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Glycoengineering of Darbepoetin-α in CHO-DG44 Cells through Overexpression of α-2,3-sialyl-transferase and CMP-sialic Acid Transporter

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ABSTRACT

Sialic acid plays a very important role in determining the circulation life span of glycoprotein in various organisms. Therefore, having a high content of sialic acid is needed by glycoprotein therapeutic agents to be able to function as desired. For example, Darbepoetin (DPO), the 5 N-linked erythropoietin showed higher bioavailability and efficacy compared to 3 N-linked erythropoietin. However, in the DPO production process, the molecular weight can vary and is highly dependent on the content of sialic acid and its production host. To improve the DPO sialic acid contents in our CHO-DG44 expressing DPO, we have engineered the cells through overexpression of α-2,3-sialyl-transferase (ST) and CMP-sialic acid transporter (CST). The DPO contained in the supernatant of the engineered cells was analyzed by Western blot and characterized by using PNGase-F or neuraminidase enzyme digestions. The results showed that, two clones, overexpressing ST or CST, were obtained. The clones showed higher molecular weight of DPO as compared to DPO expressed by the parental cells, yet retained the same protein backbone. The overexpression of these two genes does not affect cell growth. This suggests that may be these cells beneficial for therapeutic glycoproteins.

1. Introduction

Having demonstrated track record of providing reliable and efficious clinical benefit, the use of protein as a therapeutic drug has made the field of biopharmaceuticals grow very rapidly (Jayapal *et al.* 2007). However, structural instability in glycoproteins is still one of the biggest challenges in the therapeutic protein field. This is because improper conformation can negatively impact their final therapeutic efficacy (Walsh and Jefferis 2006; Varki *et al.* 2009). Oligosaccharides of proteins are often important for the recognition, signaling and interaction of events within and between cells and proteins, and have an important role in defining protein conformation pharmacological significance of glycosylation in therapeutic proteins (Li and d'Anjou 2009). Therefore, protein drugs used must be glycosylated properly in order to have optimal therapeutic efficacy. The presence of glycosylation in proteins is so great that more than two-thirds of the marketed therapies are glycoproteins (Jayapal *et al.* 2007).

Post-translational modification for addition of glycosyl residues is a crucial step for glycoproteins including human erythropoietin (hEPO), an important molecule for promoting erythrocyte generation (Walsh and Jefferis 2006). Native hEPO which has a variety of glycosyl conjugated with maximum of 3 N-link glycosyl groups presents as isoforms with broad bands around 37 kDa (Browne *et al.* 1986; Teh *et al.* 2011; Jez *et al.* 2013). As glycosylation is important to enhance its bioavailabilty, EPO molecule has been modified to bear 5 N-link glycosyl residues which known as darbepoetin (DPO) (Egrie and Browne 2001; Darling *et al.* 2002; Fan 2015; Falck *et al.* 2017). Containing higher sialic acid contents compared to native EPO (Macdougall 2002; Kianmehr *et al.* 2016), DPO has been proven to have longer *in vivo* half-life with better efficacy (Egrie *et al.* 2003).

In humans, the most common form of glycosylation occurs at asparagin residues through Asn-X-Thr/Ser

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recognition sequence (N-linked glycosylation) and at serin/threonine residues (O-linked glycosylation). The presence of terminal sialic acid in the glycoprotein molecule will help maintain EPO in the blood circulation by inhibiting its recognition by asialoglycopoprotein receptors hepatocytes (Egrie and Browne 2001; Arnold *et al.* 2007). Since, *in vivo* half-life of therapeutic glycoproteins, in particular, is determined mainly by sialic acid capping in the end sites of N-linked glycans by augmenting sialic acid content of the protein, higher *in vivo* half-life can be achieved (Ngantung *et al.* 2006).

There are several glycan molecules which modify glycoprotein including N-acetyl glucosamine (GlcAc), galactose and sialic acid (Neu5Ac) (Yin *et al.* 2015; Bydlinski *et al.* 2018). Sialic acid plays a role as teminal glycan residue to modify a glycoprotein (Fan 2015; Hossler and Khattak 2009; Goh and Ng 2017; Kwak *et al.* 2017). Thus, the more glycosyl branch presents in a glycoprotein, the more sialic acid caps it can obtain. As a biologic drug that has very high sialic acid content, recombinant human erythropoietin (rhEPO) has been widely studied. It has been reported previously that the increasing content of sialic acidin rhEPO molecules will increase its bioavailability (Su *et al.* 2010).

