a proper redox state in animal cell cultures was recognized as early as 1970 by Daniels et al. [9] who showed that a lower CRP at inoculation resulted in higher growth rates, but lower maximum cell concentration. Monitoring of CRP has been shown to be useful for on-line determination of viable cell concentration and growth phase in human hematopoietic cells [10] and hybridoma cultures [14, 21]. In addition, CRP measurements can be used in combination with oxygen uptake rate determinations to discriminate between metabolic events and culture

operational eventualities [20]. Furthermore, CRP has been used to determine the infection and harvesting times in insect cell cultures [6], and to establish the phase of the process and the time to initiate feeding in fed-batch hybridoma cultures [41]. All these applications are related to the fact that during growth almost all cells tend to reduce their environment due to accumulation of thiols [21] and other acidic compounds [45].

It is known that CRP is strongly dependent, in a complex relationship, on medium composition, pH, and dissolved oxygen tension (DOT) according to the Nernst equation [21, 26, 38]. Therefore, interpretation of CRP measurements and evaluation of its effects on cell culture can be a complex task unless it is controlled at constant values. Such difficulty can explain the lack of reports of CRP in animal cell culture. Although setting the initial redox potential of the medium to a predetermined value prior to inoculation was reported in the 1960s and 1970s [9], to our knowledge, only Hwang and Sinskey [21] have previously controlled CRP at constant values in animal cell cultures. In particular, they attained constant CRP by fresh medium addition in continuous hybridoma cultures. They observed that viable cell concentration decreased as CRP increased, however, no other kinetic or stoichiometric parameter was determined.

CRP is of paramount importance to animal cells as oxidizing levels are associated with the generation of reactive oxygen species (ROS). Oxidative stress is a second messenger that plays fundamental roles in various metabolic events [11], including signal transduction [16], cell differentiation and maturation [13, 44, 47], and induction of cell death [5, 18, 29]. On the other hand, reducing conditions have been associated with animal cell proliferation and prevention of cell death. For instance, addition of antioxidant agents to cell culture medium, such as serum, serum substitutes, dithiols, 2-mercaptoethanol, thiocarbamates, and cysteine have positive effects on culture performance [3, 8, 17, 22].

The objective of this work was to determine the effects of controlled CRP on kinetic and stoichiometric parameters, and on the type of programmed cell death in hybridoma cultures during MAb production. The results obtained were compared to reference cultures at constant DOT.

Materials and methods

Cell line and culture medium

A murine hybridoma, designated as BCF2, provided by Dr. L. Possani (IBt-UNAM), was used in this study [20]. The cell line secretes a neutralizing monoclonal antibody specific to toxin 2 of the scorpion *Centruroides noxius* Hoffman. DMEM was used as culture medium and contained 10 % (v/v) fetal bovine serum (FBS) (Gibco BRL), 4 g/l glucose, 4 mM glutamine, 3.7 g/l $NaHCO_3$, 13.2 g/l oxaloacetic acid, 0.8 mg/l crystalline insulin, 5.5 mg/l sodium pyruvate, 1 % (v/v) of antibiotic–antimycotic solution [penicillin (100,000 U/l), streptomycin (100 mg/l), amphotericin B (250 μg/l)], and 1 % (v/v) of 100× non-essential amino acid solution. All reagents except FBS were from Sigma (St. Louis, MO).

Bioreactor cultures

Cells taken from mid-exponential phase, previously subcultured at least three times in T-flasks, were used to inoculate a 1-l (700-ml working volume) Virtis (Gardiner, NY) bioreactor. All experiments initiated with 0.1×10^6 viable cell/ml. The bioreactor was maintained at 37 °C and agitated at 150 rpm with a suspended magnetic bar placed at one-fourth of liquid height. Two sets of experiments were performed; one consisting of cultures maintained at constant DOT and pH, referred hereafter as control cultures, and another set consisting of cultures maintained at constant CRP and pH. Three constant DOT concentrations, 3, 50, and 300 % (with respect to air saturation), were tested for the control cultures. In turn, six constant CRP conditions, in a broad range between −130 and +70 mV, were tested. In both sets of experiments, pH was maintained constant at 7.2.

CRP was measured with a sterilizable redox probe based on a combined platinum-reference electrode (Ingold, Wilmington, MA). Redox probes were cleaned before each culture following the procedure reported by Hwang and Sinskey [21]. Probes were initially calibrated with a standard redox buffer (Ingold, Wilmington, MA). Prior to inoculation, redox probes were kept for 3 h in culture medium maintained at 50 % DOT and pH 7.2. The measured CRP was then set to zero for direct comparison between the various cultures. CRP was maintained constant at the desired set point by automatically controlling the individual O_2 and N_2 flow rates in the inlet gas stream through individual mass flow controllers (Brooks Inst. Co., Hatfield, PA). The mass flow controllers were controlled through a Macintosh computer connected to a MacAdios 411 (GW Inst., Cambridge, MA) D/A and A/D interphase, using a proportional feedback algorithm written in Microsoft Quick Basic. Only surface aeration was used and the total gas flow rate was maintained constant at 0.6 l/min. A detailed description of the CRP control system can be found elsewhere [30]. Similarly to CRP, the DOT and pH were also controlled by automatically manipulating the relative composition of O_2 and N_2 or CO_2 and N_2, respectively, in the inlet gas stream. DOT was measured with a polarographic probe and pH with a glass gel probe

(Ingold, Wilmington, MA). A detailed description of the computerized data acquisition and control system for maintaining constant pH and DOT can be found elsewhere [20]. All proportional control constants, for CRP, DOT, and pH control loops, were estimated using semiempirical methods reported by Zieghler and Nichols and, Cohen and Coon [46].

