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Impact of sodium butyrate and mild hypothermia on metabolic and physiological behaviour of CHO TF 70R cells

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ABSTRACT

Background: To reduce costs associated with productivity of recombinant proteins in the biopharmaceutical industry, research has been focused on regulatory principals of growth and survival during the production phases of the cell culture. The main strategies involve the regulation of cell proliferation by the modulation of cell cycle control points (G1/S or G2/M) with mild hypothermia and the addition of sodium butyrate (NaBu). In this study, batch culture strategies were evaluated using CHO TF 70R cells producing the recombinant human tissue plasminogen activator (rh-tPA), to observe their individual and combined effect on the cellular physiological state and relevant kinetic parameters.
Results: NaBu addition has a negative effect on the mitochondrial membrane potential ($\Delta\Psi m$), the values of which are remarkably diminished in cultures exposed to this cytotoxic compound. This effect was not reflected in a loss of cell viability. NaBu and mild hypothermic conditions increased the doubling time in the cell cultures, suggesting that these strategies triggered a general slowing of each cell cycle phase in a different way. Finally, the individual and combined effect of NaBu and mild hypothermia produced an increase in the specific rh-tPA productivity in comparison to the control at 37°C without NaBu. Nevertheless, both strategies did not have a synergistic effect on the specific productivity.
Conclusions: The combination of NaBu addition and mild hypothermic condition causes an impact on physiological and metabolic state of CHO TF 70R cells, decreasing cell growth rate and improving glucose consumption efficiency. These results therefore provide a promising strategy to increase specific productivity of rh-tPA.

1. Introduction

The biopharmaceutical industry has focused numerous efforts to increase the productivity of its processes related to the production of therapeutic proteins and to reduce associated production costs. These strategies include the development of efficient expression systems and the improvement of media formulation and culture conditions to promote high cell densities [1,2,3,4,5], along with extending the lifespan of a culture [6]. To this date, Chinese hamster ovary (CHO) cells are the main platform for the production of a great number of recombinant therapeutic proteins. In the latter regard, scientists have been studying cell biological variables associated with the productivity of cell lines, specifically by identifying and modulating important growth and survival regulators during the productive phase of the culture [7]. Therefore, cytostatic chemical agents or overexpression of cell cycle inhibitor proteins to decrease the cell growth rate have been used. Additionally, the manipulation of the culture environmental conditions [6,8] as well as mild hypothermia have been extensively used [9,10,11,12]. Several studies have demonstrated that this condition delays cell growth, decreases the specific cell growth rate (μ) and maintains high cell viability [9]. Cell physiology is also affected, showing a delay in the progression of the cell cycle [13,14,15], a delay in the process of apoptosis [11,16,17], a reduction in the

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rates of nutrient consumption and toxic metabolite production, and a decrease in protease activity and the production of reactive oxygen species [16]. All of this is usually accompanied by an increase in the specific productivity (q) [10,18]. Other cellular proliferation control strategies involve the use of cytostatic chemical agents, including sodium butyrate (NaBu). Numerous studies have reported an increase in the expression of recombinant proteins such as rh-tPA [19,20], Mabs [20,21,22], and erythropoietin [23,24] in CHO cells exposed to NaBu at concentrations of 0.5 to 5 mM. At these concentrations, NaBu has cytotoxic characteristics [14,25,26], inhibits cell growth and genetic expression due to an increase in the accessibility of DNA transcriptional factors and induces apoptosis [24,27]. Therefore, the use of this compound in productive systems must be highly controlled to promote an increase of productivity without irreversible cytotoxic effects.

Even if various proliferation control strategies have different effects on cells, mild hypothermia as well as the use of NaBu is a known strategy used to increase recombinant protein production [6,10,18,27, 28,29,30]. Nevertheless, few studies have evaluated the simultaneous effects of these factors. Kantardjieff [31] evaluated the effect of mild hypothermia (33°C) and the addition of NaBu (2 mM) in a recombinant IgG producing CHO cell line, which overexpresses two anti-apoptotic genes. The combined effect of mild hypothermia (33°C) and NaBu addition led to an increase in the specific recombinant protein productivity (q), which indicates productivity per cell, where this value was approximately 1.2 to 6 compared to the values obtained at 33°C and 37°C without the addition of NaBu. Furthermore, proteomic and transcriptomic analyses determined that the cellular response obtained with mild hypothermia and NaBu addition showed the activation of genes involved in the secretory pathways, delay of cell cycle and alteration of protein translating mechanisms [31]. Although positive results were obtained by Kantardjieff [31], our records indicate that no other studies have considered this approach.