Sialic acid contents in an rhEPO molecule can be affected by the level of glycoconjugation enzymes in the producing host, such as sialyl-transferases (STs) which present in the golgi apparatus. STs play important roles in the biosynthesis of sialic acid (Neu5Ac) containing glycoproteins. ST mediates the addition of a cytidine 5'-monophosphate Neu5Ac (CMP-Neu5Ac) to a glycoconjugate terminated by a galactose, GlcAc or other Neu5Ac residues (Figure 1) (Takeuchisq *et al.* 1988; Zhang *et al.* 1998; Castilho *et al.* 2008; Jeong *et al.* 2008). As a substrate of STs, CMP-Neu5Ac is supplied from cytoplasm to the golgi through a CMP sialic acid transporter (CST) (Castilho *et al.* 2008). Therefore, CST is another important protein to promote sialylation of a glycoprotein. In the present study, to improve the DPO sialic acid contents, CHO-DG44 cells expressing DPO were transfected with -2,3-sialyl-transferase (ST) and CMP-sialic acid transporter (CST) genes. The results showed that, two candidate clones, overexpressing ST or CST, were obtained. The clones showed higher molecular weight of DPO as compared to DPO expressed by the parental cells.

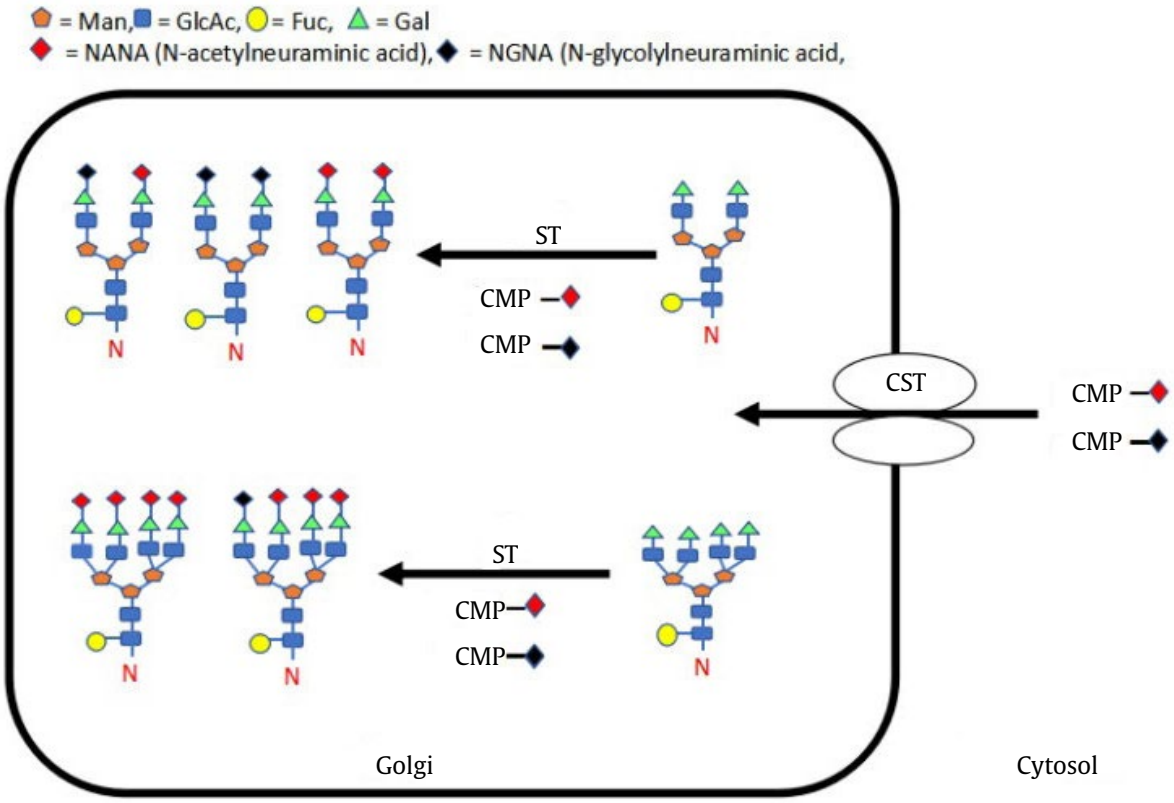

Figure 1. Post-translational modification of a glycoprotein in trans-golgi. Enzyme α-2,3-sialyl-transferase mediates addition of a CMP-sialic acid (NANA/NGNA) to a glycoconjugate terminated with galactose. CST: CMP-sialic acid transporter. ST: α 2,3-sialic acid transporter

2. Materials and Methods

2.1. Cell Culture and Reagents

CHO-DG44 cells were obtained from Life Technology (USA). The cells were transfected with bicistronic pOptiVEC plasmid harboring 5-N linked-erythropoietin DNA (CHO-DG44-DPO) and dihydrofolate reductase (DHFR) selection marker. The transfected CHO-DG44-DPO cells were grown in OptiCHO (Gibco, USA) complete medium containing 4 mM L-glutamine (Gibco, USA) in a shaker flask on an orbital shaking platform rotating at 130 rpm in a CO_2 incubator at 37°C with 5% CO_2. Plasmid DNA pJ-EPO encoding darbepoetin-α (5 N-linked rhEPO), pJ-ST encoding α-2,3-sialyl-transferase and pJ-CST encoding CMP-sialic acid transporter were synthesized and purchased from DNA 2.0. Transfection reagents FreeStyle Max and Lipofectamin-2000 were obtained from Gibco (USA), whereas G418 and metrothrexate were purchased from Sigma (USA).