Analytical methods

Cell concentration was determined by a Counter Coulter Multisizer II (Coulter Electronics, Hialeah, FL) and viability by Trypan blue exclusion. Monoclonal antibody (MAb) concentration was determined by an alkaline phosphatase sandwich ELISA technique as described elsewhere [40]. MAb integrity was analyzed by Western blots using non-reducing and non-denaturalizing conditions in 8 % acrylamide gels transferred to nitrocellulose membranes and developed with an affinity purified peroxidase labeled goat antibody anti-mouse IgG (H + L; KPL, MD). Glucose, glutamine, glutamate, and lactate were measured with a YSI-2700 enzymatic analyzer (Yellow Springs, OH). Ammonium was determined by the Kaplan technique [25]. Thiols concentration was determined by the Ellman technique [12] using cysteine as thiol standard. Apparent cell growth rates (μ), glucose, glutamine, lactate, ammonium, and thiols specific production rates as well as specific MAb production rate were calculated according to the report by Ramírez et al. [40] and Serrato et al. [42]. Oxygen uptake rate (OUR) was calculated as previously reported by Higareda et al. [20]. All these parameters were calculated during the exponential growth phase of the culture. Cell death was determined from the sub-G1 population fraction as determined by flow cytometry and cell size decrease, as described previously [31].

Results

Control system of culture redox potential

A proportional control system was designed to maintain the CRP in batch cultures at constant and predetermined values in the range of −130 mV (reducing conditions) to +70 mV (oxidizing conditions). The control system was based on manipulating the DOT through the inlet gas composition, and as shown in Fig. 1a, it was precise ($r^2 = 0.9998$) and accurate (slope = 1.0) throughout all the CRP range. Differences between the desired CRP (or set point CRP; spCRP) and measured CRP (mCRP) values had an error of <10 %. To achieve such a performance, the proportional gains of the system were calculated for each of the six constant CRP values evaluated. This was necessary due to the logarithmic relationship between CRP and DOT as established by the Nernst equation. Some gain values have been previously reported [30].

The relationship between the independent variable (average DOT) and the corresponding mCRP value for each of the six cultures tested at controlled CRP (closed circles) is shown in Fig. 1b. In this case, the *X* error bars represent the range of DOT variations needed to maintain the CRP at the specified set point. Cultures controlled at constant DOT were performed for comparison (open circles). In this case, the *X* error bars represent the experimental DOT variation range, and the *Y* error bars represent the CRP variations during each culture. Only small variations in DOT were needed to control CRP at reducing values, whereas changes of DOT in excess of 100 % were required for the oxidizing range. Notice that in the latter case, as predicted by the Nernst equation, only small changes in mCRP occurred even after large variations in DOT. In addition, under controlled DOT, the CRP variations during reducing cultures were larger than in the case of oxidant cultures. This is consistent with previous reports that show that in the latter case the oxygen and water

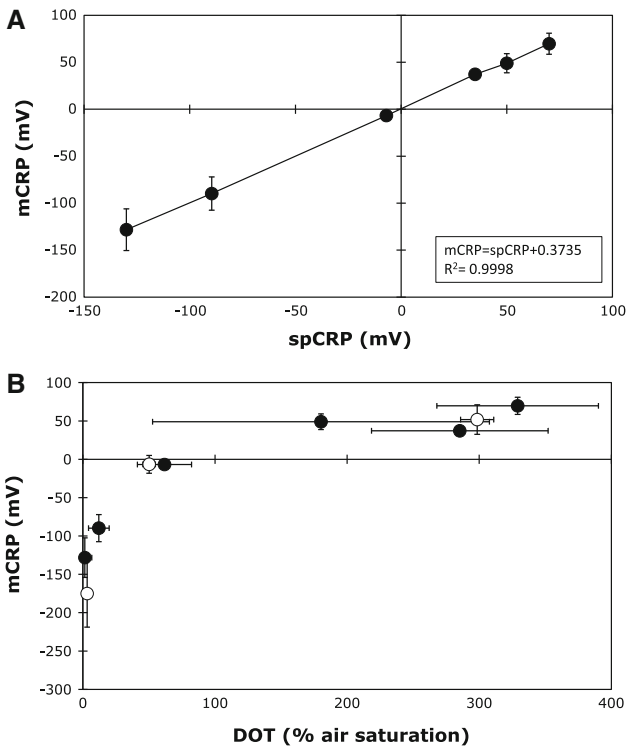

Fig. 1 Proportional control of CRP. **a** Relationship between measured CRP and its set point. **b** Relationship between CRP and DOT. *Closed circles* indicate the mode of the measured CRP in CRP controlled cultures. *Open circles* indicate the mode of DOT in DOT controlled cultures. *Y error bars* represent the CRP variation during each culture. *X error bars* for CRP data represent the DOT variation needed to maintain constant CRP; and for DOT data represent the experimental DOT variation range during constant DOT cultures

constitute the predominant oxidation–reduction couple [38, 39].

