J Human Gen Genom. 2024 May;8(1): A-10-369-4 doi: 10.6118/jhgg.8.1.115
Published online 2025 Jun 7 Research Article

Investigating the efficiency of recombinant factor 9 secretion from hFIX-producing S2 cells

Zahra Damrodi[1] , Jafar Vatandoost[2]* , Behdokht Jamali[3]

1 Department of biotechnology, Faculty of Science, Islamic Azad University, Sabzevar branch, Iran.

2 Department of Biology, Faculty of Science, Hakim Sabzevari University, Sabzevar, Iran.

3 Department of microbiology, Kherad Institute of Higher Education, Bushehr, Iran.

Corresponding Author: Jafar Vatandoost, Department of Biology, Faculty of Science, Hakim Sabzevari University, Sabzevar, Iran. Jafar.vatandoost@gmail.com.

Received 2025/05/19; **Accepted** 2025/06/6

Abstract

Background: Administration of factor IX (FIX) is the first-line treatment of hemophilia B patients and its large-scale production is of great importance to meet the needs of these patients. Therefore, this study aimed to evaluate the expression and secretion efficiency of rFIX produced by S2 cells and compare it with human embryonic kidney (HEK) cells.

Methods: *Escherichia coli* strain DH5α was used to amplify pcDNA/FIX and pMT/FIX plasmids. After plasmid isolation, HEK and S2 cells were transfected to express rFIX. rFIX activity was measured using a coagulation activity assay. Furthermore, rFIX expression level in the transfected cells was measured using an ELISA technique.

Results: The findings of this study indicated that the rFIX expression in both transfected S2 and HEK cells and both cells showed coagulation activity. Although, HEK cells showed higher rFIX secretion efficiency compared to S2 cells, hFIX-transfected S2 cells had higher rFIX expression level compared to HEK ones.

Conclusion: Overall, it was concluded that S2 cells are a more suitable option for large-scale production of rFIX due to their ability to express high level of this coagulation factor.

Keywords: Cells, Transfection, Expression, Secretion Efficiency.

1. Introduction

Hemophilia B is a relatively rare disease (one in 30000 individuals) that is associated with reduced or absent production of factor 9 (FIX) (1) and is classified based on the FIX activity as mild (FIX: 5%-30%), moderate (FIX: 1%-5%), and severe (FIX: <1%) (2). The *FIX* gene is located in the *Xq27.1* region and produces a 415-amino acid polypeptide (55 kD) that plays a pivotal role in the coagulation cascade by binding to factors VII, VIII, X, and XI and activating platelets (3, 4). Therefore, mutations in this X-linked gene are associated with defects in the coagulation cascade, and patients show symptoms such as spontaneous bleeding (5).

These patients also exhibit severe and abnormal bleeding during wounds or surgery and generally have a poor quality of life (6).Treatment approaches include treatment with plasma-derived or recombinant FIX at the time of bleeding or prophylaxis with infusions several times a week (7). New therapeutic approaches include gene therapy with the administration of AAV5 expressing variant IX, and the results of a phase III clinical study showed a reduction in disease symptoms such as decreased bleeding and improved FIX activity (36.2%) even 18 months after administration (8). However, this therapeutic approach has disadvantages such as immune response to the vector, uncertainty about long-term efficacy, and high cost (9). Therefore, FIX replacement therapy is the frontline treatment for hemophilia B. Prokaryotic cells are the most common host for recombinant protein production, however,

[DOI: 10.61882/jhgg.8.1.5]

However, this therapeutic approach has disadvantages such as immune response to the vector, uncertainty about long-term efficacy, and high cost (9).

Therefore, FIX replacement therapy is the frontline treatment for hemophilia B.

