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The Combined Effect of Sodium Butyrate and Low Culture Temperature on the Production, Sialylation, and Biological Activity of an Antibody Produced in CHO Cells

Fei Chen, Tianci Kou, Li Fan, Yan Zhou, Zhaoyang Ye, Liang Zhao, and Wen-Song Tan

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Abstract Cell cultures containing 0 ~ 5 mM sodium butyrate (NaBu) and grown at 30 and 37°C were conducted to investigate the combined effect of NaBu and low temperature on the quantity and quality of an antibody production in CHO cells. Although NaBu addition decreased cell viability by apoptosis in a dose-dependent manner at both 30 and 37°C, the onset of significant apoptosis induced by NaBu was delayed by lowering culture temperature. The highest specific antibody productivity (q_p) of 23.26 pg/cell/day was obtained in the culture containing 2 mM NaBu at 30°C; however, the highest antibody concentration of 167.84 mg/L was achieved in the culture containing 1 mM NaBu at 30°C, as the detrimental effect of further NaBu addition on cell growth compromised its beneficial effect on q_p . Moreover, protein quality with respect to the total sialic acid content and N-glycolylneuraminic acid (Neu5Gc) level was evaluated. There were no apparent changes regarding the total sialic acid content of the antibody, but manipulation of cultures with NaBu treatment or (and) low culture temperature did decrease Neu5Gc levels by 5 ~ 10%. Biological activity of the antibody was also assessed, and no obvious changes were observed. Collectively, the simultaneous application of NaBu and low culture temperature was an effective way to extend culture period and enhance final antibody concentration, without compromising the sialic acid content or biological activity.

Keywords: CHO cells, sodium butyrate, low culture temperature, sialylation, N-glycolylneuraminic acid

1. Introduction

Currently, therapeutic recombinant human proteins are predominantly produced in mammalian cells, especially Chinese hamster ovary (CHO) cells. In an effort to increase the production of recombinant proteins, both academic and industrial researchers have focused on optimizing cell line generation and process development [1]. A variety of strategies have been employed to increase recombinant protein productivity in CHO cells, including low temperature [2-5] and addition of sodium butyrate (NaBu) [6-9].

NaBu, a sodium salt of butyric acid, can enhance recombinant protein biosynthesis in mammalian cell culture [6-9], but it can also inhibit cell growth and induce cellular apoptosis [10-14]. Thus, the beneficial effect of NaBu addition on a foreign protein expression is compromised by its negative effect on cell growth. As an endeavor to reduce this defect, various strategies aimed at overcoming the cytotoxic effect of NaBu have been attempted [10-14]. Manipulations of the culture environment (chemicals such as nutrients or anti-oxidants) and the cell itself (genetics) are well documented, and to our knowledge, a shift to low culture temperature has been reported to maintain high cell viability and extend the culture period [15,16]. Thus, culture shift to low temperature may be an alternative method to alleviate NaBu-induced apoptosis and further increase the antibody production through increased specific productivity (q_p) and extended cell culture period. However, the combined effect of these stressful culture conditions on the q_p of CHO cells has not yet been clearly

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reported.

Besides their effect on production of recombinant proteins, NaBu addition or temperature shift might be able to influence the quality of recombinant glycoprotein [5,17]. Proper glycosylation, especially sialylation, is a major consideration for the production of glycoprotein therapeutics as it can affect protein stability, bioactivity, as well as immunogenicity [18]. Among > 40 naturally occurring forms of sialic acid [19], N-glycolylneuraminic acid (Neu5Gc) and N-acetylneuraminic acid (Neu5Ac) are the 2 most common forms. Neu5Gc, a derivative of Neu5Ac, which is not typically expressed in humans but is present in CHO cultures, has been suggested to be immunogenic in humans [20,21]. Thus, it is urgent to understand how cell culture conditions affect Neu5Gc levels. Extensive efforts have been made to describe the effects of environmental factors on the total sialic acid content of a glycoprotein [22,23]. However, there are very few literature reports describing the impact of culture conditions on glycoprotein Neu5Gc levels.

In this paper, we investigated the combined effect of NaBu addition and low culture temperature on production of an antibody in CHO cells. Furthermore, because the quality of foreign protein can be affected by stressful culture conditions [5,7-9,17], the sialic acid content and biological activity of purified antibody were also examined to determine their combined effect.

2. Materials and Methods

2.1. Cell line and culture maintenance

A CHO cell line secreting a humanized antibody was employed for this study. The medium for culture maintenance was EX-CELL™ 302 serum-free medium (JRH, Bioscience, USA) supplemented with 2 mM glutamine and 1.6 g/L NaHCO₃. Cells were placed on a rotary shaker with an agitation rate of 50 rpm and maintained in a humidified incubator with 5% CO₂ at 37°C. Seed cells were subcultured every 2 days at an initial cell density of 3×10^5 cells/mL. Unless otherwise stated, all materials were from Sigma (USA).

2.2. Batch cultivation

Batch suspension cultures were performed in spinner flasks (Corning, NY, USA), with a working volume of 100 mL. Exponentially growing CHO cells were harvested by centrifugation (1,200 rpm for 10 min), and inoculated in fresh EX-CELL™ 302 serum-free medium at a cell density of $3 \sim 4 \times 10^5$ cells/mL. After 2 days of cultivation, the spinner flasks were incubated at 2 different temperatures, 30 and 37°C, respectively. Simultaneously, a 1 M sterile

stock solution of NaBu was added to a final concentration of 0 ~ 5 mM on the day following temperature shift. Experiments were performed in triplicate on 2 separate occasions. Cell suspension samples were taken daily from the spinner flasks. Culture supernatants, after centrifugation, were aliquoted and kept frozen at -20°C for later analyses.

