

## The effect of different media composition and temperatures on the production of recombinant human growth hormone by CHO cells

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### Abstract

Cell lines derived from mammalian are dominant systems for the production of recombinant therapeutic proteins because of their capacity for correct protein folding, assembly and post-translational modification. In the search of an efficient method for the production of a recombinant protein using animal cell culture, we investigated the effects of different treatment including fetal calf serum concentration, glycerol and culture temperature on a Chinese hamster ovary (CHO) cell line on the production of recombinant human growth hormone (rhGH) and recombinant Chinese hamster ovary (rCHO) viability. The GH production was assessed using ELISA and western blotting methods and cell viability was determined by flow cytometry. The production of recombinant protein increased by 2-fold when stimulatory chemical such as glycerol was added in two stages, first cells were cultured without glycerol for a period of time in order to obtain enough cell density and then glycerol was added to achieve high specific productivity.. Moreover, glycerol addition increased cell viability. Low culture temperature (below 37°C) led to enhanced cellular productivity of the rhGH by 3-fold but decreased cell viability. These findings indicate that quite simple factors such as culture temperature and addition of simple chemicals may lead to the improvement of industrial process for the production of recombinant proteins such as rhGH.

**Keywords:** Chinese hamster ovary; rhGH productivity; Culture temperature; Glycerol; FCS; Cell viability

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### INTRODUCTION

Recombinant therapeutic proteins have changed the face of modern medicine and they continue to provide new and effective therapies for numerous diseases ranging from cancers to infertility (1). About 60-70% of all recombinant pharmaceutical proteins are produced in mammalian cells. Many of these proteins are expressed in immortalized Chinese hamster ovary (CHO) cells, but other cell lines, such as mouse myeloma (NSO), baby hamster kidney (BHK) and human embryonic kidney (HEK-293) cells have gained approval for recombinant protein production (2). CHO cell culture is becoming increasingly important for production of recombinant therapeutic proteins with respect to rapid growth rate, high production of recombinant proteins, and high stability of foreign gene expression (3). Increasing recombinant protein yields, and thereby, reducing production costs are a major

biotechnological targets (4). Since the productivity of mammalian cells is low as compared with that of prokaryotic hosts, there are some strategies in order to increase recombinant protein production in CHO cells, such as regulation of culture temperature (5), addition of different chemicals (6), bioreactor engineering (7), and inhibition of cell apoptosis (8, 9). Culture temperature is one of the most important parameters that could be optimized for increasing recombinant protein yields. Animal cells such as CHO are the most commonly cultured at 37°C to imitate the body environment. Since the culture temperature, as well as the medium pH (10-12) and dissolved oxygen (DO) (12-14) would affect some important cellular conditions such as growth, viability and protein synthesis, they should be studied to determine the suitable process for protein production (15). Glycerol is one of the chemical components in medium that aids in forming a solvent shell around a protein

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molecule as a protein stabilizer, and increases the viscosity of a solution for prevention of protein association (16, 17). Rodriguez and coworker also report that glycerol could stabilize the secreted interferon- $\beta$  by CHO cells and result in reduced aggregation (18). Human growth hormone (hGH) is a small, single chain peptide of 191 residues, with approximately 50% of the residues in  $\alpha$ -helical conformation. It is produced and secreted by the anterior pituitary gland and is responsible for many effects on growth, development, immunity, and metabolism (19). The first therapeutic use of exogenous hGH extracted from human pituitary glands for growth hormone deficiency was reported by Raben (20) and subsequently administered to large numbers of patients for growth hormone deficiency. When association between hGH preparations and Creutzfeldt-Jakob disease (CJD) was established in 1985, the use of pituitary-derived hGH was rapidly discontinued (21). In the present study, we examined and compared the effect of different temperatures, glycerol concentrations and different concentration of fetal calf serum on viability and recombinant protein secreted into the medium of a recombinant CHO (rCHO) cell line during rhGH production.