2.2. Transfection of pJ-ST and pJ-CST Plasmid into CHO-DG44-EPO

CHO-DG44-DPO clone#193 2.3 (parental) cells were thawed and cultured in a 125 ml shaker flask in 30 ml medium. At 48 and 24 hours before transfection, the cells were passed at 300,000 cells/ml in complete OptiCHO medium. On the day of transfection, the cells were seeded at 250,000 cells per well in a 24-well plate and placed in a CO_2 incubator. The cells were then transfected with pJ-ST, pJ-CST or cotransfected with pJ-ST/pJ-CST. FreeStyle MAX-DNA complex was prepared according to the manufacturer's protocol. Briefly, following mixing 0.3 µg DNA with 10 µl OptiPRO and 0.25 µl FreeStyleMAX with 10 µl OptiPRO, the two mixtures were then mixed and incubated at room temperature for 10 minutes. The FreeStyle MAX-DNA complex was then added to the cell suspension and incubated in CO_2 incubator without shaking. In addition, transfection was also done by using Lipofectamin-2000 (Invitrogen) in a Lipofectamin-DNA ratio 3:1. After 48 hours, 1.5 ml of fresh complete medium was added into the cell suspension and transfered onto a 6-well plate. On the following day, transfected cells were diluted at 7,000 cells/well in a 96-well plate in selection medium supplemented with 500 ng/µl (0.05%) G418 and 100 nM MTX. The cells were then maintained in selection medium and the successful transfectants were scaled up in a larger culture vessel until they reached higher cell density to be cultured in rotating shaker flasks.

2.3. Production of Recombinant rhEPO in Erlenmeyer Flasks

For rhEPO production, at 3 x 10^5 cells/ml for each clones was seeded in 10 ml OptiCHO medium supplemented with 4 mM L-glutamine in 125 ml erlenmeyer flasks in a rotating platform at 130 rpm. After seven days, 5 ml CD-FortiCHO (Gibco) was added into the culture, whereas D-glucose and L-glutamine were added every 3-4 days at final concentration of 20 mM and 8 mM, respectively. After 14 days of culture, the cells were spun at 3,000 rpm for 3 minutes to collect the supernatant for analysis of rhEPO expression and the cell pellet for analyses of ST, CST and β-actin expression levels.

2.4. Preparation of Supernatant and Cell Lysates.

At the end of culture, the cell suspension was spun 3,000 rpm for 3 minutes. The supernatant was used to characterized rhEPO expression, while the cell pellet was used to preprare cell lysates for analyses of ST, CST, and β-actin expression levels. Briefly, the cell pellet was lysed by using RIPA buffer (Roche) containing protease inhibitor cocktail (Nacalai Tesque) on ice for about 15 minutes followed by centrifugation at 14,000 rpm at 4°C for 15 minutes. The supernatant was collected and the total protein concentration was determined by BCA assay (Promega, USA).

2.5. Western Blot

For rhEPO detection, 24 µl of each supernatant of selected clones was mixed with 6 µl of 5x reducing sample buffer (Thermo Scientific, USA) and heated for 5 minutes at 95°C. Meanwhile for β-actin, ST and CST detection, about 10-20 µg of total protein was prepared from cell lysates, mixed with 5x reducing sample buffer and heated for 5 minutes at 95°C. All samples were subjected to SDS-PAGE for protein separation and then transferred onto nitrocelulose membrane by wet transfer method. Next, the membranes were incubated in 5% skim milk in PBS-T (0.05% tween-20) for 1 hour at room temperature offor overnight at 4°C. After washing with PBS-T, the membranes then were blotted with anti-EPO primary antibody (Calbiochem) 1:1,000; anti-β-actin antibody (Sigma) 1:2,000; anti-ST

antibody (Invitrogen) 1:1,000; anti-CST antibody (MyBioSource) 1:1,000) for overnight at 4°C. At the end of incubation, the membranes were washed with PBS-T, followed by incubation with alkaline phosphatase or horse radish peroxidase-conjugated secondary antibody (1:3000) for 1-3 hours at room temperature. After washing, the membrane then was incubated with the substrate (NBT-BCIP, Promega or ECL, Li-cor) for visualization of the protein bands.

2.6. Bacterial Community Analyses

To remove the N- or O- glycosyl-groups from rhEPO molecule and to confirm the protein backbone, 20 µl of supernatant of CHO-DG44-EPO clones was digested with PNGase-F enzyme. Briefly, 20 µl of supernatant was mixed with 5 µl of 5x reaction buffer, 0.2 µl PNGase-F enzyme (Sigma, USA) and incubated at 37°C for about 5 hours. The mixture then was used for Western blot for rhEPO detection.