Because of the implications of these culture strategies on recombinant protein production, and the importance of understanding the metabolic effects and productive cell behaviour, the simultaneous effects of the use of mild hypothermia and the addition of NaBu on the physiological state and relevant metabolic parameters, such as the cell cycle, the mitochondrial membrane potential ($\Delta\Psi m$), specific consumption or production rates of key metabolites and their influence on specific productivity of rh-tPA was evaluated in CHO TF 70R cells. The data generated was analysed in a manner to associate the physiologic and metabolic behaviour to the productive state of the cells exposed to the different conditions evaluated.

2. Materials and methods

2.1. Cells and culture conditions

A CHO TF 70R rh-tPA producing cell line form Pharmacia & Upjohn S.A. (Sweden) (generously donated by Torsten Björlig), was grown in batch culture in 125 ml spinner flasks. HyClone SFM4CHO culture media supplemented with 20 mM glucose and 6 mM glutamate was used for cell growth and maintenance.

The culture conditions were as follows: 37°C, 5% CO_2 and 95% humidity. To assess the effect of mild hypothermia, CHO cells were acclimatized at low temperature. The cells were initially cultured at 37°C, followed by successive cultures (every 48 h) at 35°C and then at 33°C. Gradual reduction of temperature allowed us to maintain high cell viability. The initial cell concentration was 2.5×10^5 cell/ml. Cells were counted in a haemocytometers and cell viability was determined with the trypan blue dye exclusion test.

To select a cell culture conditioned to NaBu, four NaBu concentrations were tested (0.25; 0.5; 1; and 3.5 mM) [14,32,33,34]. Due to the cytotoxic effect of NaBu, its addition was made at 48 h after initiation of the culture to avoid an early loss of cell growth.

2.2. Metabolic assays

The concentration of rh-tPA was performed with a commercially available ELISA immunoassay (TriniLIZE rh-tPA antigen kit) from Trinity Biotech. The glucose and lactate concentrations were determined with an YSI 2700 automatic biochemistry analyser (Yellow Spring Instruments).

2.3. Cell cycle analysis

The percentage of cells in different phases of the cell cycle was evaluated using flow cytometry (Beckman Coulter FC500). The protocol was standardized based on the Stalk Institute protocols (http://fccf.salk.edu/protocols/cellcycle.php). Cells (1×106 cells/ml) were briefly washed twice with PBS and re-suspended in a polypropylene tube in which 200 μl PBS had been added and 1.8 ml cold ethanol was added dropwise with agitation. Cells were fixed for 1 h at 4°C, then washed twice with PBS and 1 ml of a propidium iodide solution (40 μg/ml of propidium iodide in 3.8 mM sodium citrate) was added to the remaining pellet. The pellet was incubated for 3 h at 4°C with 50 μl of stock RNase A solution. Samples were collected at medium velocity, with a total of 20,000 events per sample. The analysis was performed with the Cylchred software (cell cycle analysis software from Cardiff University), allowing the percentage of cells in the G1/G0, S or M phases of the cell cycle to be determined.

2.4. Analysis of the mitochondrial membrane potential

This assay was performed using cytometric techniques, via the use of the JC-1 probe (5,5′, 6, 6′- tetrachloro – 1,1′, 3, 3′ tetraethyl benzimidazolyl carbo cyanine iodide), which is a cationic dye that accumulates in the mitochondria. The protocol was standardized based on Cossarizza & Salvioli (2000) [35] and Prince et al. [36]. Approximately 0.5×106 cell/ml were treated with 10 μg/ml of JC-1 (Life technology, CAS 47729-63-5), then incubated in the dark for 10–15 min at 37°C. Additionally, a control sample of 0.5×106 cell/ml was treated with 100 nM of valinomycin (Santa Cruz Biotechnology, CAS 2001-95-8) for 30 min at 37°C in the dark before the addition of the JC-1 dye. Both samples were carefully homogenized, centrifuged at $300 \times g$ for 5 min, and washed with PBS. The cells were analysed in a Beckman Coulter flow cytometer at a medium rate.

2.5. Statistical analysis

A two-way ANOVA was performed to compare the parameters of different cell culture conditions, using the statistical tools in Microsoft Excel. A principal component analysis (PCA) was performed using SIMCA-P (Umetrics, Sweden).

3. Results and discussion

3.1. Selection of culture conditions

To determine the effect of NaBu addition to cell cultures, a series of batch cultures were created using the CHO TF 70R rh-tPA producing cell line. Considering the opposite effect of adding NaBu on cell viability and qrh-tPA (Table 1), different NaBu concentrations (0.25, 0.5, 1 and 3.5 mM) were tested. The appropriate NaBu concentration was defined as one that significantly improves qrh-tPA and affects minimally the cell viability. A decrease in cell viability by below 70% after 72 h of culture was considered to be a detrimental effect of the addition of NaBu. According to this criterion, a concentration of 0.5 mM NaBu was selected to perform the following experiments.

Table 1
Effect of NaBu concentration on cell viability and specific rh-tPA productivity in CHO TH 70R cell culture at 37°C at 72 h of culture.