## MATERIALS AND METHODS

### *Cell line and culture media*

The rCHO cells producing recombinant human growth hormone (rhGH) were used in this study. The CHO cells were purchased from Royan Institute, Isfahan, Iran. They were established by transfection of a vector (pSeqTag, Invitrogen, USA) containing human growth hormone gene. The stable rCHO cell line producing rhGH was selected at 400  $\mu$ g/ml gentamicin (Sigma, USA) and cultivated in Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/Ham's F12) (Gibco, USA) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco, USA), 1% Penicillin/Streptomycin (Gibco, USA) and 200  $\mu$ g/ml gentamicin. Cells were incubated in a humidified incubator, with 5% CO<sub>2</sub> at 37°C.

### *Effects of FCS, glycerol and temperature*

rCHO were weaned off from 10% (v/v) FCS by stepwise decreasing the serum

concentration by 50% in every other passage. In this way the serum concentration could be easily reduced from 10% (v/v) to 1.25% (v/v). The influence of different concentrations of glycerol on rhGH production was also examined. For these experiments, glycerol was added to medium to a final volume of 0.5 to 2.0% simultaneously with inoculated cells (day 0). Additionally, cells were supplied with 1% glycerol in 2 and 4 days following inoculation. The stock solution of glycerol was 50% (v/v in water) and was sterilized by autoclave. To study the effect of culture temperature on rhGH production, cells were incubated at 31°C, 34°C and 37°C, respectively. In all treatments, the basal medium was DMEM/Ham's F12 supplemented with 10% FCS, 1% P/S and 200  $\mu$ g/ml gentamicin. For every test condition, cells were inoculated at  $2.5 \times 2 \times 10^5$  cells/ml and each experiment was repeated in triplicate in 12-well tissue culture plates (The cell density and viability were determined by trypan blue exclusion). At day 8, media were collected by centrifugation of cells (1500 rpm 5 min) and supernatants were stored at -20°C until they were analyzed for rhGH concentrations using enzyme-linked immunosorbent assay (ELISA) kits.

### *ELISA assay*

The concentration of secreted rhGH in the medium was quantified by ELISA using a commercially available kit (Radim, Italy) according to manufacturer's protocols. Briefly, 20  $\mu$ l of each samples was added to the pre-coated wells, then 200  $\mu$ l of enzyme-linked conjugates were dispensed into each well and incubated for 60 min at 37°C. 200  $\mu$ l freshly prepared substrate solution was added to the wells and incubated for 20 min at 37°C. Between each step, wells were washed three times with washing buffer. After the reaction stopped, the absorbance was measured at 450 nm with an automated ELISA reader. The concentration of rhGH was calculated based on the hGH ELISA standard curve.

### *Propidium iodide staining for cell viability assay*

Cells ( $5 \times 10^5$ /ml) were cultured in T25 flask in total volume of 5 ml media. After 3 days, supernatant was removed and cells were pelleted

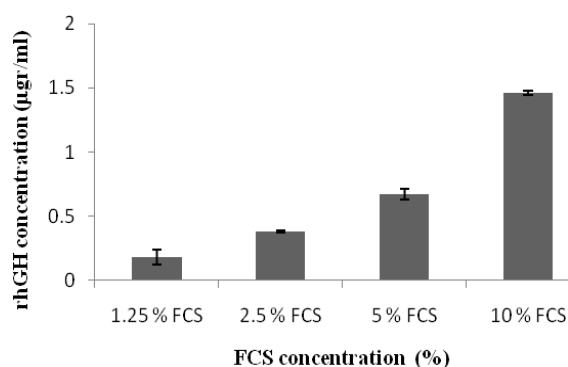
by centrifugation at 400 g (1500 rpm) for 10 min. Then, cells were resuspended in 1ml PBS and 100  $\mu$ l of cell suspension was incubated with 5 $\mu$ l of 5 mg/ml Propidium iodide (PI, Sigma, USA) at the dark at 37°C for 10 min. Cells were washed with 2ml PBS and centrifuged at 600 g for 6 min at room temperature. The cell pellet was resuspended in 1ml PBS and cell viability was measured on the FL-2 channel by flow cytometer (Partec, Germany).

