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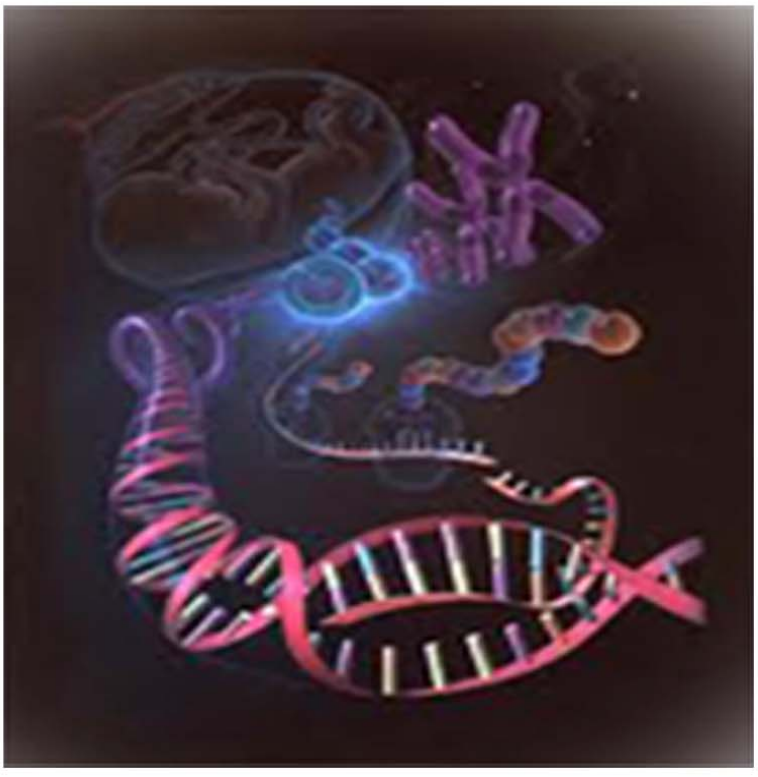

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Research Paper

INFLUENCE OF VARIOUS FACTORS ON ERYTHROPOIETIN PRODUCTION EXPRESSED IN CHINESE HAMSTER OVARY CELLS

Indrapal Reddy Addela[1*], Ravinder Rudravaram[2], Lakshmi Narasu M[3] and Ramesh B[3]

Corresponding Author:* ***Ravinder Rudravaram,* ✉ *ravinder.dr@gmail.com*

The erythropoietin was expressed in Chinese Hamster Ovary (CHO) cells and culture techniques were developed, purification process was developed. Different culture conditions were tried to evaluate the influence of various factors on erythropoietin production expressed in Chinese Hamster Ovary Cells. The productivity was checked at different harvests and found that the EPO expression was stable upto 10 harvest, sialidase activity was checked, effect of addition of sodium butyrate was studied on the productivity of Erythropoietin (EPO).

Keywords: Chinese Hamster Ovary cells, Erythropoietin, Glycopeptides, Sialic acids

INTRODUCTION

Erythropoietin (EPO) is 30 kDa glycoprotein hormone stimulator of erythropoiesis produced in the fetal liver and subsequently in the adult kidney (Schuster *et al*., 1992). The protein back bone of the mature hormone consists of 165 aminoacids. Protein glycosylation is of paramount importance to the efficacy and manufacturing of therapeutic glycoproteins (Patrick Hossler *et al*., 2009). The glycan structures are important because they can effect many of the biological properties of the glycoprotein including pharmacokinetics, bioactivity , secretion, *in vivo* clearance, solubility, receptor recognition and antigenicity (Takeuchi *et al*., 1988). Glycosylation of the proteins takes on the form of oligo-saccharides attached to either the side chain of aspargine (N-linked) or serine/threonine (O-linked) with the former being the most prominent (Silva *et al*., 1996). Glycans have a very prominent role towards affecting therapeutic efficacy and determining the in vivo half life (Warren, 1993). Erythropietin promotes the proliferation and differentiation of erythroid precursors and leads to an increased expression of the antiapoptotic proteins (Elliott *et al*., 2003) and inhibition of apoptosis (Tilbrook and Klinken, 1999)) and by controlling the dynamic balance between erythropoiesis and erythrocyte loss in order to maintain red cell volume (Moritz *et al*., 1997; and Lacombe and Mateux, 1998).