2.7. Digestion of rhEPO with Neuraminidase

To investigate the contribution of sialic acid contents to rhEPO molecular weight, 20 µl of the supernatant was incubated with neuraminidase and incubated at 37°C for 5 hours. The samples were then subjected for Western blot analyses.

3. Results

The bicistronic expression of CHO-DG44-DPO and DHFR selection marker on pOptiVEC plasmid was driven by CMV promoter. Transfection obtained from this vector creates parental clone (clone 193 2.3) that successfully expressed rhEPO proteins with molecular weight approximately 45 kDa (Figure 2).

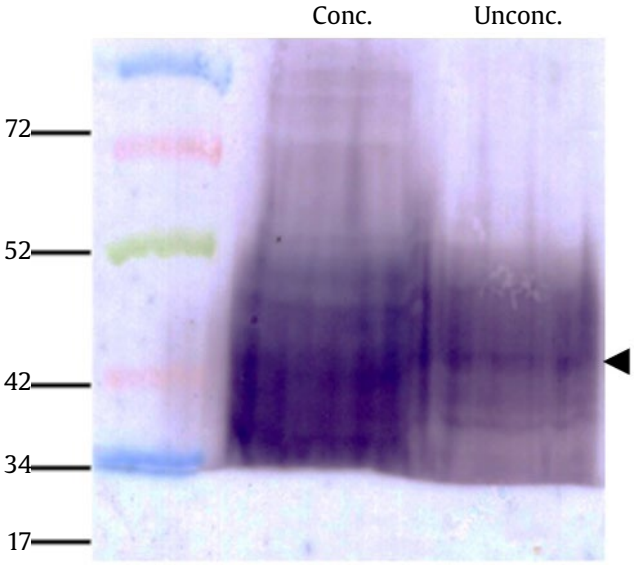

Figure 2. Profile of rhEPO produced by parental CHO-DG44-DPO during 14 days culture in an erlemeyer flask. Black arrow head indicates the main EPO band around 45 kDa. Conc: conditioned medium of CHO-DG44-DPO concentrated by amicon. Unconc: unconcentrated sample

To increase the efficiency of producing rhEPO with higher molecular weight which in line with the enrichment of the glycosylation modification and sialic acid capping, we performed transfection of parental CHO-DG44-DPO cells either with ST or CST plasmids or both ST/CST plasmid cotransfection. Overexpression of ST/CST in CHO-DG44-DPO is expected to increase the amount of CMP-Neu5Ac in golgi and enhance sialylation process by ST enzyme.

The transfection of CHO-DG44-DPO cells with plasmids harboring ST or CST or combination of both plasmids were performed using either FreeStyle Max or Lipofectamin 2,000 reagents in a 24-well plate without shaking. Twenty four hours after transfection, the cells were transferred onto a 6-well plate. Then, after 48 hours, the cells were diluted to 70,000 cells/ml to be cultured in 8 plates of a 96-well plate. During limiting dilution culture in 96-well plates, the cells were maintained in selection medium containing 500 ng/µl G418 and 100 nM MTX without shaking. The surviving and growing clones were then scaled up to larger culture vessels until a large number of cells were enough for culturing in a shaker flask and rhEPO production. From this study, five clones that grow rapidly have been obtained, they were ST2L, ST3L, ST2FS, and CST4L, CST8FS clones, each transfected with plasmids containing ST or CST, respectively. The data shown in Figure 3, shows that all of these

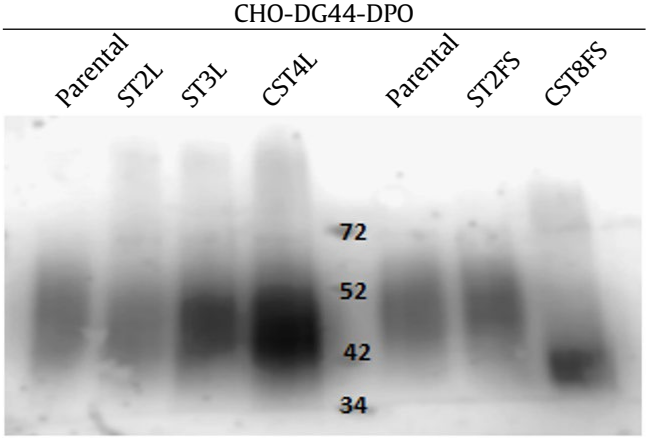

Figure 3. Transfection of ST/CST plasmids in CHO-DG44-EPO cells. Detection of rhEPO in supernatant of surviving clones in 6-well plate after limiting dilution and screening with G418. rhEPO was detected as isoforms around 42-52 kDa. Parental: CHO-DG44-DPO cells. ST2L, ST3L, ST2FS: clones transfected with ST plasmid. CST4L, CST8FS: clones transfected with CST plasmid

clones still maintained rhEPO expression. One late growing clone, ST/CST1FS which was cotransfected with plasmids harboring ST and CST genes also survived and scaled up into a shaker flask. Later, clone ST3L did not survive and was replaced with ST3FS. Finally, there were six clones which grew well and maintained in shaker flasks. Most of the clones did not survive during scaling up process or shaking in an orbital shaker.