## RESULTS

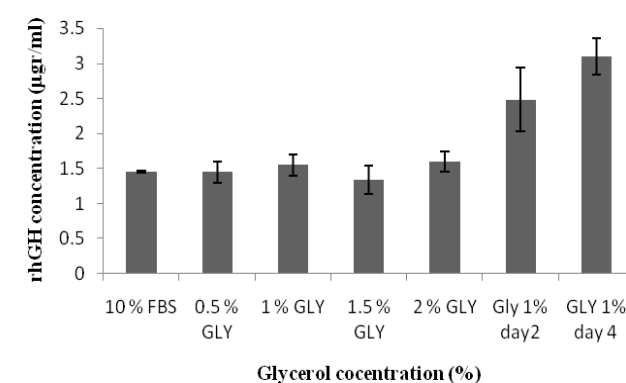
In this study, the effect of FCS concentration, glycerol addition and different temperatures were assessed on rhGH production and cell viability.

### Effects of FCS on rhGH production

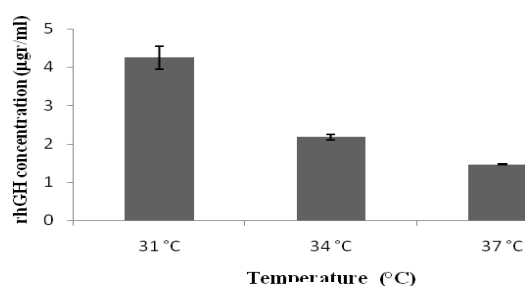
Effects of different concentrations (1.25, 2.5, 5 and 10 V/V%) of fetal calf serum on production of rhGH were studied. The FCS levels showed a dramatic effect on rhGH production as higher concentrations of FCS caused higher levels of rhGH production in a dose dependent manner (Fig. 1A).