[1] Sudershan Biotech, Hyderabad, India.

[2] Sowbhagya Biotech (P) Limited, Plot # 79, Phase - II, IDACherlapally, Nacharam, Hyderabad, Andhra Pradesh 500051, India.

[3] Department of Biotechnology, JNTUH, Hyderabad, India.

Although Chinese Hamster Ovary cells (CHO) cells are the most prevalent for producing glycoprotein therapeutics, the other cell lines Baby Hamster Kidney (BHK), Mouse myeloma (NSO) and Human retinal cells (PERC.6) (10) are being developed as high producing cell lines.

MATERIALS AND METHODS

Materials

All the equipments and instruments required for analysis or manufacturing are procured from reputed manufacturers. All the chemicals used were of analytical/reagent grade obtained from merck/sigma. All the biochemicals, enzymes and other required were obtained from Sigma/Bio-Rad/Invitrogen. Cell culture flasks/pipettes etc were purchased from Nunc/Corning. All the glassware used was procured from Borosil/ Schott Duran.

Methods

The erythropoietin gene was cloned into TOPO vector, the presence of gene was verified by sequencing. The plasmid containing EPO gene was transformed into CHO cells. The best expressing clone was selected, cell banks were made and characterized for identification, purity, cell viability, mycoplasma contamination etc and stored in liquid nitrogen.

One vial from working cell bank was taken and thawed in a water bath set at 37 degree centigrade and transferred 1 mL of cells into T75 flasks containing 30 mL of growth medium and incubated at 37°C, 5% CO_2 in a CO_2 incubator. After attaining about 90~95% confluence (about 48 h) the flask was trypsinized and subcultured into 5 T75 flaks containing growth media and incubated as before.

After attaining confluence one flask was used for checking the erythropoietin productivity at different passages (2,4,6,8 and 10). The erythropoietin productivity was checked by HPLC method using C8 column and mobile phase A (0.1% TFA in water) and Mobile Phase B (0.1% TFA in 90% acetonitrile and 10% Water for injection), flow used was gradient with 1 mL/ minute flow rate, UV set at 215 nm.

Second flask was used to check the erythropoietin productivity at different harvests. The growth media from the flask was discarded and flask was washed two-three times with sterile PBS and 30 mL of growth medium was added and incubated as before. After completion of 24 h the media was harvested and fresh media was added to the flask. This was repeated upto 10 harvests and the EPO productivity was tested by HPLC method.

The third flask was used check sialidase activity in harvests and use of $CuCl_2$ in order to reduce sialidase activity to produce high sialated EPO. $CuCl_2$ at different concentrations (5, 10, 15, 20ìM) was added to production media and incubated and harvests 1 to 6 were collected. Sialic acid content was estimated using N-acetyl neuraminic acid as standard. To 100µL of each standards and samples 1 mL of resorcinol reagent was added and incubated at 100°C for 30 min. Cooled on ice and 2 mL of a mixture of 12 volumes of butanol and 48 volumes of butyl acetate was added and mixed vigorously and allowed to separate 2 phases. The upper layer was removed and absorbance was measured at 580 nm. Final concentration was calculated.

In another study effect of sodium butyrate (shown to unbind the chromatin structure, thus making it more accessible to RNA polymerase and promoting mRNA replication) at different

concentrations (0.1,0.2,0.4 and 0.8M) was studied. The same cultureing process was carried as in the above processes.

RESULTS AND DISCUSSION

The cell line used for this study was well characterized and the two tier cell banking system was used for further expansion and manufacturing process. Master and working cell banks were characterized as per ICH guidelines.