	Sodium butyrate (NaBu) concentrations				
	Control	0.25 mM	0.5 mM	1 mM	3.5 mM
Cell viability (%)	82.33 ± 1.23	79.15 ± 1.76	72.9 ± 5.7	64.1 ± 3.53	22.62 ± 0.87
qrh-tPA (ng/10^6 cell * h)	1.31 ± 0.32	2.74 ± 0.04	3.07 ± 0.07	4.3 ± 0.17	2.42 ± 0.39

In this condition, adequate cellular viability and a high specific productivity of rh-tPA are obtained (Table 1). In contrast, the addition of 1 and 3.5 mM of NaBu caused a drastic loss of cell viability after 60 h.

Mild hypothermia was established at a temperature of 33°C according to results reported in previous studies performed by our research group [37,38], and an increase in cell growth, cell viability and the specific productivity of recombinant proteins was achieved, which was appropriate for this study. Based on such evidence, the experimental design considered two factors with two levels each. The first factor was NaBu (0 and 0.5 mM) and the second factor was temperature (33°C and 37°C).

3.2. Effect of mild hypothermia and NaBu on cell viability and cell growth

To evaluate the effect of mild hypothermia and NaBu on cell viability and growth, cells were cultured in spinner flask at 37°C (CC-37) and at 33°C (CC-33), previously adapted to low temperature. For each of these conditions 0.5 mM of NaBu was added (NaBu-37 and NaBu-33, respectively) after 72 culture. Fig. 1 shows the cell growth profile and cell viability in the different cultures. Fig. 1a shows that control CC-37 achieved a maximum cell concentration of $3.6 \times 10^6 \pm 0.4 \times 10^6$ cell/ml at 144 h, while CC-33 achieved a maximum cell concentration of $2.5 \times 10^6 \pm 0.2 \times 10^6$ cell/ml. The NaBu cultures had a lower cell concentration at 144 h compared to the control. The NaBu-37 culture had a cell concentration of $1.5 \times 10^6 \pm 0.2 \times 10^6$ cell/ml, and NaBu-33 had a concentration of $0.9 \times 10^5 \pm 0.02 \times 10^6$ cell/ml. These data indicate that both mild hypothermia and NaBu slowed cell growth, a phenomenon that was exacerbated when these conditions were applied simultaneously (NaBu-33). Table 2 shows that, along with mild hypothermia (CC-33), the addition of NaBu (NaBu-37) decreases the specific cell growth rate in 24% and 36% respectively, in comparison to the control (CC-37). Additionally, a major decrease in the specific cell growth rate (approximately 60%) was observed in the NaBu-33 culture. Previous studies have widely reported a decrease in the specific cell growth rate under mild hypothermic culture conditions or with the addition of NaBu [32,39,40], which would explain the lower cell concentrations in cultures CC-33 and NaBu-33. Cell viability values (Fig. 1b) were above 95% for the controls CC-37 and CC-33. An acute loss of cell viability was observed in NaBu-37 after 96 h, decreasing to 89% at 144 h. The loss of cell viability in NaBu-33 was significantly less severe (93% at 144 h). Slikker et al. [16] reported that a lower culture temperature resulted in the overexpression of antioxidant and anti apoptotic molecules such as glutathione peroxidase and bcl-2, respectively. This could explain the greater cell viability in cultures incubated at 33°C (CC-33 and NaBu-33) in relation to the cultures incubated at 37°C (CC-37 and NaBu37). The decrease in cell viability and the inhibition of cell growth due to the addition of NaBu has been related to an increase in the percentage of cells that die by apoptosis induced by the alteration of gene expression implicated in this process [25,41] and the inhibition of deacetylated histones (HDAC), which inhibit the progression of the cell cycle [14].

3.3. Effect of mild hypothermia and NaBu on cell metabolism

The effect of mild hypothermia and NaBu on glucose consumption and lactate production is shown in Fig. 2. The glucose concentration (Fig. 2a) at the end of culturing was remained above 4 mM in all the evaluated conditions, and therefore glucose did not limit the cell growth [42]. Mild hypothermia (CC-33) and NaBu addition (NaBu-37) significantly increased the specific glucose consumption rate from 10% to 34% compared to the control. The effect of both factors was evaluated statistically using a two-way ANOVA (Table 3). The combined action of these factors (NaBu-33) did not have a synergistic effect on glucose consumption ($P = 0.4611$, Table 3). The increase in glucose consumption in the presence of NaBu agrees with the information reported by Carinhas et al. [43], who observed an increase in cellular metabolic activity due to the addition of 0.75 mM of NaBu in a CHO cell culture. This was attributed to an increase of the carbon flux to glycolysis, the TCA cycle and amino acid metabolism, and consequently, a sustained energetic state matching the high production of recombinant protein was produced [43].