Prokaryotic cells are the most common host for recombinant protein production, however, the lack of proper protein folding and post-translational modifications are among the limitations of their use (10). For this reason, mammalian cells such as rat ovary cells have been considered for recombinant protein production (11). However, the production cost is higher and the time required for protein production is longer. In addition, possible contamination with viruses is a major limitation for large-scale production of recombinant proteins (12). Insect-based recombinant protein expression systems such as the Schneider 2 (s2) cell line derived from *Drosophila melanogaster* seem to lack the above-mentioned disadvantages and have been introduced as suitable hosts for the production of non-conjugated proteins (13). Considering that several copies of the expression vectors are inserted into the chromosomes of S2 cells, they are ideal for large-scale recombinant protein production (14). Therefore, in this study, the efficiency of recombinant FIX production in *FIX*-overexpressed S2 cells and HEK cell line were compared.

2. Materials and Methods

2.1. Preparation of bacteria and plasmids

Escherichia coli strain DH5α was used as a host for the amplification of pcDNA/FIX and pMT/FIX plasmids. The former plasmid was used for the transfection of HEK cells and the latter for the transfection of S2 cells. For this purpose, *E. coli* strain DH5α was cultured in LB medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl with pH adjusted to 7 and autoclaved. After overnight incubation, the bacteria were swapped to a plate containing LB agar medium supplemented with ampicillin and incubated at 37°C for 18 hours to allow the recombinant bacteria to grow. Then, using a plasmid extraction kit (Cinnagen, Iran), plasmids were separated from the bacteria by centrifugation at 5000g for 60 seconds according to the manufacturer's instructions.

2.2. Cells culture

Both cell lines used in the present study were obtained from the Pasteur Institute of Iran, Tehran. HEK cells (10^5 cells/mL) were cultured in DMEM/F12 (1:1) medium (Gibco, USA) supplemented with 10% FBS. DMEM medium contained 10 g/L DMEM, 3.7 g/L $NaHCO_3$, 120 mg/L penicillin, and 220 mg/L streptomycin. HamF12 medium contained 10.6 g/L HamF12, 1.2 g/L NaHCO3, 120 mg/L penicillin, and 120 mg/L streptomycin. HEK cells were incubated at 37°C and 5% CO_2, and S2 cells at 27°C.

2.3. Transfection procedures

HEK cells were trypsinized one day before transfection and incubated at a cell density of 10^4 cells/mL for 24 hours at 37°C with 5% CO_2.

The transfection mixture contained 100 µL of 2.5 M $CaCl_2$ and 5 µg of DNA diluted to 1 ml with 0.01X TE. This mixture was mixed with 2X HEPES buffer and incubated for 20 min at room temperature. Then, 100 µL of calcium phosphate and the transfection mixture were added to 1 ml of culture medium and mixed gently, and the cells were incubated for 6 hours at 37°C with 5% CO2.

The transfection of S2 cells was performed in the same manner as HEK cells, except that the cells were incubated at 27°C without CO_2.

2.4. Recombinant FIX expression

On days 1, 2, and 3 of cell culture and after adding vitamin K1, samples of the culture medium were collected and after counting and lysing the cells, they were centrifuged and the supernatant was collected and stored in a freezer at -20°C. 500 µg/ml $CuSO_4$ was used to induce rFIX expression in S2 cells. rFIX expression was measured using a coagulation activity assay. For this purpose, 100 µL of the collected culture medium was mixed with 100 µL of deficient FIX plasma and 100 µL of aPTT and incubated for 3 min at 37°C. Then, 100 µL of 25 mM calcium chloride was added and the coagulation time was measured according to the standard chart using the Diagnostic Stago Start Coagulation Analyzer.

The expressions of FIX in S2 and HEK cells were measured using an ELISA kit (Cedarlane, Canada) according to the manufacturer's instructions.

2.5. Statistical analysis

The results were presented as means±SD and an unpaired T-Test was used to analyze the data. All analyses were done in GraphPad Prism V.8 software.