EPO Productivity at Different Passages

One cryovial was taken from liquid nitrogen and thawed to room temperature using water bath previously set at 37°C and after complete thawing the cryovial was centrifuged at 10000 rpm for 5 min. The supernatant was discarded and 1 mL of fresh media was added and resuspended the pellet, cell count was taken by trypan blue stain. The cells were diluted to 10 x 10^6 cells and seeded into two T75 flasks containing growth medium and incubated at 37°C, 5% CO_2 until about 90~95% confluence was achieved (about 48 h). The spent media was discarded and flask was washed for 2-3 times with sterile phosphate buffer saline, pH 7.2. One flask was trypsinized used trypsin EDTA solution and cell count was taken and cells were diluted to 10 x 10^6 cells and seed into two T75 flasks containing growth medium and incubated at 37°C, 5% CO_2 until about 90~95 % confluence was achieved, To other flask 30 mL of fresh production media was added and incubated at 37°C, 5% CO_2 for 24±2 h. The spent media was harvested and the erythropoietin concentration was determined using HPLC method. The above process was done for 10 passages but only 2,4,6,8 and 10th passage was shown (Table 1). From the following we concluded that there was no decrease in productivity in mg/L and Productivity/ x10^6/24 h as number of passages increase which concludes that the cell line was stable up to 10 passages and is suitable for manufacturing process in large scale production.

Table 1: Average Productivity of EPO at Different Passages

Passages	Average Productivity (mg/L)	Cells
2	53	54×10^6
4	48	52.4×10^6
6	55	58×10^6
8	50	51×10^6
10	49	51×10^6

One vial of working cell bank was taken and sub cultured in the same manner as described above after reaching the confluence the growth media was discarded, cells were washed with PBS and 30 mL of growth media was added, after 24 h the media was collected and analyzed for EPO content. The same process was carried out to check productivity up to 10 harvests. From the following data we concluded that the productivity up to 6 harvests was good and then after the productivity was decreasing, this may be due to the decrease of cell number (Table 2). We know from our previous experience the EPO productivity depends on number of cells and EPO production is directly proportional to number of cells at different harvesting stages.

Table 2: Productivity of EPO at Different Harvests

Harvest	Productivity (mg/L)
1.	48
2.	53
3.	51
4.	47
5.	46
6.	44
7.	22
8.	18
9.	9.4
10.	4.3

The other experiment was carried out to study to reduce the sialidase activity in harvest in order to produce high sialated EPO. The same process is carried out up to 6 harvests except addition of 20 μM of $CuCl_2$ in production medium. In process analysis (cell morphology, cell count, and glucose estimation) was done and the cell condition was comparable to normal batches. After 6 harvests the cell count was 450±50 x 10^6 and average productivity up to 6 harvests was 47.69 mg/mL (Table 3). The sialic content was about 10.25 moles of sialic acids/mole of EPO against 10.18 in flasks which was not added with $CuCl_2$, there was little increase in sialic acid content but we could not conclude that this increase was due to addition of $CuCl_2$ which may be due analytical errors; we need to carry out some more experiments.

Table 3: Average Productivity of EPO at Different Harvests

Harvest	Productivity (mg/L)
Harvest 1	48.46
Harvest 2	47.83
Harvest 3	53.21
Harvest 4	51.76
Harvest 5	44.78
Harvest 6	40.12
Average Productivity 47.69 mg/ L	

When different concentrations of sodium butyrate was added to the culture there was an increase in the 14.3% productivity of erythropoietin as compared to normal if number of cells is taken into consideration. It may be worthwhile to add sodium butyrate to culture medium if the cost benefit ratio is taken into consideration.

Table 4: Effect of Sodium Butyrate on EPO Production

Average Productivity	Normal (mg/ml)	0.1mM SB (mg/ml)	% of Increase
Up to 5 Harvest	13.17	14.07	6.8
Average Cell count (million)	49.6	46.4	–
Productivity: mg/L per million cells			
Up to 5 Harvest	0.265	0.303	14.3

CONCLUSION

From the above experiments we concluded that the cell line developed for the production of EPO was working good and the cell line was stable upto 10 passages and the EPO production was good upto 6 harvests and the effect of CuCl2 on sialidase inhibition has to be established. The % productivity of erythropoietin was increased by addition of sodium butyrate.

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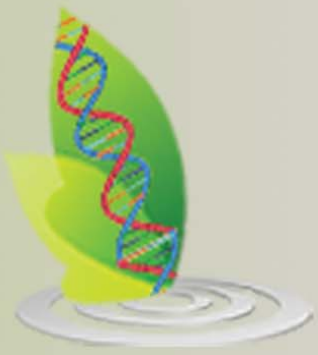

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Hyderabad, INDIA. Ph: +91-09441351700, 09059645577
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