The surviving clones, ST2L, ST2FS, ST3FS, CST4L, CST8FS and ST/CST1FS together with parental CHO-DG44-DPO were then seeded at 3 x 10^5 cells/ml in erlenmeyer flasks in 10 ml OptiCHO medium with 4 mM L-glutamine without G418 in a rotating platform at 130 rpm. Final concentration of 20 mM D-glucose and 8 mM L-glutamine were added every 3-4 days, whereas 5 ml CD-FortiCHO was added at day 7. The supernatant and cell pellet were both collected at day 14 (Figure 4A). To determine the cell growth, the cell density was observed and counted every 3-4 days. The results showed that each clones showed different cell growth profile (Figure 4B). Western blot data of each clone also showed different profile which may reflect the rhEPO isoforms. The CHO-DG44-DPO parental clone shows a band with the size of around 42-45 kDa. The ST3L and CST8FS clones have main bands below 42 kDa, while the ST2FS and ST/CST1FS clones show bands that are almost similar to that of parental cells. Interestingly, clone CST4L and ST3FS showed more intense bands at higher molecular weight (below 52 kDa) in comparison to the band of the parental cells (Figure 4C). Detection of ST, CST and β-actin levels in cell lysates indicated that clone ST3L, ST2FS, ST3FS and ST/CST1FS showed higher expression of sialyl transferase than those of parental cells. Interestingly, CST4L also showed increasing ST expression eventhough it was not transfected with ST plasmid. Moreover, clone CST8FS, CST4L and ST/CST1FS showed increased CST expression compared to that of parental cells. These data indicated that overexpression of ST and CST were confirmed in the selected clones (Figure 4D).

Next, we compare the rEPO from CHO-DG44-DPO with those from epoetin-α which contains 3 N-link glycosyl groups and possesses lower molecular weight than DPO. In general, rhEPO bands of CHO-DG44-DPO appeared to be about 42 kDa to 52 kDa, whereas purified epoetin-α bands of Hemapo appeared to be at about 42 kDa to 47 kDa. As we can see in Figure 5, clone CHO-DG44-DPO ST3FS secreted rhEPO isoforms with the main band at about 50 kDa, showing higher molecular weight than epoetin-α bands. In addition, clone CST4L also possesses rhEPO size which similar to that of clone ST3FS whereas parental CHO-DG44-DPO ST3L, ST2FS and ST/CST1FS clones only show less amount of rhEPO with the same molecular weight. This data indicated that in clone ST3FS and CST4L, rhEPO sialylation may be enhanced by ectopic expression of ST or CST in CHO-DG44-DPO.

To confirm the rhEPO of the expressed clones, enzyme digestion by PNGase-F was carried out to remove the N- and O- glycosyl-groups from rhEPO molecule. The supernatant was incubated with PNGase-F and then used for rhEPO detection by Western blot. As seen in Figure 6A, rhEPO backbone without glycosyl conjugates appeared to be at about 20 kDa for all samples. It suggests that all rhEPO samples demonstrated to have the same protein backbone eventhough they showed different profiles of rhEPO isoforms. Meanwhile, digestion of rhEPO molecule with neuraminidase affects the profile of rhEPO isoform bands to be shifted to lower molecular weight in comparison to non-treated samples, from about 40-52 kDa to 34-45 kDa indicating the sialic acid removal. In addition, this sialic acid digestion still results in a variety of rhEPO molecular weight in which clone ST3FS and CST4L showed the most intense rhEPO band at higher molecular weight at about 45 kDa compared to parental cells or other clones (Figure 6B).