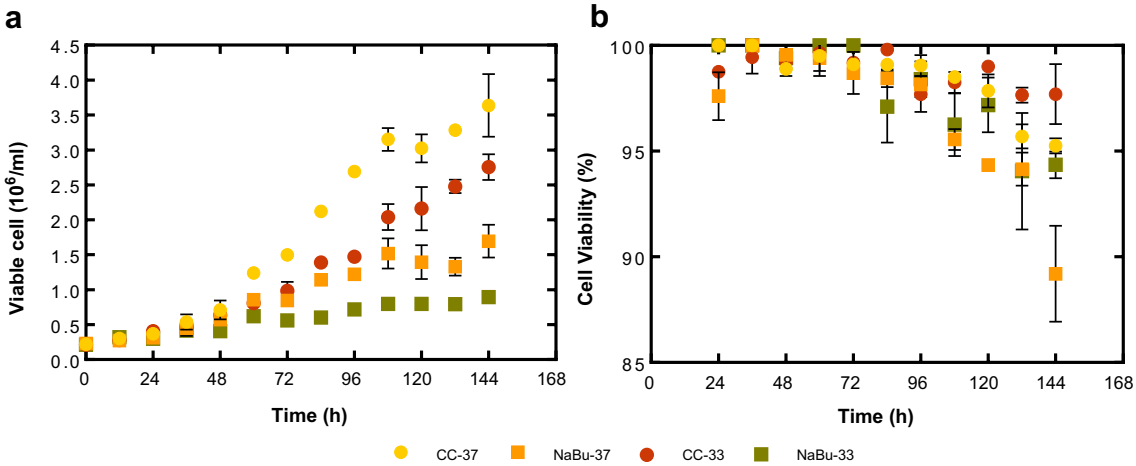

Fig. 1. Viable cell profile (a) and viability (b) of rh-tPA producing CHO TF 70R cell exposed to mild hypothermia and/or addition of 0.5 mM of NaBu.

Table 2
Culture parameters rh-tPA producing CHO TF 70R cell exposed to mild hypothermia and/or addition of 0.5 mM of NaBu.

	CC-37	CC-33	NaBu-37	NaBu-33
μ (h^{-1})	0.025 ± 0.001	0.019 ± 0.0009	0.016 ± 0.004	0.010 ± 0.0004
T_D (h)	28 ± 1.53	36 ± 1.70	43 ± 0.95	68 ± 2.83
q_{glc} (mmol 10^{-6} cell h^{-1})	107.35 ± 6.15	118.55 ± 2.62	144.35 ± 11.67	163.35 ± 1.63
q_{lac} (mmol 10^{-6} cell h^{-1})	123.05 ± 6.58	129.2 ± 6.79	144.85 ± 10.54	178.8 ± 0.14
Yg/L (mol/mol)	1.15 ± 0.13	1.09 ± 0.03	1.01 ± 0.16	1.095 ± 0.01
$q_{rh\text{-}tPA}$ (ng 10^{-6} cell h^{-1})	1.70 ± 0.06	2.14 ± 0.04	3.37 ± 0.05	3.22 ± 0.11

A maximum concentration of 12.5 mM lactate was achieved in CC-37 and CC-33 after 120 h (Fig. 2b). In NaBu-37, the maximum concentration was lower (11.1 mM); while in culture NaBu-33 the lactate concentration was 8.8 mM. Additionally, at the end of these two cultures lactate profile shows a metabolic shift, from production to consumption. NaBu at 37°C (NaBu37) and 33°C (NaBu33) significantly increased the specific lactate production rate by 17% and 45%, respectively (Table 2 and Table 3), which was in accord with the greater glucose consumption mentioned earlier.

3.4. Effect of mild hypothermia and NaBu on rh-tPA production

The individual and combined effects of NaBu and mild hypothermia on rh-tPA production are presented in Fig. 3. A maximum rh-tPA concentration of 74 mg/l was achieved in the control (CC-37); while in the mildly hypothermic culture with or without NaBu addition, the maximum concentration was approximately 110 mg/l. After this value was reached, a decrease in rh-tPA concentration was observed in all of the cultures. The decrease was greater with the addition of NaBu. This was related to the high loss of viability shown in these cultures beginning at 96 h (Fig. 1b) and was probably also related to a higher concentration of proteases in the culture media [14,24,25].