**A**

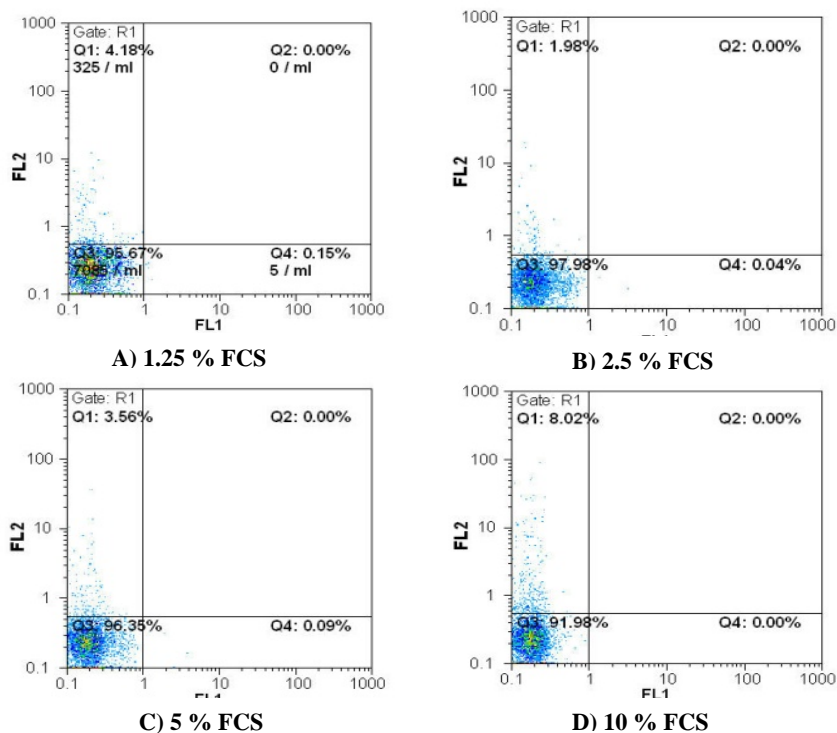


**B**

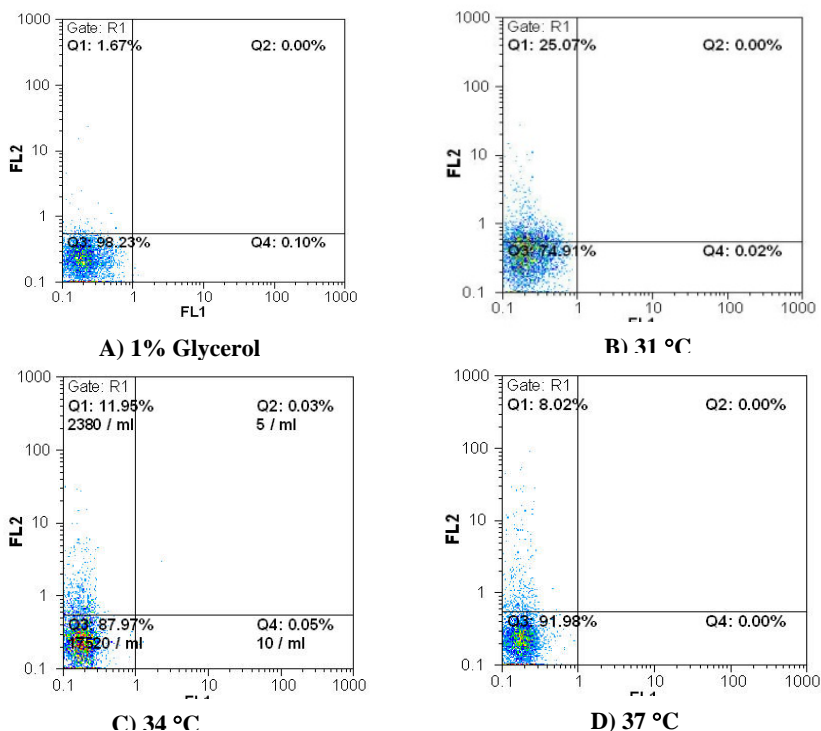


**C**

**Fig. 1.** The effect of different treatments on the concentration of rhGH, produced by CHO. In all treatments, at day 8, media were collected by centrifugation of the cells. The level of rhGH assayed by ELISA; A: effect of FCS concentration, B: effect of glycerol addition and C: effect of temperature.



**Fig. 2.** Effect of FCS concentration on the cell viability. After growth in different FCS concentration, cell pellet stained with propidium iodide and cell viability was measured on the FL-2 channel by flow cytometer.



**Fig. 3.** Effect of glycerol and different temperature on cell viability. After growth in different condition, cell pellet stained with Propidium iodide and cell viability was measured on the FL-2 channel by flow cytometer.

The viability of cells also was examined at different media conditions. As demonstrated in Fig. 2, rCHO cells viability was higher when FCS was set at 5% and then decreased compared to control condition (10% FCS and 37°C). Whereas addition of 1%

glycerol increased cell viability and decreased FL2 by 1.67%, lowering culture temperature to 34 and 31°C, decreased cell viability by 12 and 25%, respectively (Fig. 3). The viability percentages of different treatments are expressed in Table 1.

**Table 1:** Comparison between cell viability (FL2 percentage) under different conditions

Treatment	Death percentage
FCS 1.25%	4.18
FCS 2.5%	1.98
FCS 5%	3.56
FCS 10%	8.02
Glycerol 1%	1.67
Temperature 31°C	25.07
Temperature 34°C	11.95
Temperature 37°C	8.02

## DISCUSSION

In the present study, we demonstrated that using different media compositions, simple additives and changing temperatures could easily enhance production of recombinant protein by CHO cells.

Serum is an important supporting and promoting cell growth component in cell culture medium. The gradual reduction of serum concentration increases the chances of successful adaptation of cells to a serum-free environment. It is reasonable that increased serum concentrations may increase the amount of rhGH production. In contrast, it was shown in this study that, an increase in FCS may enhance cell death through the reduction of nutrient and elementary factors in medium which takes place following cell proliferation. On the other hand, the concentration of FCS seems to be critical for cell viability. we noticed that as the concentration of FCS decreased from 2.5% (v/v) to 1.25% (v/v) the cell mortality increased. High concentration of serum in the medium can interfere with recombinant protein purification process.