Distinctive patterns in CRP, particularly during low DOT conditions, were observed between cultures controlled at constant DOT and those controlled at constant CRP. As shown in Fig. 2a, CRP decreased during the exponential growth phase for cultures maintained at 3 and 50 % DOT. The capacity of the cells to reduce the culture medium was inversely dependent on the DOT. In contrast, the CRP control algorithm was sufficient to offset the cellular reducing capacity under all the CRP range tested (Fig. 2b). It has to be noted that in the case of +50 mV, the control gain was not the best for the proportional control, nonetheless, the average of CRP data was +50 mV and the mode value was +49 mV. In some cultures, nutrient exhaustion during the stationary growth phase caused a perturbation in the CRP control (for instance, culture at −130 mV). DOT profiles during the most reducing culture (−130 mV) and at 3 % DOT are shown in Fig. 2c. As shown, oxygen limitation was more severe in the CRP-controlled cultured. No anoxic conditions were reached in the case of 3 % DOT controlled culture. In the case of the −130 mV controlled culture, DOT remained below 2 % during the first 105 h.

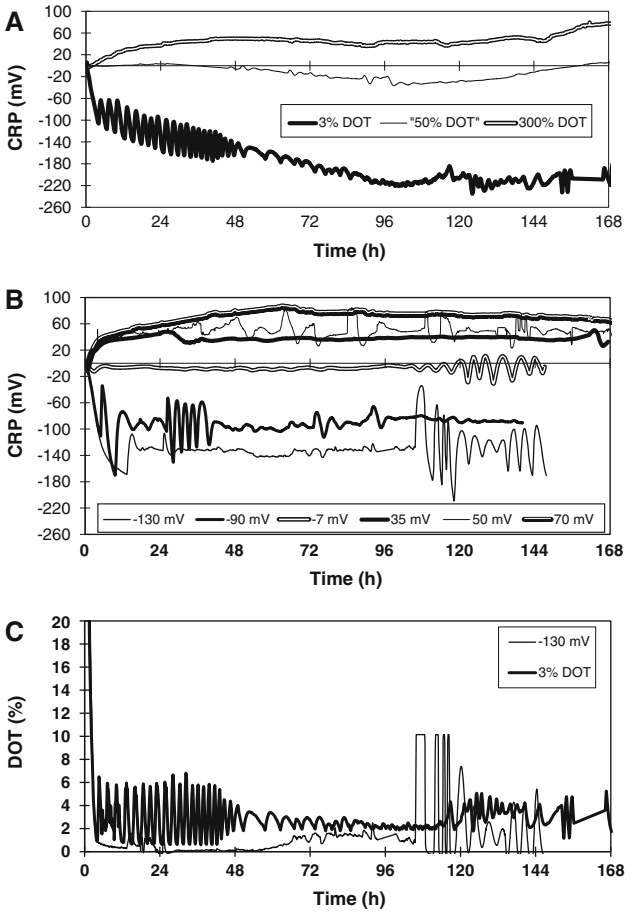

Fig. 2 CRP behavior at different conditions. **a** CRP at constant DOT. A major decrease on the CRP value is observed at low DOT caused by the presence of reducing compounds. **b** Controlled CRP cultures. **c** DOT profiles for the lowest DOT-controlled (3 %) and lowest CRP-controlled (−130 mV) cultures. Six cultures in the range of −130 to +70 mV were developed up to 168 h

Effect of CRP on cell growth and MAb productivity

The maximum viable cell concentration in cultures controlled at constant CRP (Fig. 3a, closed circles) increased as CRP decreased, even though the mean DOT was only 1.8 % at the lowest CRP tested (Fig. 1b). Such a result shows the negative impact that an oxidizing culture medium has on cell growth. A similar behavior was observed for the constant DOT cultures (Fig. 3a, open circles). However, in this case the highest maximum viable cell concentration attained at the lowest DOT tested (3 %) was slightly lower than the highest value attained for constant CRP cultures, even though the reducing conditions were lower (−175 vs. −130 mV for the case of constant CRP). This suggests that highly reducing CRP conditions could also compromise the culture performance. It has been previously reported [39] that hypoxic condition (<10 %) can result in increasing cell viability but can also affect specific growth rate and product generation. However,