2.3. Routine analytical methods

Cell concentration during the culture was estimated using a hemocytometer. Viable cells were distinguished from dead cells by the trypan blue dye exclusion method. The activity of lactate dehydrogenase (LDH) in the medium was measured with an enzyme test kit according to the manufacturer's protocol (Nanjing JianCheng Bioengineering Institute, Nanjing, China).

Quantification of the antibody was detected by a reversed phase HPLC method. The antibody concentration was quantified using reference standards (Shanghai CP Guojian Pharmaceutical Co., Ltd, Shanghai, China). q_p was calculated in the range showing linear correlation between antibody concentration and the integral of viable cell concentration (IVCC) between day 2 and the end of the culture [24].

2.4. Analysis of cell cycle

To assess the combined effect of NaBu addition and low culture temperature on cell cycle distribution, cellular DNA was analyzed by flow cytometry. Briefly, $\sim 1.0 \times 10^6$ cells taken from the cultures were centrifuged, washed twice with PBS, fixed using ice-cold 70% (v/v) ethanol and stored at -20°C. Before measurement, the fixed cells were washed twice with PBS, resuspended in PBS containing 50 µg/mL RNase A, and incubated for 30 min at room temperature. Then, propidium iodide (PI, Invitrogen, USA) was added to a final concentration of 50 µg/mL and the preparation was incubated at room temperature in the dark for an additional 30 min. Flow cytometric analysis was carried out using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Cellular debris and doublets were gated out using a bitmap surrounding the PI-stained single cells.

2.5. Analysis of cell apoptosis

The percentage of CHO cells undergoing apoptosis was determined using the Apoptosis Detection kit (Invitrogen, USA) according to the manufacturer's instructions. Briefly, $\sim 1.0 \times 10^6$ cells were harvested, washed twice with cold PBS, and suspended in PI dyeing reagent for 5 ~ 15 min at room temperature in the dark. The stained cells were immediately analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.6. Determination of sialic acid content of purified antibody

The antibody secreted by CHO cells was purified as described by Gawlitzek *et al.* [25]. A single peak was obtained and confirmed in comparison with the antibody standard (Shanghai CP Guojian Pharmaceutical Co., Ltd, Shanghai, China). Purified antibody was stored at -20°C for later analysis.

Quantification of sialic acid content of the antibody was conducted using a reversed phase HPLC method [26]. Briefly, the sialic acids were released from the Protein-A purified samples in a NaHSO_4 solution (0.25 M, 80°C , 20 min) and derivatized in the same solution with o-phenylenediamine-2HCl (OPD) (10 mg/mL, 80°C , 40 min). The derivatives were then separated on a C-18 reversed-phase Ultrasphere-ODS column and the sialic acids were monitored by a HPLC fluorescence detector (Waters, USA). The following settings were used on the detector: excitation wavelength, 230 nm; emission wavelength, 425 nm. The peak areas were quantified by linear regression of a standard curve. The sialic acid content was determined by comparison to a standard curve obtained using a sialic acid

standard.

2.7. Determination of biological activity of antibody

The biological activities of antibody were tested using a standard method according to the Pharmacopoeia of the Peoples Republic of China. Briefly, $100\ \mu\text{L}$ of exponentially growing L929 cells at a density of $\sim 1.2 \times 10^5$ cells/mL were inoculated into a 96-well microplate (Corning, NY, USA) in a humidified 5% CO_2 incubator at 37°C . After 24 h of cultivation, the antibody obtained from the cultures and the antibody standard, were added together with $60\ \mu\text{g}/\text{mL}$ actinomycin D and $60\ \text{U}/\text{mL}$ $\text{TNF-}\alpha$ to the L929 cells. The 96-well microplate was further incubated for 18 h. Then, $10\ \mu\text{L}$ chromogenic agent (Cell Counting Kit-8, Dojindo Molecular Technologies, Japan) was added to measure the absorbance at 490 nm in a microplate reader.