In general, the specific rh-tPA productivity was improved by both mild hypothermia and the presence of NaBu, in comparison to the control (CC-37, Table 2). Additionally, it is important to mention that the combined use of mild hypothermia and NaBu had a significant effect on qrh-tPA ($P = 0.049$, Table 3) showing an 89% increase in comparison to the control. This behaviour of the specific rh-tPA productivity agrees with that reported by Kantardjieff et al. [31] for CHO cells producing recombinant IgG. In this case, cells exposed to 33°C and 2 mM NaBu had a specific productivity 6-fold greater than that obtained in a control at 37°C without NaBu. The productivity increase was associated with an increase in the cellular secretory capacity, which was demonstrated via a transcriptomic and proteomic analysis of functional proteins involved in the secretion process. Importantly, the cell line used by Kantardjieff [31] overexpressed two types of anti-apoptotic genes, which would explain the tolerance to a higher NaBu concentration and therefore higher recombinant protein production. However, the rh-tPA producing CHO cells exposed to 1 mM NaBu and 37°C had a 4-fold increase in specific productivity in comparison to the control but suffered a dramatic loss of viability at 72 h (Table 1). NaBu and mild hypothermia do not act synergistically to promote increased production. As previously mentioned, both strategies are related to diverse cellular processes involved in increasing the production of recombinant proteins, and one of these strategies might be possibly favoured over another. Therefore, it would be relevant to assess the molecular response expressed in the presence of mild hypothermia and/or NaBu to identify which pathways are most affected by each of these strategies under culture conditions that allow variables such as the specific growth rate or changes in the culture medium to be studied separately. This can be performed using a continuous culture.

3.5. Effect of mild hypothermia and NaBu on progression of the cell cycle

Another important effect of the use of a cell proliferation control strategy such as mild hypothermia and NaBu exposure, is the modulation of cell cycle. Both strategies have been related to an increase in the percentage of cells in the G1/G0 phase of the cell cycle, which has been associated with an increase in the recombinant protein production [19,44]. In this context, the distribution of cells in the various cell cycle phases (G1/G0, S, and G2/M) was evaluated (Table 4). Mild hypothermia significantly changed the distribution of cells in the various phases in CC-33 compared to CC-37 (Table 5). An increase from 30.7% to 42.2% in the percentage of cells in the S phase and a decrease from 57.5% to 44.9% of cells in G1/G0 phase were observed in CC-37 and CC-33, respectively (Table 4). Additionally, a decrease from 30.7% to 24.3% in the percentage of cells in the S

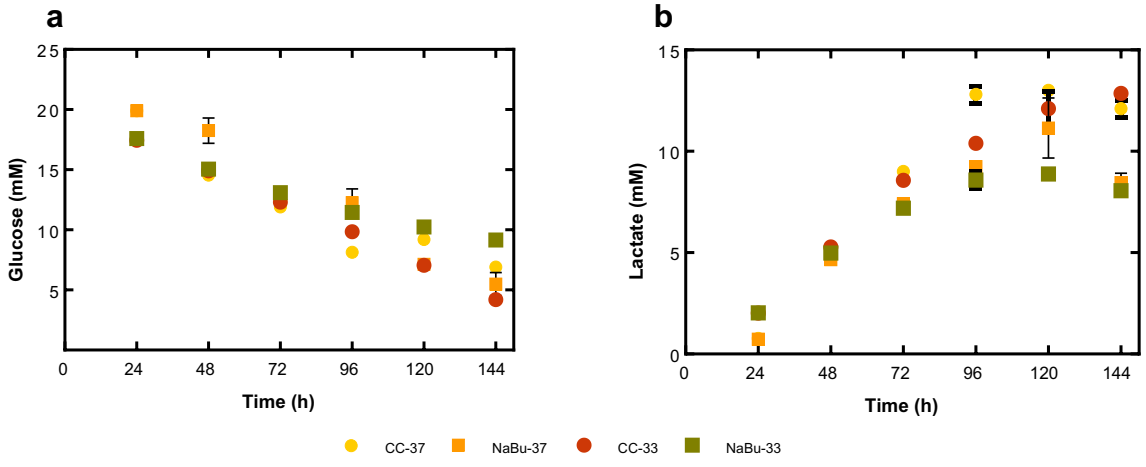

Fig. 2. Profile of glucose consumption (a) and lactate generation (b) in rh-tPA producing CHO cell exposed to mild hypothermia and/or addition of 0.5 mM of NaBu.

Table 3
Results of the two-way ANOVA expressed as the *P*-value. Temperature effect, NaBu effect and the combined effect of both strategies on the culture parameters.

	P-value of cell culture parameters			
	t_D	q_{glc}	q_{lac}	$q_{rh\text{-}tPA}$
Temperature effect	0.0002*	0.001*	0.016*	0.016*
NaBu effect	0.00005*	0.0344*	0.002*	0.002*
Combined effect	0.003*	0.4611	0.049*	0.049*

* Data with statistically significant differences (significance level of $P < 0.05$).

phase and from 57.5% to 53.2% in the G1/G0 phase were observed in CC-37 and NaBu-37, respectively, which indicated a change in the distribution of cells in the various cell cycle phases. On the other hand, a synergistic effect of mild hypothermia and NaBu (NaBu-33) was observed, reflected in an increase of 20% of cells in the G1/G0 phase and a decrease of 60% of cells in the G2/M phase.