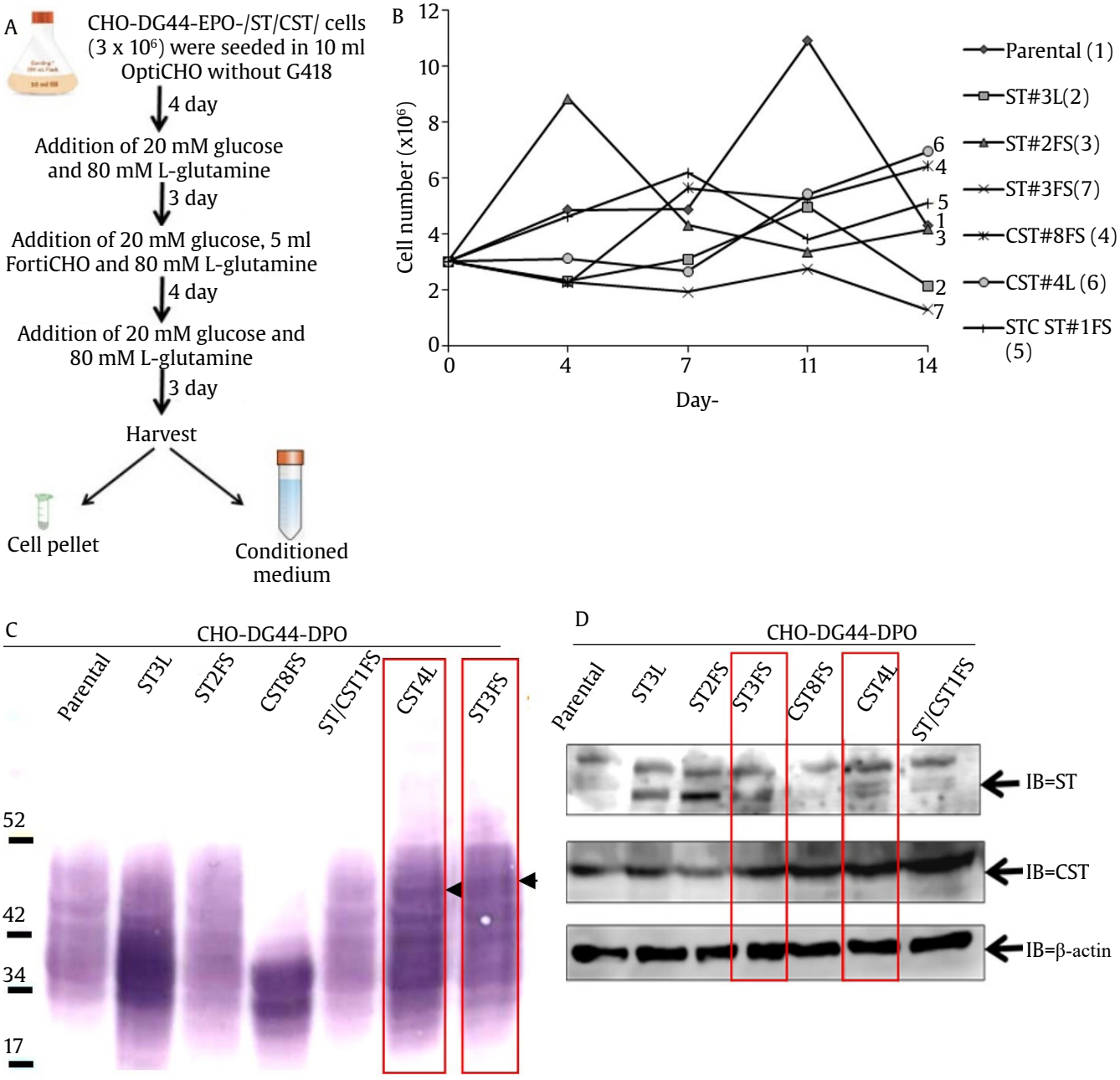

Figure 4. rhEPO production in the engineered CHO-DG44-DPO clones. (A) Production of rhEPO in shaker flasks. The selected clones were seeded in shaker flasks and incubated for 14 days. Every 3-4 days, the cultures were supplemented with glucose and L-glutamine at final concentration 20 mM and 80 mM, respectively. At day 7, the cultures were added with 5 ml FortiCHO containing L-glutamine. At the end of incubation, both conditioned medium and cell pellet were collected for further analyses, (B) growth curve of engineered CHO-DG44-DPO clones during fermentation process, (C) the conditioned media were subjected for western blot analysis for rhEPO detection. Black arrow heads indicate rhEPO isoform with higher molecular weight which may contain higher sialic acid contents, (D) detection of ST, CST, and β-actin expression by western blot. Red boxes indicate candidate clones which may produces rhEPO with higher sialic acid contents

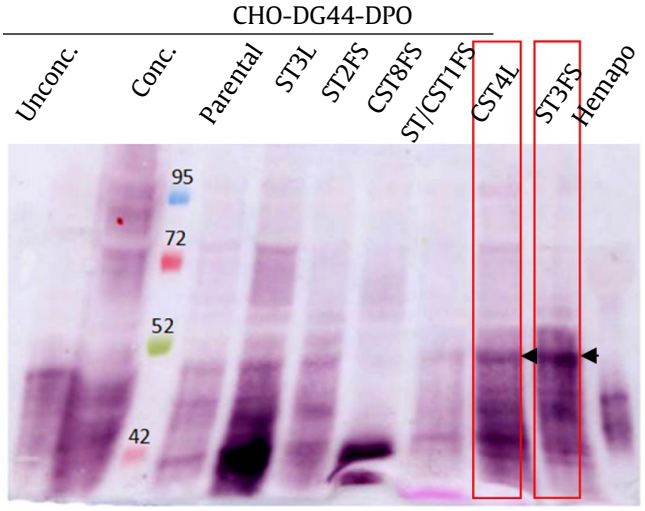

Figure 5. Comparison of rhEPO production in engineered CHO-DG44-DPO clones and Hemapo. Conc (concentrated) and Unconc (unconcentrated) samples were prepared from previous experiments using parental cells. Hemapo: Epoetin-α with 3 N-link glycosylation. Red boxes indicate candidate clones which may produces rhEPO with higher sialic acid contents