3. Results

3.1. The combined effects of NaBu and low temperature on cell growth and antibody production

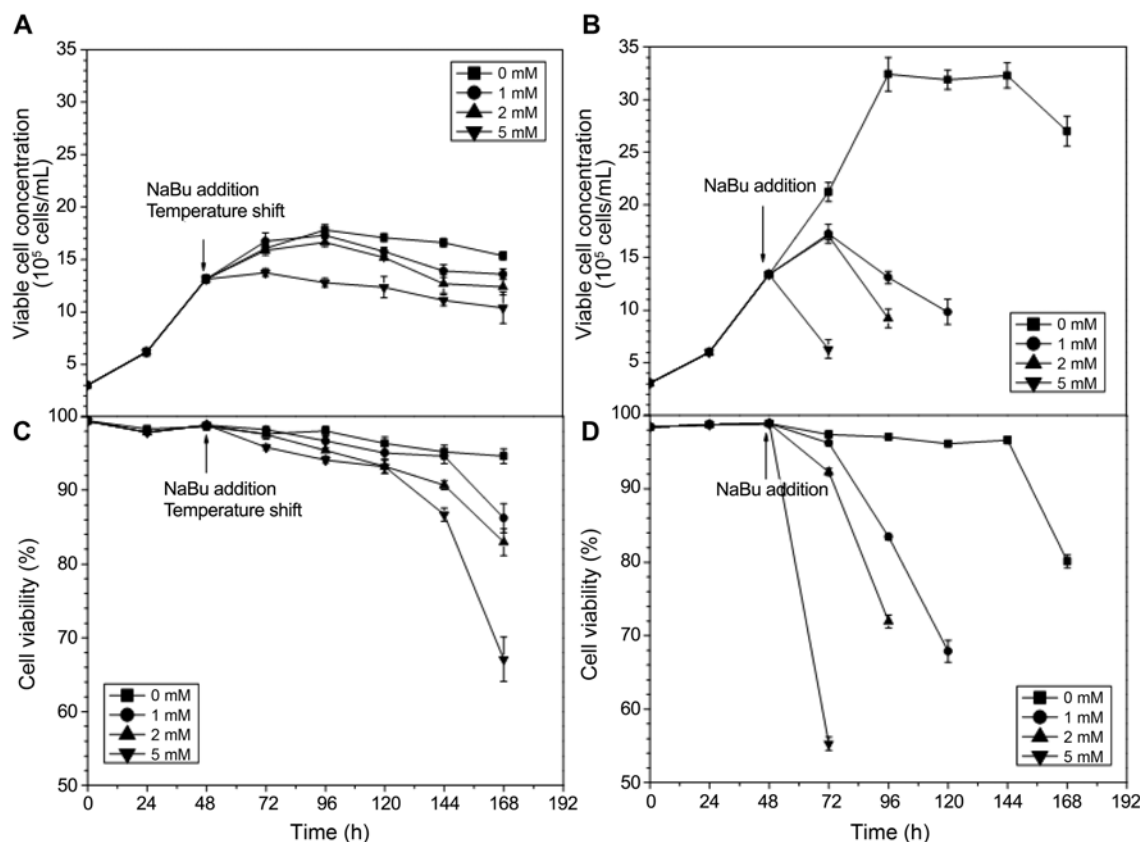


Fig. 1. The combined effect of NaBu addition and low culture temperature on cell growth and viability: (■) 0 mM; (●) 1 mM; (▲) 2 mM; and (▼) 5 mM. (A) Viable cell concentration at 30°C . (B) Viable cell concentration at 37°C . (C) Cell viability at 30°C . (D) Cell viability at 37°C . Arrows indicate the timing of NaBu addition or temperature shift. The error bars represent the standard deviations calculated from the data obtained from 3 independent experiments.

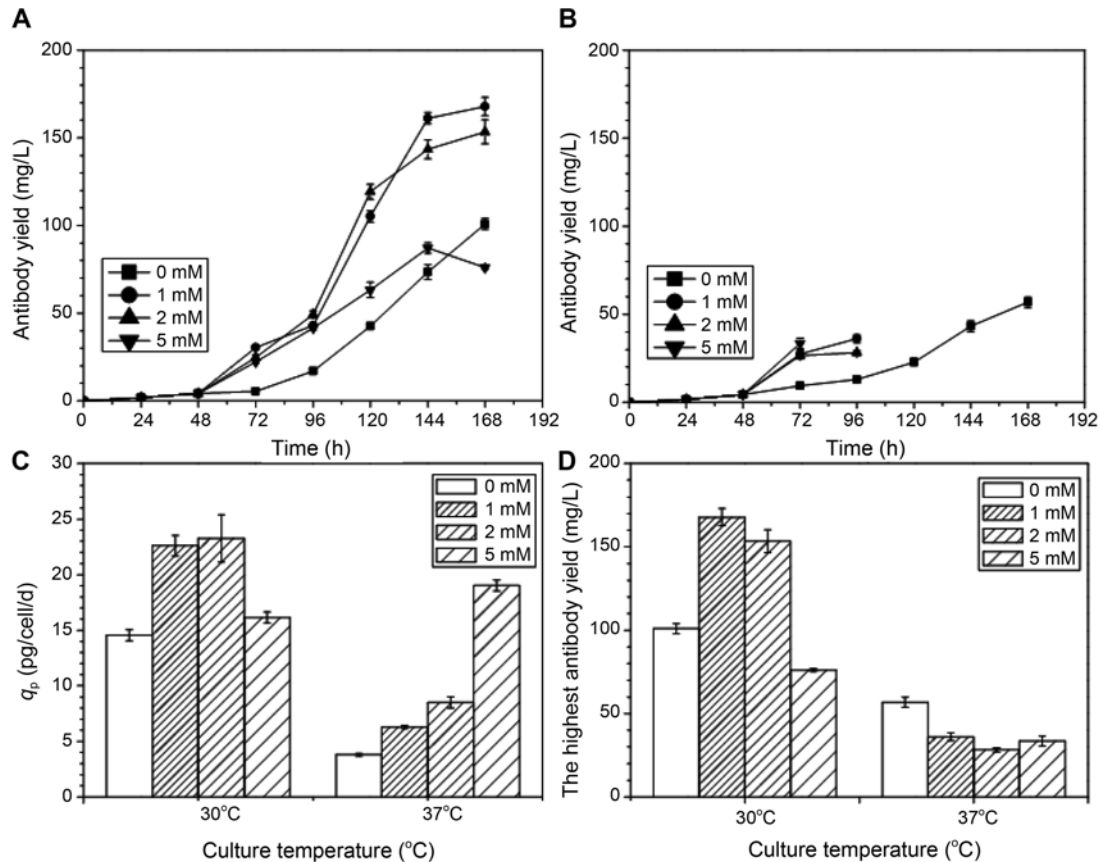


Fig. 2. The combined effect of NaBu addition and low culture temperature on antibody production: (■) 0 mM; (●) 1 mM; (▲) 2 mM; (▼) 5 mM. (A) Antibody concentration at 30°C. (B) Antibody concentration at 37°C. (C) Specific antibody productivity at 30 and 37°C. (D) The final antibody concentration at 30 and 37°C. The error bars represent the standard deviations calculated from the data obtained from 3 independent experiments.