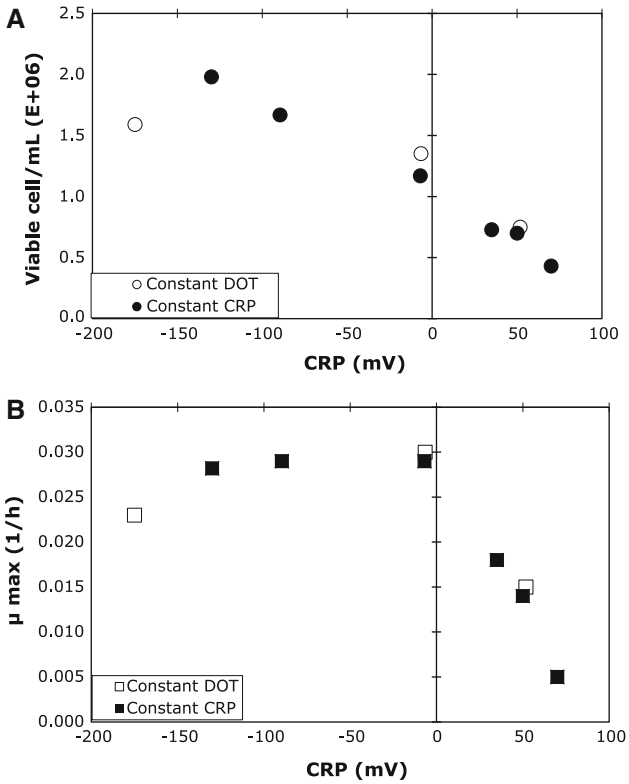

Fig. 3 CRP effect on cell growth. **a** Maximum viable cell concentration. **b** Specific growth rate

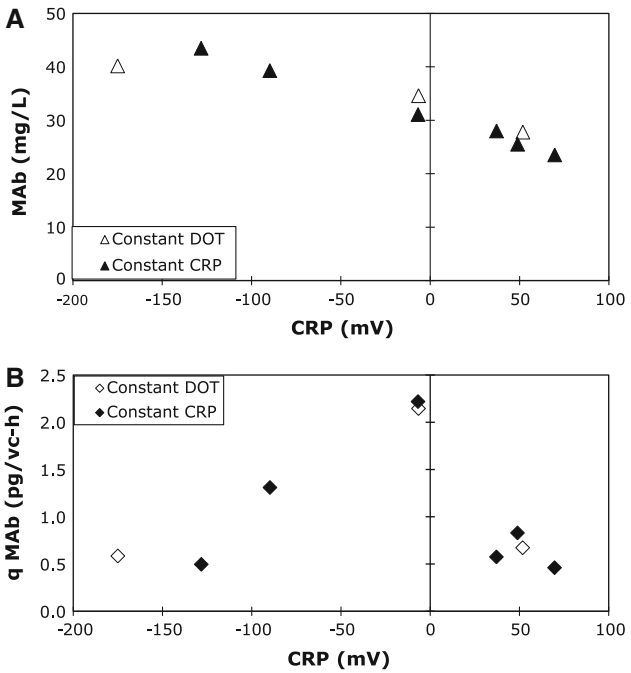

Fig. 4 Effect of CRP on monoclonal antibody productivity. **a** Effect on MAb concentration. **b** Effect on MAb productivity

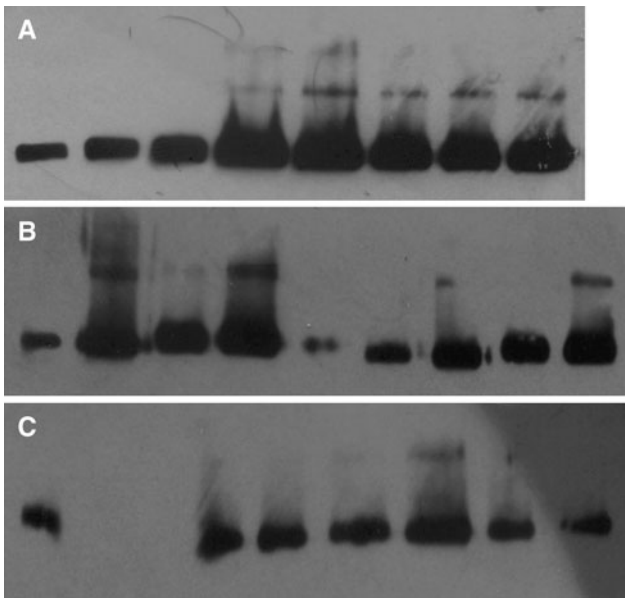

Fig. 5 Effect of CRP on monoclonal antibody stability. Western blot using non-denaturalizing and non-reducing polyacrylamide gels at different CRP. **a** Constant CRP at −130 mV. (*1*) Std. (*2*) 24 h. (*3*) 48 h. (*4*) 73 h. (*5*) 98 h. (*6*) 124 h. (*7*) 137 h. (*8*) 147 h. **b** Constant CRP at −7 mV. (*1*) Std. (*2*) 26 h. (*3*) 47 h. (*4*) 70 h. (*5*) 81 h. (*6*) 99 h. (*7*) 117 h. (*8*) 129 h. (*9*) 147 h. **c** Constant CRP at +70 mV. (*1*) Std. (*2*) 16 h. (*3*) 24 h. (*4*) 48 h. (*5*) 66 h. (*6*) 72 h. (*7*) 96 h. (*8*) 120 h. (*9*) 145 h

effects under controlled CRP have not been established. As shown in Fig. 3b, the highest maximum specific growth rate (μ) was 0.3 h^{-1} and remained relatively constant between a CRP of −130 to −7 mV. A similar μ has been previously reported for this cell line [20, 42], indicating that no significant oxidative damage existed under such CRP range. The μ sharply decreased for CRP values above −7 mV, whereas it decreased slightly below −130 mV. Others have also observed a decrease in μ for cultures maintained below 5 % DOT [33, 34, 36, 37], which has been attributed to an incomplete oxidation of glutamine.