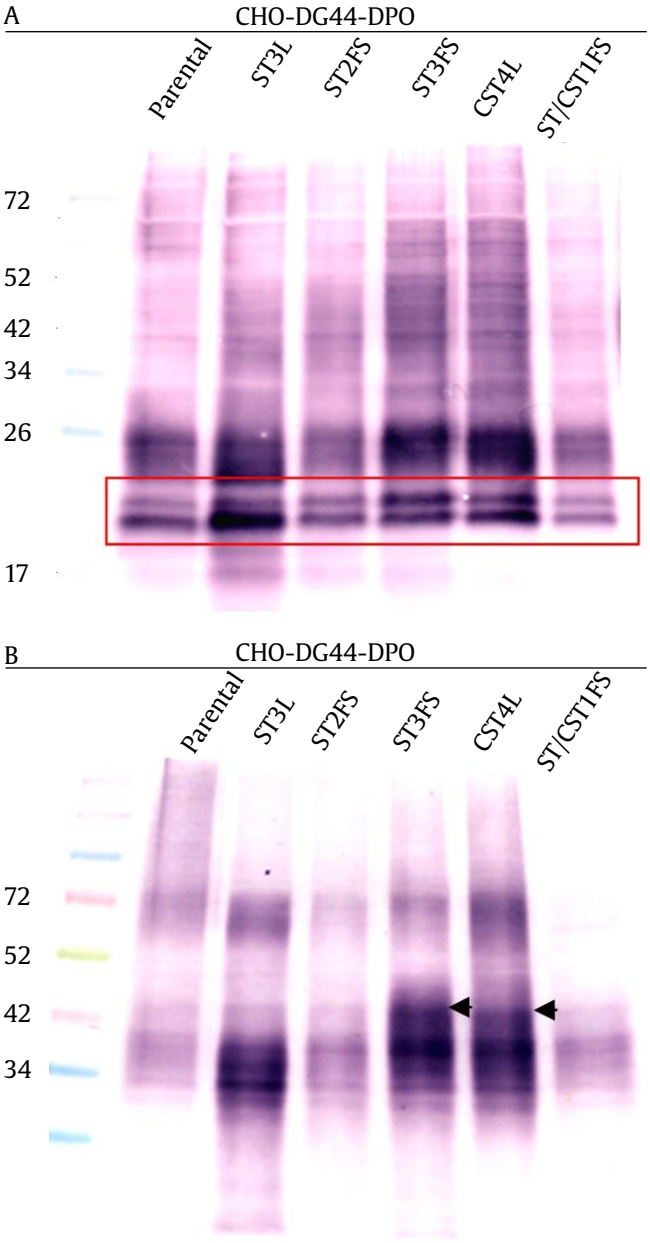

Figure 6. Enzymatic digestion of rhEPO. (A) Digestion of rhEPO with PNG-ase F. Red box indicate rhEPO backbone without glycosyl groups, (B) digestion of rhEPO with neuraminidase. Arrow heads indicate rhEPO isoform with higher molecular weight

4. Discussion

Human erythropoietin (hEPO) is a glycoprotein containing 166 amino acids which mainly secreted by kidney cells (Koeffler and Goldwasser 1981). For decades, rhEPO has been industrially manufactured by using chinese hamster ovary (CHO) cells as production host for clinical treatment of anemia patients with renal failure, cancer or AIDS (Eschbach *et al.* 1984; Lin *et al.* 1985; Abels 1992; Elliott *et al.* 2003). Those rhEPOs are available in different types which include epoetin-α, epoetin-β or recently darbopoetin-α (Elliott *et al.* 2003). Compared to other EPOs, DPO has two additional N-linked glycosyl groups which enhance its *in vivo* bioavailability eventhough it has a lower affinity for its receptor *in vitro* (Kianmehr *et al.* 2016). Thus, the longer half-life of DPO, the future of its clinical application will also be more prospective.

N-glycan moiety is a complex structure which involves the modification of asparagine or threonine residues of protein by different enzymes including glycosylation enzymes found in golgi apparatus. An important enzyme α2,3-sialyl transferase (ST) facilitates the addition of CMP-sialic acid to the galactose residue of the glycan. Native rhEPO can be modified by up to 14 sialic acids, whereas 2 additional N-linked in DPO enable ST to added more sialic acids up to 22 molecules to DPO glycans. Overexpression of ST enzyme has been reported to increase sialylation of rhEPO (Takeuchisq *et al.* 1988; Castilho *et al.* 2008; Caldini *et al.* 2003). However, each N-linked glycan can bear zero to 4 sialic acids performing asialo-, mono-, di-, tri- or tetra-sialylated N-linked glycans. Thus, the level of sialylation of a glycoprotein is not only affected by ST enzymatic

activity, but also by CMP-sialic acids availability as its substrate (Caldini *et al.* 2003; Wang 2005).