To determine the combined effects of NaBu and low culture temperature on antibody production in CHO cells, the cultures were performed in batch cultivation mode as described in Materials and Methods. Fig. 1 showed cell growth and cell viability profiles of the cultures subjected to different NaBu concentrations (0, 1, 2, and 5 mM) at both 30°C (Fig. 1A) and 37°C (Fig. 1B). In the absence of NaBu, culture temperature shift from 37 to 30°C suppressed cell growth, but cell viability remained high for a longer culture period. However, NaBu treatment inhibited cell growth and decreased cell viability in a dose-dependent manner regardless of culture temperature. At 24 h post-treatment with NaBu at 37°C, cell viability in the culture without NaBu addition was 97.41%, while cell viabilities with addition of 1, 2, and 5 mM NaBu were 96.23, 92.30, and 55.30%, respectively. By comparison, the cultures at 30°C experienced the better cell growth: cells retained viabilities of at least 85% during the course of 144 h. The onset of significant cell death induced by NaBu addition was delayed, indicating the effect of low culture temperature on NaBu tolerance. Thus, combining

NaBu addition with low culture temperature was more beneficial for antibody production than NaBu addition alone, as a result of an extended culture period.

Despite its negative effect on cell growth, protein production benefited from NaBu addition, demonstrated by a higher specific productivity and a higher product yield at 30°C (Fig. 2). NaBu addition at 30°C affected q_p in a different fashion from that at 37°C. At 37°C, the highest q_p of 19.03 pg/cell/day was obtained with 5 mM NaBu addition, while the highest q_p of 23.26 pg/cell/day was achieved with addition of 2 mM NaBu at 30°C (Fig. 2C). In all experimental groups, the highest antibody yield of 167.84 mg/L (Fig. 4D) was achieved in the culture with 1 mM NaBu treatment at 30°C. The presence of NaBu up to 5 mM did not further increase antibody production, perhaps because the cytotoxic effect of NaBu on cell growth outweighed its beneficial effect on q_p .

3.2. The combined effect of NaBu and low temperature on cell apoptosis

To investigate whether the rapid decrease in cell viability in

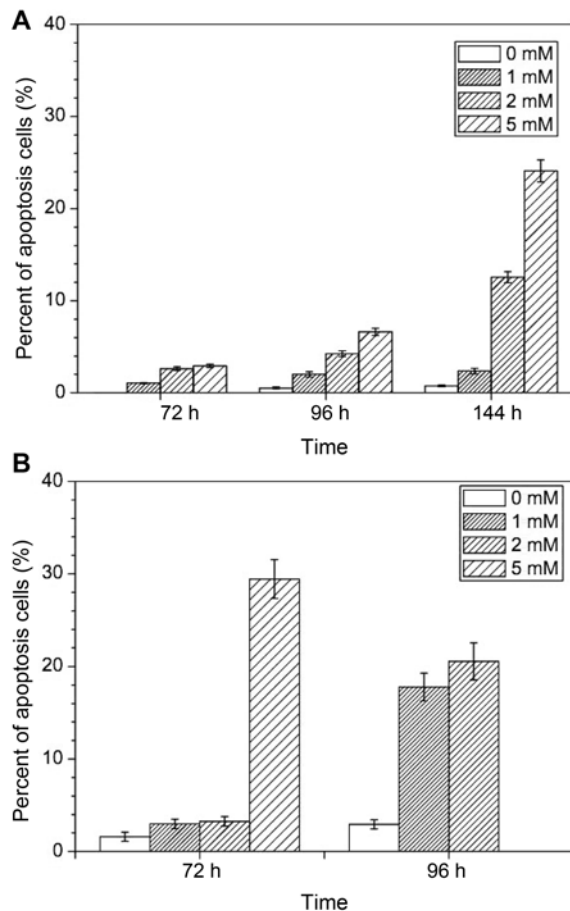


Fig. 3. The combined effect of NaBu addition and low culture temperature on cell apoptosis. (A) 30°C. (B) 37°C. The error bars represent the standard deviations calculated from the data obtained from 3 independent experiments.

the presence of NaBu was associated with apoptotic cell death, cells were sampled during the culture for apoptosis measurement. The percent of apoptotic cells as a function of cultivation time was shown in Fig. 3. Reduction in temperature to 30°C did not synergize with NaBu addition to increase apoptosis. Rather, a ~24 h delay of the onset of significant apoptosis (>10%) was afforded by lowering culture temperature. This “protection” by lowering culture temperature was reflected in the extended culture period, which explained the phenomenon that the culture with 1 mM NaBu addition at 30°C demonstrated significantly increased final antibody concentration.

To further determine the combined effect of NaBu treatment and low culture temperature on cell death, the LDH activity was also investigated. The increase in LDH activity is considered to be an indication of cell lysis [27]. As anticipated, the increasing LDH activity correlated well with the changes in cell viability. The LDH activity increased in a dose-dependent manner with NaBu addition at both 30 and 37°C (Fig. 4). At 37°C, the LDH activity increased

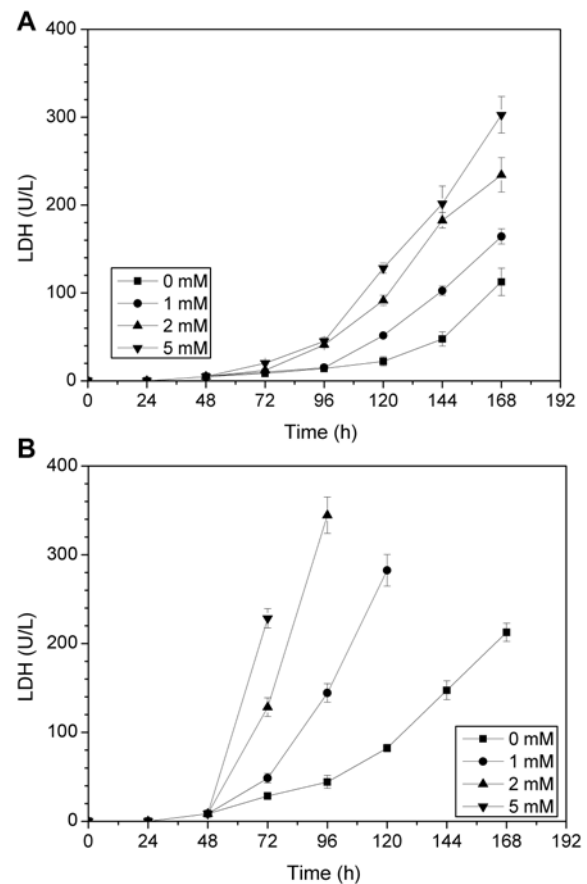


Fig. 4. The combined effect of NaBu addition and low culture temperature on LDH activity: (■) 0 mM; (●) 1 mM; (▲) 2 mM; (▼) 5 mM. (A) 30°C. (B) 37°C. The error bars represent the standard deviations calculated from the data obtained from 3 independent experiments.