We found that simple process variables, such as low temperature, enhances the cellular productivity of rhGH. Our result is consistent with several other published studies (15, 22). Weidemann and coworkers (23) have described that a low culture temperature (below 37°C) for a recombinant BHK cell line suppressed the cell growth and glucose consumption, but did not affect the cellular productivity of recombinant antithrombin III. Reduced culture temperature is hypothesized to improve recombinant protein production via cell arrest. Also, it has been observed that, at lower culture temperatures, the growth rate, glucose consumption, lactate production, and

ammonia production are reduced. Increased protein production rates are not the only benefit attributed to lower culture temperatures, product quality has also been observed to be temperature dependent. Higher product quality at lower culture temperatures has been attributed to lower protease activity and lower activity of other deleterious temperature-sensitive enzymes (22).

We found that at low temperature, cell viability was decreased, whereas Furukawa and Yoon showed that rCHO cell culture at low temperature improved cell viability and reduced contamination by endogenous CHO cell proteins for a longer period of time (4, 15). Ludwig and coworker reported that, for an anchorage-dependent BHK cell line, the growth rate decreased by 40% for cells cultivated at 33°C compared to cells grown at 37°C (22). In contrast, Ducommun and coworker observed nearly identical growth rates for CHO cells maintained in a packed bed cultured at 37 and 33.5°C (24). Clark and coworker indicated that CHO cell viabilities are not significantly different, regardless of the culture temperature (22). It is possible that the slowly ramped temperature profile did not significantly alter the cell metabolism, as compared to a one step change in temperature. These results indicated that a gradual decrease of temperature does not always result in increased cell viability.

Liu and coworker demonstrated that addition of 1% glycerol had the highest stimulatory effect on macrophage colony-stimulating factor (M-CSF) production (8). They demonstrated the addition of glycerol could increase the M-CSF production but inhibit the cell proliferation, and then a two stage process was developed in the next sections to maximize the production of M-CSF by CHO cells. The strategy is to culture the cells without glycerol for a period of time in order to obtain enough cell density and then glycerol is added to achieve high specific productivity.

Glycerol promotes cystic fibrosis transmembrane conductance regulator protein folding and enhances its synthesis in mouse embryonic fibroblast cell line (NIH 3T3) (25). Additionally, glycerol aids the formation of a solvent shell around a protein molecule as a

protein stabilizer, and increases the viscosity of a solution for prevention of protein association (16, 17). Rodriguez and coworker also report that glycerol could stabilize the secreted interferon by CHO cells and result in reduced aggregation (18).

Brown and coworker claims that glycerol acts as a chemical chaperone and promotes recombinant protein synthesis in NIH 3T3 cells (25). Glycerol is also known to stabilize proteins against chemical and thermal denaturation (26), increase the refolding yield of proteins and prevents human interferon aggregation during synthesis (8).

### CONCLUSION

In conclusion, these findings will greatly contribute to a more economic industrial process for the production of rhGH. As a result, recombinant protein yields can be increased significantly by lowering the culture temperature and supplementing the cell culture medium with glycerol after cell growth. Lowering culture temperature, which is easily executed in cell culture processes, has become a popular method for increasing the production levels of rCHO cells in commercial processes.