The effect of CRP on MAb production, either under controlled CRP or controlled DOT cultures, is shown in Fig. 4 (closed and open triangles, respectively). Both control strategies yielded similar results. Similarly to maximum viable cell concentration, maximum MAb concentration also decreased with increasing CRP (Fig. 4a). As shown in Fig. 4b, the maximum specific MAb production rate (q_{MAb}) showed a maximum at intermediate CRP values (−7 mV), whereas it decreased by as much as four times at lower or higher CRP. The q_{MAb} observed here are within the range of other hybridoma cells [33, 36, 37]. An optimum DOT for q_{MAb} was originally observed by Miller et al. [33] and is now well documented. The results shown in Fig. 4 indicate that there is also an optimum CRP for q_{MAb}, which is different for cell growth and could indicate different redox and energy balances needed for optimum protein synthesis or growth requirements.

To determine if the redox conditions affected MAb integrity, production kinetics under low (−130 mV), intermediate (−7 mV) or high (70 mV) CRP, were followed by Western blots (Fig. 5). It can be observed that even under the most reducing condition disulfide bonds were not disrupted and the complex MAb structure was preserved (Fig. 5a). A slight aggregation was observed in all cases (upper bands in the gels), which could be caused by non-specific binding between MAb molecules.

Effect of CRP on cell metabolism

To evaluate the effect of CRP on cell metabolism, the specific consumption rates of glucose and glutamine (q_{glc} and q_{gln}, respectively), the specific lactate production rate (q_{lac}), and the yield of lactate on glucose ($Y_{lac/glc}$) were determined (Fig. 6). In reducing cultures, q_{glc} remained relatively constant at about 30×10^{-11} mmol/cell-h (Fig. 6a). Such values were in accordance with those previously reported [36, 37]. However, q_{glc} increased by as much as twofold under oxidizing conditions. Interestingly, at 300 % DOT, q_{glc} remained within the same range as those obtained at reducing cultures. The q_{gln} showed a similar behavior to q_{glc} (Fig. 6b). That is, q_{gln} remained relatively constant at around 5×10^{-11} mmol/cell-h in all cultures at constant DOT and in cultures controlled at

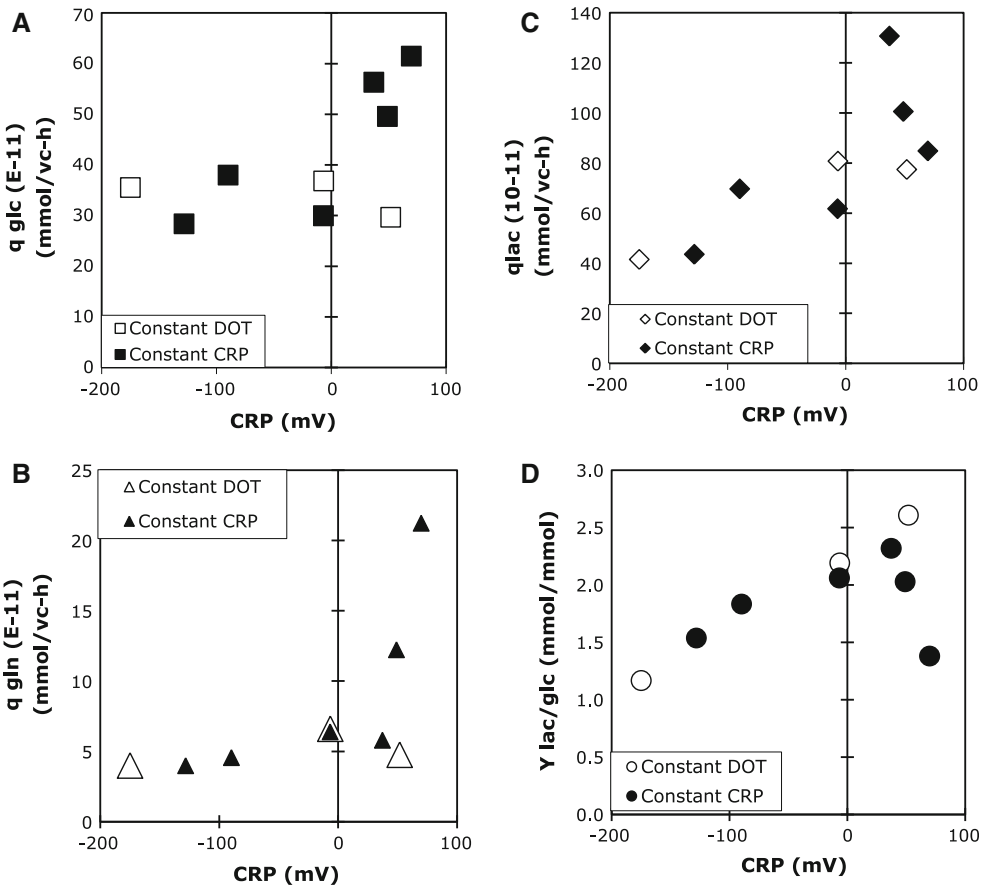

Fig. 6 Effect of CRP on cell metabolism. **a** Specific glucose consumption rate. **b** Specific glutamine consumption rate. **c** Specific lactate production rate. **d** Lactate/glucose yield