rapidly once after NaBu treatment, and increased sharply toward the end of cultivation. However, its activity gradually increased at 30°C, indicating that cellular membrane integrity was improved by lowering culture temperature. At the termination of culture, the LDH activities at 37°C with 0 ~ 5 mM NaBu addition were 212.68, 282.46, 344.43, and 228.44 U/L, respectively, while those at 30°C were 112.58, 164.19, 234.29, and 302.58 U/L, respectively.

3.3. The combined effect of NaBu and low temperature on cell cycle distribution

To further investigate cell growth, we studied the distribution of the cells regarding their cell cycle phase. As the protein may be preferentially produced during G1 cell cycle phase [28], we stated the percentage of G1 phase cells in total cells (Fig. 5). An ~8% increase in the proportion of cells at G1 phase was observed with a temperature shift from 37 to 30°C. However, there was no significant difference among cell cultures with 0 ~ 5 mM NaBu addition at either 37 or 30°C. These results demonstrated

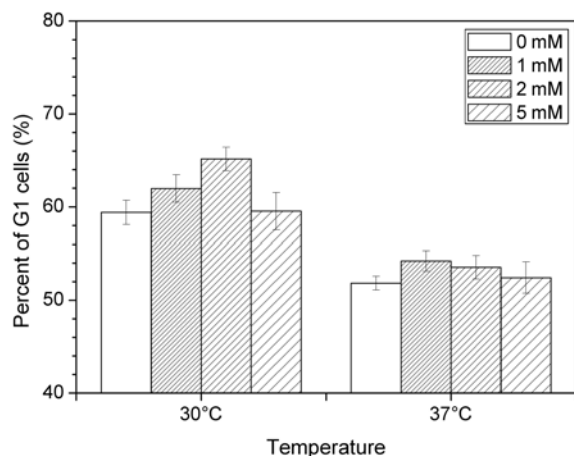


Fig. 5. The combined effect of NaBu addition and low culture temperature on percentage of cells at G1 phase. The error bars represent the standard deviations calculated from the data obtained from 3 independent experiments.

that lowering culture temperature solely increased the percentage of G1 phase cells, while combining NaBu with low culture temperature did not further increase the percentage of G1 phase cells.

3.4. The combined effect of NaBu and low temperature on sialylation of antibody

To determine the combined effect of NaBu addition and low culture temperature on the quality of antibody produced by CHO cells, both the total sialic acid contents and glycoprotein Neu5Gc levels were investigated. As shown in Fig. 6, the total sialic acid content remained more or less at the same level among all culture conditions; however, NaBu treatment or lowering culture temperature did reduce Neu5Gc levels. The harvest Neu5Gc levels were lower when culture temperature was shifted from 37 to 30°C; for example, the Neu5Gc level decreased from 17.70 to 12.00% in the absence of NaBu. Moreover, Neu5Gc levels further decreased from 12.00 to 7.32% when 1 mM NaBu was added to the former culture at 30°C. Thus, NaBu addition in combination with low culture temperature was able to further decrease Neu5Gc levels, indicating an improvement in antibody quality.

3.5. The combined effect of NaBu and low temperature on biological activity of antibody

For therapeutic use of an antibody, biological activity is one of the most important criteria for development of an antibody production process. Therefore, we investigated in detail the combined effect of NaBu addition and low culture temperature on the biological activity of antibody. The biological activity was evaluated by the median effective dose (ED_{50}) test. Whereas, the data presented in Table

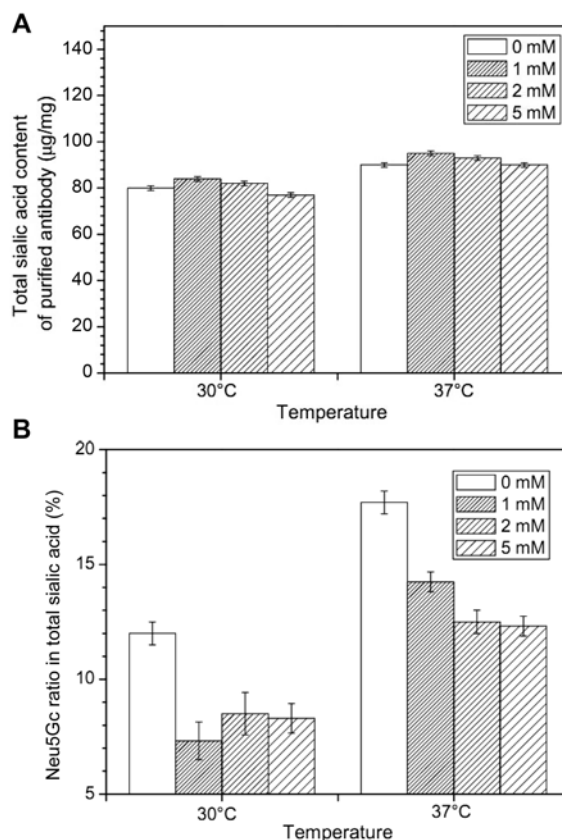


Fig. 6. The combined effect of NaBu addition and low culture temperature on the total sialic acid content and Neu5Gc levels of purified antibody. (A) Total sialic acid content. (B) Neu5Gc levels. The final value of total sialic acid content was calculated as a ratio of μg sialic acid per mg antibody. Neu5Gc level was calculated as a fraction of Neu5Gc in the total sialic acid of the antibody.