All together, these results indicate that although there was no retardation of the cell cycle in G1/G0 with mild hypothermia (33°C) nor in the presence of NaBu, there was a decrease in the specific growth rate. In this way, an increase in the length of each cell cycle phase could explain the increase in the cell doubling time (tD) of CC-33 (36 h) in comparison to CC-37 (28 h). This behaviour was previously reported in myeloma [45] and hybridoma [46], cells in which the anti-apoptotic gene Bcl-2 was overexpressed. In myeloma cells, a retardation of the cell cycle was observed at a lower dilution rate due to a greater time for the progression of the S and G2/M phases, while in hybridoma cells, a lower dilution rate led to an increase in the doubling time\, due to the extension of the progression of the G1 phase. On the other hand, because mild hypothermia (CC-33) resulted in a major change in the cell percentage in the various cycle phases than NaBu addition (NaBu-37), and because of the lower difference between the tD values (Table 2), each of these variables presumably affects the duration of each cell cycle phase in a different way. The combined effect of mild hypothermia and NaBu on the duration of the cell cycle phases was magnified; the tD value was nearly tripled in comparison to CC-37 (Table 2). Hendrick et al. [19] evaluated the correlation between the exposure of rh-tPA-producing CHO cells to 1 mM NaBu and mild hypothermia (32°C) individually and established a clear relationship between increased rh-tPA production and the maintenance of cells in the G1/G0 phase of the cell cycle for a longer time in cultures exposed to NaBu. The control (37°C without NaBu) had 43% of cells in the G1 phase, and after NaBu was added, this value increased to 54%. However, it is important to mention that the effect observed by Hendrick et al. [19] was limited to cultures exposed to NaBu because the thermal shock data were not

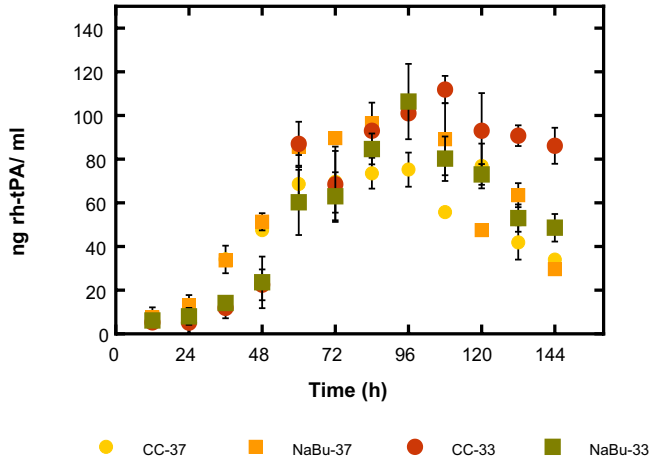

Fig. 3. Profile generation of rh-tPA in CHO TF 70R cells exposed to mild hypothermia and/or addition of 0.5 mM of NaBu.

Table 4
Distribution of cells in the cell cycle phases of CHO TF 70R cells exposed to mild hypothermic conditions (33°C) and NaBu (0.5 mM), expressed as a percentage (%).

	CC-37	NaBu-37	CC-33	NaBu-33
G1/G0 (%)	57.5	53.2	44.9	71.8
S (%)	30.7	24.3	42.2	23.4
G2/M (%)	11.8	22.6	12.9	4.8

presented. Additionally, mild hypothermia was achieved in our study by previous conditioning of the cultures and not by an abrupt temperature change, as it is usually performed in cultures exposed to mild hypothermia.

Recently, Chen et al. [20] evaluated the combined effect of NaBu and mild hypothermia on the production, sialylation and biological activity of an antibody produced by CHO cells. They analysed the effect of these control strategies of cellular proliferation on the distribution of cells in the cell cycle via the evaluation of the percentage of cells in G1 phase and concluded that neither mild hypothermia nor the presence of NaBu promoted an arrest of the cells in the G1 phase. It should be mentioned that they did not assess the arrest of cells in other phases of the cell cycle.

3.6. Effect of mild hypothermia and NaBu on mitochondrial membrane potential

Studies have shown the generation of an oxidative environment as a result of cellular stress conditions [47,48]. In response, a survival mechanism exists that aims to reduce reactive oxygen species by decoupling oxidative phosphorylation, which could be reflected in a loss of the mitochondrial membrane potential [49]. To determine if mild hypothermia and/or NaBu disturbed the cellular oxidative metabolism, the percentage of cells that preserved the mitochondrial membrane potential in the cultures was measured. Fig. 4 shows that in CC-37 and CC-33, over 90% of the cells maintained the mitochondrial membrane potential until 144 h, indicating that mild hypothermia did not significantly affect the mitochondrial membrane potential. On the other hand, in NaBu-37 and NaBu-33, a notable loss of the mitochondrial membrane potential was observed after 72 h of culture and the effect was not diminished by mild hypothermia. Therefore, the loss of the mitochondrial membrane potential in these cultures could be attributed solely to the presence of NaBu. Nevertheless, it is important to mention that, although a great loss in the mitochondrial membrane potential was observed in NaBu cultures, cultures exposed to these conditions maintained a cell viability of approximately 90%. Previous studies have found the induction of autophagy and apoptosis in CHO cell cultures exposed to NaBu [41,48,50]. Lee and Lee [41] reported the occurrence of autophagy in CHO cells as a cellular survival mechanism in cultures treated with NaBu, possibly because of the elimination of inefficient mitochondria to prevent an increase in cellular oxidative stress that could generate apoptotic signals. Because appropriate mitochondrial function is essential for cellular survival, the selective elimination of a subset of dysfunctional mitochondria is a necessary process, but it also