constant CRP between −130 and 35 mV. Such values are in accordance with other reports [33, 34, 36, 37]. Similarly to q_{glc}, q_{gln} sharply increased by about fourfold in constant CRP cultures under the highest oxidizing conditions. The very large DOT variations needed to maintain the CRP in constant values (see Fig. 1b) could be the cause of the observed results for q_{gln} and q_{glc}. Nevertheless, an explanation from a physiological perspective of such observation requires further studies. Maximum lactate concentration remained relatively constant between 42 and 52 mM under all conditions tested (data not shown). Similar maximum lactate concentrations have been previously reported for this same cell line [20, 42]. That maximum lactate concentration did not increase even under the most reducing condition (culture at 3 % DOT) indicates that fully aerobic metabolism was maintained in all cases. As shown in Fig. 6c and d, both q_{lac} and $Y_{lac/glc}$ increased as oxidant conditions increased to 35 mV and thereafter sharply decreased at higher CRP values. As discussed later, such a behavior can be indicative of an oxidative stress to cell metabolism [39]. Maximum ammonium concentrations remained between 3 and 4 mM in most cultures and decreased to 2 mM only under the most oxidizing conditions (above +35 mV; data not shown). It is known that such concentrations are not inhibitory for growth. However, the specific ammonia production rate increased by as much as two times in oxidizing cultures (data not shown).

The effect of CRP on thiol production is shown in Fig. 7a. As expected, the maximum concentration of thiols reached its highest value under the most reducing conditions and decreased linearly as CRP increased. Likewise, the specific production rate of thiols (q_{thiols}) also decreased linearly, from 68×10^{-11} to 23×10^{-11} µmol/cell-h, as CRP increased from −175 to 70 mV (Fig. 7b). Such a behavior shows that the concentration of thiols is an indicator of the redox condition, as they are unstable and readily oxidized as previously reported [11, 17, 21]. Media reduction is a typical phenomenon observed in many cultures due to the generation of reducing chemical species [21, 45]. Such species maintain optimum intracellular redox potential and direct the exchange of some amino acids that participate in the production of glutathione and other redox molecules. Oxygen plays a critical role in the nature of such molecules, which are unstable and readily oxidize. This means that at low CRP, the presence of such molecules is high but as CRP increases their oxidation rate increases, causing a diminution in their concentration.

The effect of CRP on specific oxygen uptake rate (q_{OUR}) was determined as such a parameter also yields direct information on cell metabolism (Fig. 7c). The q_{OUR}

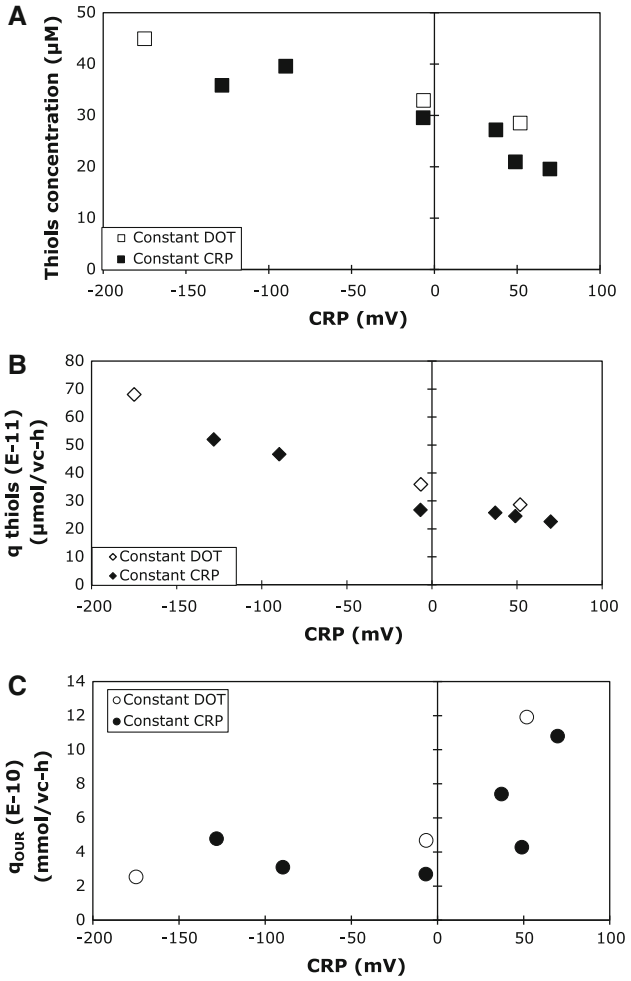

Fig. 7 Effect of CRP on thiols production and OUR. **a** Maximum thiols concentration. **b** Specific thiols consumption rate. **c** Oxygen uptake rate