1 was calculated by comparison to the antibody standard and experimental values as a ratio of ED_{50} (standard): ED_{50} (sample), of which the ED_{50} of antibody standard was ~ 1.16 ng/mL . As shown in Table 1, there were no significant differences with regard to the value of ED_{50} (standard): ED_{50} (sample) in the culture subjected to either NaBu addition or temperature shift. These results demon-

Table 1. The combined effect of NaBu addition and low culture temperature on the biological activity of antibody

NaBu concentration (mM)	In vivo biological activity of antibody (%)	
	Culture temperature(°C)	
	30	37
0	84.34 ± 0.43	90.01 ± 0.90
1	83.23 ± 0.40	87.23 ± 1.02
2	83.34 ± 0.45	87.03 ± 0.89
5	80.23 ± 0.19	81.54 ± 1.23

The final value of biological activity of antibody was calculated by comparison to the antibody standard and experimental values as a ratio of ED_{50} (standard): ED_{50} (sample), of which the ED_{50} of antibody standard was ~ 1.16 ng/mL . Values are means $\pm$ SD.

strated that combining NaBu addition with low culture temperature did not deteriorate the quality of antibody produced by CHO cells.

4. Discussion

In an effort to increase the production of recombinant proteins in recombinant CHO cells, the effects of various environmental factors on cell growth and recombinant protein production have been extensively investigated [2-9, 16]. Studies regarding the effects of NaBu [6-14] or temperature [2-5] on recombinant protein production have been independently discussed in depth. However, to our best knowledge, the combined effect of NaBu addition and low culture temperature on antibody production in CHO cells had not yet been reported. Thus, this motivated us to systematically investigate their combined effect on the production, sialylation, and biological activity of antibody in CHO cells.

NaBu addition increased q_p at 37°C, whereas cells demonstrated a dramatic reduction in viable cell density at the same time. The opposing effects of NaBu on q_p and cell viability led to no elevation; and instead, a decline in final antibody concentration (Fig. 2D). This phenomenon was consistent with those observed in many CHO cell lines [10-14, 29]. Our application of lowering culture temperature prolonged culture period and further elevated q_p , which resulted in an enhancement of final antibody concentration. Additionally, q_p and final antibody concentration could be further enhanced by 60.4 and 66.3%, respectively, through the addition of 1 mM NaBu at 30°C. Therefore, an additional effect was exhibited when using NaBu and low temperature in combination.

One of the limitations of NaBu as a tool to improve recombinant protein productivity is that NaBu can lead to significant cell apoptosis, which consequently results in premature termination of production cultures [10-14]. In agreement with previous research [10-14], NaBu addition was found to induce apoptotic cell death in a dose-dependent manner (Fig. 3). Accordingly, to solve this problem, NaBu-induced anti-apoptotic engineering is an emerging area of research [10-14]. Reduction in culture temperature as an anti-apoptosis method was successfully applied in our study, though its effect was merely a delay rather than a complete inhibition of apoptosis. However, adoption of lowering culture temperature for an industrial-relevant process is acceptable due to its easy execution in a cell culture process. Thus, culture at low temperature in coordination with NaBu supplementation would be a promising method for future application.

Although the molecular mechanism responsible for the

effects of NaBu on increased q_p has not yet been clearly elucidated, one of the most convincing arguments is that NaBu could loosen chromatin packing as a result of histone hyperacetylation, through inactivation of histone deacetylase (HDAC) [30, 31]. Conversely, reduced culture temperature influences q_p in a different fashion. It has been suggested that lowering culture temperature causes a shift in the proportion of cells from S to G1, which is partially responsible for increased q_p [28, 32]. It is well known that cells at G1 phase do not need to devote cellular resources to biomass production, thus subsequently can be employed for recombinant protein synthesis. However, an analysis of cell cycle characteristics had not been previously conducted regarding the combined effects of NaBu addition and low culture temperature for correlation with cell cycle events. The obtained data demonstrated that NaBu addition did not increase the accumulation of cells at G1 phase, whereas combining NaBu addition with low culture temperature elevated the proportion of cells at G1 phase (Fig. 5).

In an effort to apply NaBu and low temperature to commercial antibody production in CHO cells, product quality in regard to glycoprotein sialylation and biological activity is a concern. Documented reports have shown that culture process variables can considerably affect the sialic acid levels of recombinant proteins [22, 23]. A higher sialylation of biopharmaceutical proteins is advantageous because glycoproteins lacking terminal sialic acid are generally ineffective *in vivo* because of rapid clearance by asialoglycoprotein receptors [33, 34]. In our study, no changes were observed in the total sialic acid content of antibody obtained from the cultures with NaBu addition or a temperature shift. However, the amount of Neu5Gc as a percentage of total sialic acids did decrease. Sialic acids are a class of monosaccharide consisting of Neu5Ac and its derivatives, especially Neu5Gc. Neu5Gc is generated by hydroxylation of CMP-Neu5Ac to CMP-Neu5Gc, catalyzed by CMP-Neu5Ac hydroxylase (CMAH) in most animal species [35]; however, a significant exception is humans, which cannot produce Neu5Gc due to a genetic mutation in the human CMAH gene. Despite traces of Neu5Gc being detected in human serum due to the assimilation of dietary sources such as red meat and milk [20, 36], Neu5Gc containing molecules can elicit immunogenicity issues in humans [21]. Thus, understanding factors that affect Neu5Gc in manufacturing is of significant importance when assessing the equivalency of different cell culture parameters. Our results of lower Neu5Gc content in the culture with NaBu addition are similar to those of Li *et al.* [37]. Furthermore, the simultaneous application of NaBu addition and low culture temperature further decreased the Neu5Gc level, which would not be expected to lead to an

increase in immunogenicity.