Table 5
Two-way ANOVA expressed as a *P*-value. Temperature effect, NaBu effect and combined effect of both strategies in the distribution of CHO TF 70R cells in the cell cycle phases.

	P-value of cell culture parameters		
	G1/G0	S	G2/M
Temperature effect	0.215	0.011*	0.003*
NaBu effect	0.005*	0.0004*	0.365
Combined effect	0.001*	0.006*	0.002*

* Data with statistically significant differences (significance level of $P < 0.05$).

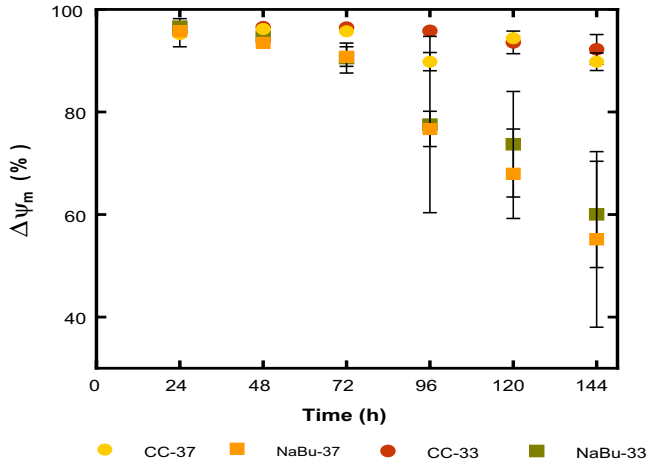

Fig. 4. Effect of mild hypothermia and NaBu on the mitochondrial membrane potential of CHO TF 70R cells.

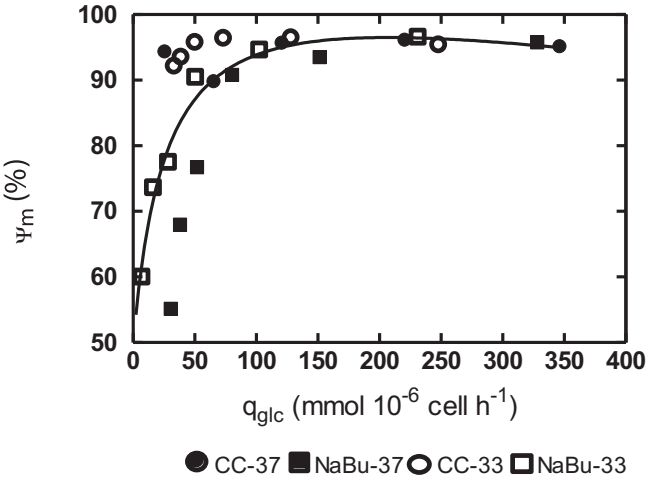

Fig. 6. Relation of glucose specific consumption rate and mitochondrial membrane potential.

can gradually decrease the mitochondrial membrane potential in the cells, as a consequence of a low mitochondrial mass [50,51]. This phenomenon could explain the results presented in our study, but additional studies should be performed to corroborate this result.

3.7. Glucose consumption and loss of mitochondrial membrane potential

To establish putative relationships between behavioural parameters of cell cultures in various growth stages, biostatistical modelling based on PCA was performed. The results are shown in Fig. 5, in which each condition studied had been placed in a different quadrant. The first component (PC1) discriminates the presence of NaBu, and the second component (PC2) separates clearly different temperatures. The first component explains over 70% of the experimental variation. Interestingly, PC1 contrasts mainly the specific glucose consumption rate and mitochondrial membrane potential. Similarly, the specific lactate production is correlated to mitochondrial membrane potential. This is a consequence of the direct relationship between lactate production and glucose consumption. Fig. 6 shows the specific relationship between mitochondrial membrane potential and specific glucose consumption rate in all cell culture conditions evaluated. A clear correlation between the mitochondrial membrane potential and the specific glucose consumption rate can be observed, adjusting adequately to a saturation curve ($r2 = 0.96$). Starting from that adjustment, at a specific glucose consumption rate of 10 nmol/106 cell/h, the loss of the mitochondrial membrane potential can be estimated to be approximately 50%. Likewise, over a specific glucose consumption rate of 100 nmol/106 cell/h, the mitochondrial membrane potential was maintained at greater than 90%, independent of the culture conditions. In this context, previous studies have related the state of mitocondrial membrane potential to the metabolic efficiency of the cell culture [52]. Borth [52] evaluated the response of hybridoma cells exposed to increasing glucose concentrations in a glutamine-free media in relation to the mitochondrial membrane potential at 6 h of growth, to elucidate the relationship between glucose consumption and mitochondrial activity. A linear relationship between the fluorescence level of