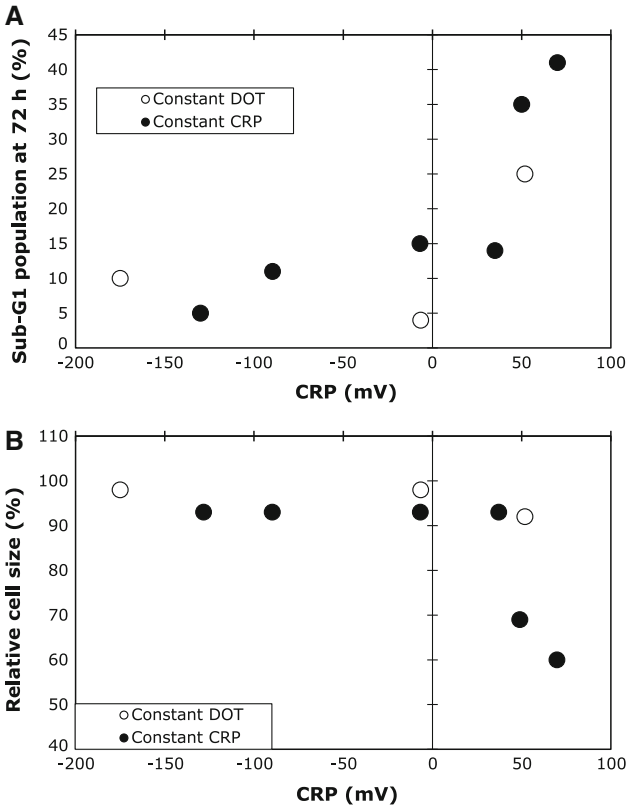

Fig. 8 Effect of CRP on programmed cell death. **a** Percentage of Sub-G1 population at 72 h (representing the time during exponential growth phase). It is observed that in the case of the most oxidant conditions, the percentage of apoptosis is high even though essential nutrients (glucose and glutamine) are not limited in the culture. **b** Percentage of cell size decrease during the exponential phase of the cultures corroborates the premature presence of apoptosis in the cultures

followed a similar trend as that observed for q_{glc} and q_{gln}, as it remained relatively constant during reducing conditions and increased by as much as fourfold under oxidizing conditions. The q_{OUR} values obtained under reducing conditions were within those commonly reported [36, 37], and their relatively constant values indicate that oxygen was above the saturation constant for these cultures. The increased q_{OUR} under oxidizing conditions is in agreement with previous reports [36, 39] and has been interpreted by such authors as a metabolic adaptation to high DOT conditions. In all cases, glutamine depletion coincided with a sudden decrease in oxygen consumption rate (data not shown), in agreement with previous reports [20].

Effect of CRP on cell death

Apoptosis has been previously shown to be the main programmed cell death pathway in this cell line [31]. Accordingly, to determine if high CRP could induce this type of death, apoptosis was quantified. The sub-G1 peak population fraction, indicative of apoptotic cells, was determined at 72 h (exponential phase of the cultures) by flow cytometry. As shown in Fig. 8a, the apoptotic population was not higher than 15 % in reducing cultures; a value considered as background. In such cases, an increase in the apoptotic death was only observed when glucose or glutamine was depleted, which coincides with previous reports [31, 32]. In contrast, an increase in the sub-G1 peak was observed in oxidative cultures even when key nutrients (glucose and glutamine) were present at non-limiting concentrations, indicating that oxidative stress was a potent apoptotic inducer [5, 18, 27]. These results were also corroborated by the percentage of cell size decrease, which has also been shown to be indicative of apoptotic death [31]. Accordingly, the percentage of cell size decrease at 72 h with respect to the maximum cell size was calculated and is shown in (Fig. 8b). The highest cell size decrease (40 %) was observed in the most oxidant culture, whereas the lowest cell size decrease (2 %) occurred under the most

reducing condition. All cultures at constant DOT showed a similar cell size decrease. Staining with fluorescent dyes and determination of the characteristic DNA ladder pattern of 180-bp multiples by agarose gels [31] were also performed in all samples and corroborated the results shown in Fig. 8 (data not shown).

Discussion

Redox potential is a direct measure of electron transfer, and plays an important role in many biological reactions. For instance, different physiologic events are regulated by this variable, which in turn is directly dependent on the cell culture environment [5, 11, 13, 16, 18, 29, 44, 47]. Accordingly, culture performance is closely related to CRP, but its effect on animal cell metabolism and physiology has been scarcely studied under controlled conditions. In this work, a novel CRP control system, based on the automatic variation of the inlet gas composition through a proportional feedback algorithm, was developed. Such a system proved to be precise and accurate and was able to maintain the CRP at constant and predetermined values in the range of −130 to +70 mV (Fig. 1a). To achieve the desirable CRP control, the DOT had to be changed logarithmically, in accordance to the Nernst equation (Fig. 1b). This meant that at reducing values, a small DOT variation caused large CRP changes, whereas at oxidative values, large DOT changes only caused a small variation in CRP. Considering such a relationship, CRP could be helpful to study culture performance, cell metabolism, and physiology at precisely controlled reducing values where DOT determinations by traditional methods are difficult and/or inaccurate due to technical limitations of present-day probes below DOT values of 5 %. With the unique control system developed in this work, it was possible to determine the effect of CRP on different culture parameters. In addition, CRP is a novel variable that could provide useful insights on culture performance due to its direct influence on metabolic reactions.