The altered expression of Neu5Gc does reveal some clues as to how cells respond to the synergistic effect of NaBu addition and low culture temperature regarding sialylation. The donor of Neu5Gc, CMP-Neu5Gc, is synthesized in the cytosol from CMP-Neu5Ac by the enzyme CMP-Neu5Ac hydroxylase, in the presence of NADH [38,39]. However, the supply of NADH may become limited due to the decrease in specific glucose and glutamine consumption rate under NaBu-supplemented and low temperature culture conditions [40]. In fact, in our experimental regime, the specific glucose and glutamine consumption rates were significantly reduced, especially under NaBu-supplemented and low culture temperature (30°C) dual-stress conditions (data not shown). Thus, it could be concluded that combining NaBu addition with low culture temperature decreased Neu5Gc levels due to a limited supply of NADH.

5. Conclusion

NaBu has been widely reported to increase the specific productivity of recombinant proteins; however, there are few papers reporting that NaBu significantly increases the final protein concentration, because NaBu can also induce apoptotic cell death. Our successful use of lowering culture temperature in combination with NuBu addition not only significantly increased the q_p but also the final concentration of antibody in CHO cells. In addition, combining NaBu addition with low culture temperature did not deteriorate the protein quality with respect to either sialylation or biological activity. Therefore, lowering culture temperature in combination with NaBu addition would be a promising method for future application to achieve both higher q_p and higher final antibody concentration, while not comprising either the sialic acid content or biological activity.

References

1. Wurm, F. M. (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat. Biotechnol.* 22: 1393-1398.
2. Furukawa, K. and K. Ohsuye (1998) Effect of culture temperature on a recombinant CHO cell line producing a C-terminal α -amidating enzyme. *Cytotechnol.* 26: 153-164.
3. Fogolin, M. B., R. Wagner, M. Etcheverrigaray, and R. Kratje (2004) Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. *J. Biotechnol.* 109: 179-191.
4. Yoon, S. K., J. Y. Song, and G. M. Lee (2003) Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese Hamster Ovary cells. *Biotechnol. Bioeng.* 82: 289-298.
5. Ahn, W. S., J. J. Jeon, Y. R. Jeong, S. J. Lee, and S. K. Yoon (2008) Effect of culture temperature on erythropoietin production and glycosylation in a perfusion culture of recombinant CHO cells. *Biotechnol. Bioeng.* 101: 1234-1244.
6. Virginie, M. B., S. Hisiger, C. Durand, M. Perrier, and M. Jolicoeur (2009) Na-butyrate sustains energetic states of metabolism and t-PA productivity of CHO cells. *J. Biosci. Bioeng.* 108: 160-167.
7. Mimura, Y., J. Lund, S. Church, S. Dong, J. Li, M. Goodall, and R. Jefferis (2001) Butyrate increases production of human chimeric IgG in CHO-K1 cells whilst maintaining function and glycoform profile. *J. Immunol. Methods.* 247: 205-216.
8. Yoon, S. K., J. K. Hong, and G. M. Lee (2004) Effect of simultaneous application of stressful culture conditions on specific productivity and heterogeneity of erythropoietin in Chinese Hamster Ovary cells. *Biotechnol. Prog.* 20: 1293-1296.
9. Sung, Y. H., Y. J. Song, S. W. Lim, J. Y. Chung, and G. M. Lee (2004) Effect of sodium butyrate on the production, heterogeneity and biological activity of human thrombopoietin by recombinant Chinese hamster ovary cells. *J. Biotechnol.* 112: 323-335.
10. Kim, N. S. and G. M. Lee (2000) Overexpression of *bcl-2* inhibits sodium butyrate-induced apoptosis in Chinese hamster ovary cells resulting in enhanced humanized antibody production. *Biotechnol. Bioeng.* 71: 184-193.
11. Kim, N. S. and G. M. Lee (2001) Inhibition of sodium butyrate-induced apoptosis in recombinant Chinese Hamster Ovary cells by constitutively expressing antisense RNA of caspase-3. *Biotechnol. Bioeng.* 78: 217-228.
12. Sung, Y. H., S. J. Hwang, and G. M. Lee (2005) Influence of down-regulation of caspase-3 by siRNAs on sodium-butyrate-induced apoptotic cell death of Chinese hamster ovary cells producing thrombopoietin. *Metab. Eng.* 7: 457-466.
13. Sung, Y. H., J. S. Lee, S. H. Park, J. Koo, and G. M. Lee (2007) Influence of co-down-regulation of caspase-3 and caspase-7 by siRNAs on sodium butyrate-induced apoptotic cell death of Chinese hamster ovary cells producing thrombopoietin. *Metab. Eng.* 9: 452-464.
14. Oh, H. K., M. K. So, J. Yang, H. C. Yoon, J. S. Ahn, J. M. Lee, J. T. Kim, J. U. Yoo, and T. H. Byun (2005) Effects of *N*-Acetylcysteine on butyrate-treated Chinese Hamster Ovary cells to improve the production of recombinant human interferon- β -1a. *Biotechnol. Prog.* 21: 1154-1164.
15. Chuppa, S., Y. S. Tsai, S. Yoon, S. Shackelford, C. Rozales, R. Bhat, G. Tsay, C. Matanguihan, K. Konstantinov, and D. Naveh (1997) Fermentor temperature as a tool for control of high-density perfusion cultures of mammalian cells. *Biotechnol. Bioeng.* 55: 328-338.
16. Kaufmann, H., X. Mazur, M. Fussenegger, and J. E. Bailey (1999) Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. *Biotechnol. Bioeng.* 63: 573-582.
17. Kim, N. S., K. H. Chang, B. S. Chung, S. H. Kim, J. H. Kim, and G. M. Lee (2003) Characterization of humanized antibody produced by apoptosis-resistant CHO cells under sodium butyrate-induced condition. *J. Microbiol. Biotechnol.* 13: 926-936.
18. Sethuraman, N. and T. A. Stadheim (2006) Challenges in therapeutic glycoprotein production. *Curr. Opin. Biotechnol.* 17: 341-346.
19. Lamari, F. N. and N. K. Karamanos (2002) Separation methods for sialic acids and critical evaluation of their biologic relevance. *J. Chrom. B.* 781: 3-19.
20. Irie, A., S. Koyama, Y. Kozutsumi, T. Kawasaki, and A. Suzuki (1998) The molecular basis for the absence of N-glycolylneuraminic acid in humans. *J. Biol. Chem.* 273: 15866-15871.
21. Varki, A. (2001) N-glycolylneuraminic acid deficiency in humans. *Biochimie.* 83: 615-622.