### ACKNOWLEDGMENT

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### REFERENCES

- Jayapal KP, Wlaschin KF, Hu W, Yap MGS. Recombinant protein therapeutics from CHO cells- 20 years and counting. *Chem Eng Prog.* 2007;103:140.
- Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat biotechnol.* 2004;22:1393-1398.
- Ryu JH, Kim MS, Lee GM, Choi CY, Kim BS. The enhancement of recombinant protein production by polymer nanospheres in cell suspension culture. *Biomaterials.* 2005;26:2173-2181.
- Yoon SK, Hong JK, Choo SH, Song JY, Park HW, Lee GM. Adaptation of Chinese hamster ovary cells to low culture temperature: cell growth and recombinant protein production. *J biotechnol.* 2006;122:463-472.
- Fox SR, Patel UA, Yap MGS, Wang DIC. Maximizing interferon production by chinese hamster ovary cells through temperature shift optimization: Experimental and modeling. *Biotechnol bioeng.* 2004;85:177-184.
- Kim DY, Lee JC, Chang HN, Oh DJ. Effects of supplementation of various medium components on Chinese hamster ovary cell cultures producing recombinant antibody. *Cytotechnology.* 2005;47:37-49.
- Nienow AW. Reactor engineering in large scale animal cell culture. *Cytotechnology.* 2006;50:9-33.
- Liu CH, Chen LH. Enhanced recombinant M-CSF production in CHO cells by glycerol addition: model and validation. *Cytotechnology.* 2007;54:89-96.
- Arden N, Betenbaugh M. Regulating apoptosis in mammalian cell cultures. *Cytotechnology.* 2006;50:77-92.
- Borys MC, Linzer DIH, Papoutsakis ET. Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells. *Nat Biotechnol.* 1993;11:720-724.
- Hosoi S, Murosumi K, Sasaki K, Satoh M, Miyaji H, Hasegawa M, *et al.* Optimization of cell culture conditions for G-CSF (granulocyte-colony stimulating factor) production by genetically engineered Namalwa KJM-1 cells. *Cytotechnology.* 1991;7:25-32.
- Ozturk SS, Palsson BO. Growth, metabolic, and antibody production kinetics of hybridoma cell culture: 2. Effects of serum concentration, dissolved oxygen concentration, and medium pH in a batch reactor. *Biotechnol prog.* 1991;7:481-494.
- Reuveny S, Velez D, Macmillan J, Miller L. Factors affecting cell growth and monoclonal antibody production in stirred reactors. *J immunol methods.* 1986;86:53-59.
- Lin AA, Kimura R, Miller WM. Production of tPA in recombinant CHO cells under oxygen limited conditions. *Biotechnol bioeng.* 1993;42:339-350.
- Furukawa K, Ohsuye K. Effect of culture temperature on a recombinant CHO cell line producing a C-terminal -amidating enzyme. *Cytotechnology.* 1998;26:153-164.
- Mishra R, Seckler R, Bhat R. Efficient Refolding of Aggregation-prone Citrate Synthase by Polyol Osmolytes. *J Biol Chem.* 2005;280:15553.
- Fagain CO. Use of stabilizing additives in Stabilizing Protein Function Springer. 1997:70-79.
- Rodriguez J, Spearman M, Huzel N, Butler M. Enhanced Production of Monomeric Interferon by CHO Cells through the Control of Culture Conditions. *Biotechnol prog.* 2005;21:22-30.
- Rezaei M, Zarkesh-Esfahani SH. Optimization of production of recombinant human growth hormone in Escherichia coli. *J Res Med Sci.* 2012;17:681-685
- Raben M. Growth hormone. 2. Clinical use of human growth hormone. *The N Engl J Med.* 1962;266:282.

21. Catzel D, Lalevski H, Marquis CP, Gray PP, Van Dyk D, Mahler SM. Purification of recombinant human growth hormone from CHO cell culture supernatant by Gradiflow preparative electrophoresis technology. *Protein Express Purif.* 2003;32:126-134.
22. Clark KJR, Chaplin FWR, Harcum SW. Temperature Effects on Product Quality Related Enzymes in Batch CHO Cell Cultures Producing Recombinant tPA. *Biotechnol prog.* 2004;20:1888-1892.
23. Weidemann R, Ludwig A, Kretzmer G. Low temperature cultivation—a step towards process optimisation. *Cytotechnology.* 1994;15:111-116.
24. Ducommun P, Ruffieux PA, Kadouri A, Von Stockar U, Marison IW. Monitoring of temperature effects on animal cell metabolism in a packed bed process. *Biotechnol bioeng.* 2002;77:838-842.
25. Brown CR, Hong-Brown LQ, Biwersi J, Verkman AS, Welch WJ. Chemical chaperones correct the mutant phenotype of the F508 cystic fibrosis transmembrane conductance regulator protein. *Cell Stress Chaperone.* 1996;1:117-125.
26. Timasheff SN. Protein hydration, thermodynamic binding, and preferential hydration. *Biochemistry.* 2002;41:13473-13482.