CRP can be associated with different cellular events such as intracellular redox state and generation of reactive oxygen species (ROS), which are important in product generation and on cell growth [44, 47]. It was observed that reducing conditions had a beneficial effect on culture performance, as reflected by the highest concentrations attained of maximum viable cells, monoclonal antibody, and thiols, as well as the highest q_{thiol}. In addition, the lowest values of q_{glc}, q_{gln}, q_{lac}, $Y_{lac/glu}$, and q_{O2}, were found under reducing conditions, and apoptotic death in these cultures only occurred after limitation of an essential nutrient (i.e., glucose and glutamine). Altogether, these results showed that constant and low CRP values support high MAb production without excessive production of waste by-products, such as lactate, and without a high consumption of essential nutrients. The high thiols concentrations obtained at reducing conditions could indicate a better intracellular transport of cysteine and the concomitant maintenance of an optimum intracellular redox potential by glutathione production. This is supported by the fact that 2-mercaptoethanol stimulates cystine uptake, and increases MAb production due to dithiol formation with cysteine [22]. Cysteine transport plays a key role in protein synthesis and glutathione (L-g-glutamyl-L-cysteinyl-glycine) generation [3], as it has been demonstrated that the production of this amino acid in the cell is limited and depends on medium supply. At reducing CRP, cysteine is not oxidized to cystine, allowing the exchange of this amino acid by cell-specific systems such as the Na-ATP dependent ASC [2, 43, 48]. Consistent with our results, it has been shown that some antioxidants, such as thiols (e.g., thiocarbamates, cysteine, 2-mercaptoethanol) are effective inhibitors of apoptosis [22]. The addition of antioxidant agents, such as serum substitutes, prevents apoptotic death triggered by serum limitation, by maintaining a proper CRP and controlling the ROS [8]. Therefore, the results presented here confirm the relation between CRP, thiols concentration, and apoptosis.

As CRP increased, a linear decrease in the maximum concentration of viable cells, monoclonal antibody, and thiols, as well as an increase in the various nutrient consumption rates was observed. Such a behavior indicates that a more oxidant environment is detrimental to culture performance. The only parameter that showed the highest value at intermediate CRP (around 0 mV) was the maximum q_{MAb}, indicating that different optimum CRP values exist for cell growth and MAb production. Different DOT values for MAb production and cell growth have been reported previously and have been related to differences in the energy requirements for each anabolic function [36, 37]. It has been demonstrated that loss of growth is a consequence of thiols oxidation [8, 17]. It should be noted that at the highest CRP tested, a rapid increase was observed in the various parameters related to nutrient consumption (q_{glc}, q_{gln}, q_{lac}, $Y_{lac/glu}$, and q_{O2}). This behavior can result in higher ATP production, reflecting a possible cellular response for offsetting the oxidative damage. For instance, higher ATP demands are needed to replenish intracellular GSH, which can be depleted under oxidative stress [2, 43, 48].

It is well documented that survival of different mammalian cells requires a particular balance between oxidative and antioxidative species [11], and the ability of a cell to maintain such a balance will determine if it proliferates or dies [5, 27]. In addition, it has been shown that oxidative stress regulates different programmed cell death patterns

such as apoptosis, autophagy, and necroptosis [7, 15, 19, 27, 28]. Accordingly, the premature appearance of apoptosis observed in this study in oxidative cultures, even in the presence of essential nutrients, corroborates the role of high CRP as inductor of this type of programmed cell death. Knowledge of the effect of constant CRP on cell physiology and metabolism could be useful even in engineered cell lines where programmed cell death is delayed or eliminated [24, 27].

In this study, we presented the control of CRP by manipulation of DOT, but CRP can also be controlled by manipulating other independent variables, such as pH or by addition of different antioxidants [20]. It has been reported that some antioxidants such as glutathione, cysteine, or 2-mercapthoethanol can influence culture performance [21, 22]; for instance, by increasing the growth rate and decreasing the production of ROS, which have detrimental effects on cell metabolism. Thus, such antioxidants can be used to control CRP independently of DOT.

Conclusions

The findings reported here showed the effect of controlled CRP on hybridoma culture performance. Knowledge of the influence of CRP on cell metabolism could aid in the design of improved culture strategies, for instance, by establishing reducing conditions through CRP control instead of controlling DOT at low values that help to establish more accurate references, a task that is technically difficult due to measurement limitations at very low DOT values. Accordingly, CRP can be initially controlled at reducing values to promote cellular growth, improve nutrient utilization, and delay cell death; and subsequently switched to moderated oxidant conditions to promote MAb production.

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