To increase the sialic acid contents of rhEPO, overexpression of CMP-sialic acids synthase had been carried out (Son *et al.* 2011). Nevertheless, the abundance of CMP-sialic acids in cytosol does not significantly improve rhEPO sialylation. The CMP-sialic acids need to be transported from cytosol to golgi by CMP-sialic acid transporter (CST) before they can be used for sialylation by ST. Moreover, engineering of CHO-K1 cells by overexpression of three different genes, human ST, CHO CST, and mutant GNE/MNK increases more sialic acid conjugations to rhEPO compared to the control cells. Eventhough there are no significant difference of di- and tri-sialylated N-linked glycans between the engineered and control cells, the level of tetra-sialylated N-linked glycans is twice higher in ST/CST overexpressing cells compared to the control cells.The mutated GNE/MNK enzyme is chosen to prevent feedback inhibitory effect of GNE/MNK by CMP-sialic acid (Son *et al.* 2011).

In this study, we tried to stably express 5 N-linked DPO in cGMP-banked CHO-DG44 and successfully obtained CHO-DG44-DPO. Unfortunately, the EPO profile of our DPO was not significantly different from that of epoetin-α (Hemapo®) profile which contains 3 N-link glycosyl groups. To overcome this deficiency, we enginereed our CHO-DG44-DPO through overexpression of ST, CST or co-expression of ST/CST to enhance our rhEPO profile by increasing the total rhEPO molecular weight which associate with the increase of sialic acid contents. To our knowledge, this is the first research to express two key genes, ST and CST, simultaneously for glycosylation in CHO-DG44 cell lines. In this research, we have obtained two clones, ST3FS and CST4L which ectopically expressed ST or CST. Both of these clones showed improved rhEPO profiles in comparison to that of parental cells and epoetin-α. Interestingly, two candidate clones, ST3FS and CST4L showed to have better rhEPO profile compared to that of ST/CST co-expression as seen in clone ST/CST1FS. These two clones showed more dense rhEPO band at higher molecular weight compared to rhEPO band of parental cells as well as epoetin-α (Figure 5). ST3FS overexpressed ST, but not CST. Meanwhile, we found that CST4L which overexpressed CST, also showed higher ST expression level compared to parental cells. This makes both candidate clones expressed higher ST level than parental cells demonstrating the importance of ST for improving rhEPO profiles as also reported by Jeong *et al.* (2008). In addition, single CST overexpression may be less important than single ST overexpression eventhough CST overexpression may enhance ST effect. Moreover, PNGase-F digestion confirmed that rhEPO expressed by the engineered cells retained similar rhEPO backbone likes the one produced by parental cells, whereas neuraminidase digestion confirmed the higher rhEPO molecular weight in ST3FS and CST4L clones (Figure 6). This study is in line with previous studies which shows that sialyltransferase is one of limiting factor enzymes in glycosylation. Sialyltransferase overexpression has been shown to increase the degree of sialylation in HEK and CHO cells (Weikert *et al.* 1999; Fukuta *et al.* 2000). In addition to ST, as one of the key genes of sialylation, overexpression of CST alone was able to enhance the degree of sialylation of IFN gamma which was resembled by this present study in which it increased the EPO sialylation profile. Furthermore, purification of rhEPO from supernatant and glycan analyses are urgently needed to confirm the post-translational modification and enrichment of sialic acid contents which happened in the candidate clones.

Using CHO cells showed that overexpression of ST increased the siaylation of TNFR-IgG by 33% (Fukuta *et al.* 2000). Working on a different enzyme, Wong *et al.* (2006) was able to enhance IFN gamma in as much as 4-16% by overexpressing CST in CHO cells. Using HEK 293 cells Chitlaru *et al.* (1998) was able to enhance the sialylation of human acetylcholinesterase about 62% by overexpressing alpha 2,6 sialyltransferase in HEK cells. However, by expressing the same gene in CHO cells, Bragonzi *et al.* (2000) was only able to increase sialylation content of IFN gamma by only 4%. On the other hand, Sam *et al.* (2018) have shown that simultaneous overexpression of alpha (2,6) sialyltransferase and GNE/MNK in CHO-DG44 cells was capable of enhancing considerable amount of sialyation by approximately 42%. Some of the above approaches prove that increasing sialic acid in therapeutic proteins is possible as has been done by some previous researchers. However, the data shows that this approach is strongly influenced by the protein of interest, glycosylation key genes and the type of cell lines.

Conflicts of Interest

The authors declare that there is no conflict of interest in this research.

Acknowledgements

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