22. Lipscomb, M. L., L. A. Palomares, V. Hernandez, O. T. Ramirez, and D. S. Kompala (2005) Effect of production method and gene amplification on the glycosylation pattern of a secreted reporter protein in CHO cells. *Biotechnol. Prog.* 21: 40-49.
23. Zanghi, J. A., A. E. Schmelzer, T. P. Mendoza, R. H. Knop, and W. M. Miller (1999) Bicarbonate concentration and osmolality are key determinants in the inhibition of CHO cell polysialylation under elevated pCO₂ or pH. *Biotechnol. Bioeng.* 65: 182-191.
24. Renard, J. M., R. Spagnoli, C. Mazier, M. F. Salles, and E. Mandine (1988) Evidence that monoclonal antibody production kinetics is related to the integral of viable cells in batch systems. *Biotechnol. Lett.* 10: 91-96.
25. Gawlitzek, M., T. Ryll, J. Lofgren, and M. B. Sliwowski (2000) Ammonium alters N-glycan structures of recombinant TNFR-IgG: Degradative versus biosynthetic mechanisms. *Biotechnol. Bioeng.* 68: 637-646.
26. Anumula, K. R. (1995) Rapid quantitative determination of sialic acids in glycoproteins by high-performance liquid chromatography with a sensitive fluorescence detection. *Anal. Biochem.* 230: 24-30.
27. Wagner, A., A. Marc, J. M. Engasser, and A. Einsele (1992) The use of lactate dehydrogenase (LDH) release kinetics for the evaluation of death and growth of mammalian cells in perfusion reactors. *Biotechnol. Bioeng.* 39: 320-326.
28. Sunley, K. and M. Butler (2010) Strategies for the enhancement of recombinant protein production from mammalian cells by growth arrest. *Biotechnol. Adv.* 28: 385-394.
29. Hendrick, V., P. Winnepenninckx, C. Abdelkafi, O. Vandeputte, M. Cherlet, T. Marique, G. Renemann, A. Loa, G. Kretzmer, and J. Werenne (2001) Increased productivity of recombinant tissular plasminogen activator (t-PA) by butyrate and shift of temperature: A cell cycle phases analysis. *Cytotechnol.* 36: 71-83.
30. Jiang, Z. and S. T. Sharfstein (2008) Sodium butyrate stimulates monoclonal antibody over-expression in CHO cells improving gene accessibility. *Biotechnol. Bioeng.* 100: 189-194.
31. Yee, J. C., M. L. Gatti, R. J. Philp, M. Yap, and W. S. Hu (2008) Genomic and proteomic exploration of CHO and Hybridoma cells under sodium butyrate treatment. *Biotechnol. Bioeng.* 99: 1186-1204.
32. Moore, A., J. Mercer, G. Dutina, C. J. Donahue, K. D. Bauer, J. P. Mather, T. Etcheverry, and T. Ryll (1997) Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultures. *Cytotechnol.* 23:47-54.
33. Takeuchi, M., N. Inoue, T. W. Strickland, M. Kubota, M. Wada, R. Shimizu, S. Hoshi, H. Kozutsumi, S. Takeuchi, and A. Kobata (1989) Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA.* 86: 7819-7822.
34. Yamaguchi, K., K. Akai, G. Kawanishi, M. Ueda, S. Masuda, and R. Sasaki (1991) Effect of site-directed removal of N-glycosylation sites in human erythropoietin on its production and biological properties. *J. Biol. Chem.* 266: 20434-20439.
35. Malykh, Y. N., L. Shaw, and R. Schauer (1998) The role of CMP-N-acetylneuraminic acid hydroxylase in determining the level of N-glycolylneuraminic acid in porcine tissues. *Glycoconj. J.* 15: 885-893.
36. Tangvoranuntakul, P., P. Gagneux, S. Diaz, M. Bardor, A. Varki, and E. Muchmore (2003) Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc. Natl. Acad. Sci. USA.* 100: 12045-12050.
37. Borys, M. C., N. G. Dalal, N. R. Abu-Absi, S. F. Khattak, Y. Jing, Z. H. Xing, and Z. J. Li (2010) Effects of culture conditions on N-Glycolylneuraminic acid (Neu5Gc) content of a recombinant fusion protein produced in CHO cells. *Biotechnol. Bioeng.* 105: 1048-1057.
38. Shaw, L. and R. Schauer (1988) The biosynthesis of N-glycolylneuraminic acid occurs by hydroxylation of the CMP-glycoside of N-acetylneuraminic acid. *Biol. Chem. Hoppe Seyler* 369: 477-486.
39. Shaw, L. and R. Schauer (1989) Detection of CMP-N-acetylneuraminic acid hydroxylase activity in fractionated mouse liver. *Biochem. J.* 263: 355-363.
40. Grampp, G. E., T. K. Blumen, K. Kelly, P. Derby, L. A. Sleeman, and D. Hettwer (1994) Environmental control of sialic acid composition in glycoproteins secreted by mammalian cells. *Presented at the Cell Culture Engineering IV meeting.* San Diego, CA.