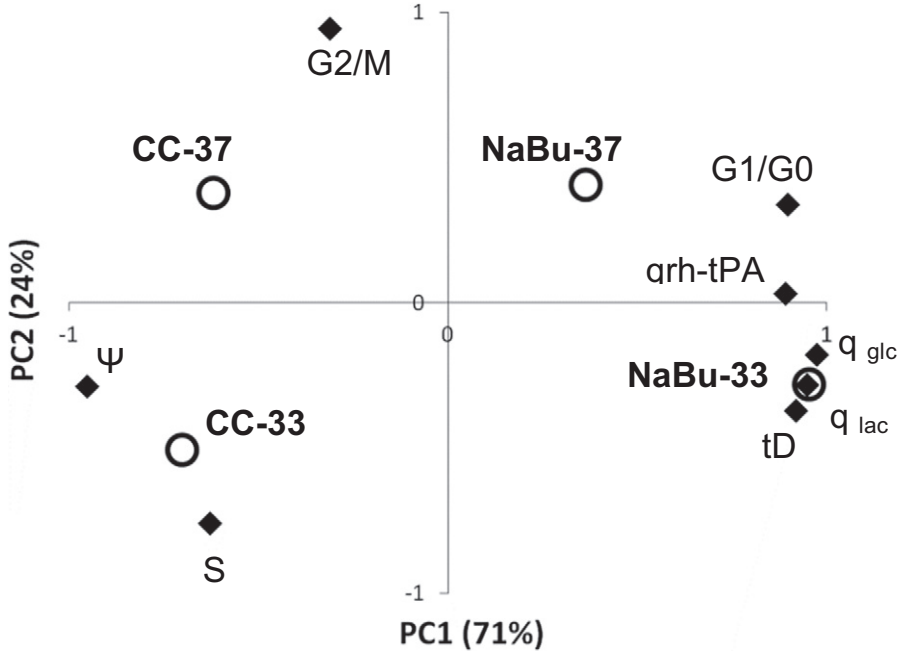

Fig. 5. Principal component analysis. Cell culture parameters on growth stage were used as variables and culture conditions as observations.

rhodamine 123 and the specific glucose consumption was evident over this time period.

According to the exposed results, the loss in the mitochondrial membrane potential would be evidence a decrease in metabolic activity, in terms of the specific consumption of glucose, but would not be related to cell growth or cell viability. In this respect, it is interesting to note that the higher productivity levels of rh-tPA are achieved under NaBu addition conditions. In this condition the higher specific rates of glucose consumption are observed, which would lead, on the one hand, to the maintenance of a high mitochondrial membrane potential (notwithstanding the adverse effects of NaBu) and, on the other hand, to improve productivity of the protein of interest.

4. Conclusions

In CHO TF 70R cells that were adapted to mild hypothermia (33°C) or exposed to NaBu, the general cell growth was slower, which was reflected in an increase of the doubling time, and affected the duration of each cell cycle phase differently. The increase of the specific productivity of rh-tPA could not be associated with the increase in the percentage of cells in any specific cell cycle phase.

NaBu had a negative impact on the mitochondrial membrane potential ($\Delta\Psi m$), which was not countered by the mild hypothermia. On the other hand, the loss of $\Delta\Psi m$ was correlated with an increase in the specific glucose consumption rate. The detrimental effect of NaBu on the mitochondria/energetic status is reflected by the increase in glucose uptake. In the context of the conditions evaluated, the loss of $\Delta\Psi m$ did not reflect an important loss of cell viability.

Mild hypothermia and the presence of NaBu did produce an increase in the specific productivity of rh-tPA individually and together. It would expect that the combination of both approaches promoted a synergic effect, enhancing the rh-tPA specific productivity rather than the sum of the individual effect of each condition, but it was not the case. Higher specific glucose consumption was observed in cultures exposed to NaBu, but this was not reflected in a greater cell growth neither in lactate production. So, a greater and more efficient consumption of glucose would explain the increase in the rh-tPA specific productivity.

We believe that greater effort should be made to deepen our understanding of the molecular networks underlying the response of cells to mild hypothermia and/or the presence of NaBu. We are currently determining the transcriptomic response of CHO TF 70R cells in a continuous culture under such conditions.

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