3. Results

3.1. Transfected S2 cells coagulation activity and rFIX expression

After transfection of S2 cells, the coagulation activity of FIX in the culture medium and cell lysate was examined by one-stage coagulation assay at 24, 48, and 72 hours. The results showed a significant decrease in the coagulation time of the culture medium collected from the transfected cells compared to the control, indicating improved coagulation activity. As shown in Figure 1a, the coagulation activity increased with time and reached its peak at 72 hours after transfection of S2 cells. Furthermore, the expression levels of rFIX in S2 cell lysates showed an increasing trend over time, while the culture medium showed a constant trend over time, indicating the production of rFIX by S2 cells (Figure 1b).

[DOI: 10.61882/jhgg.8.1.5]

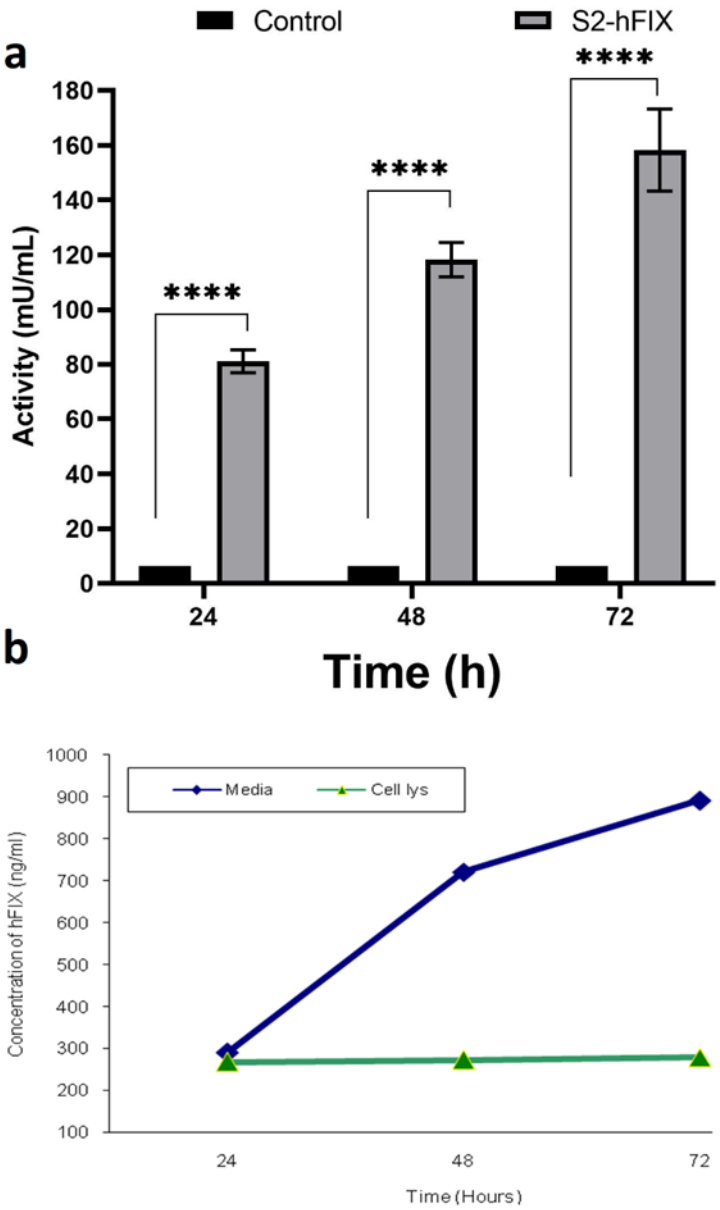


Figure 1. Coagulant activity of S2 cells transfected with *FIX* (a) and measurement of rFIX expression in culture medium and lysate of S2 cells (b) during 24, 48, and 72 hours after transfection (n=3). **** shows a significant difference at a probability level of P<0.0001.

3.2. Transfected HEK cells coagulation activity and rFIX expression

The coagulation activity of HEK-rFIX is shown in Figure 2a. As can be seen, the coagulation activity of transfected HEK cells showed a significant increase compared to the control over time. Also, the expression levels of rFIX in these cells showed an increasing trend after the transfection process in the period of 24 to 72 hours (Figure 2b), indicating the success of the transfection process of HEK cells for the production of rFIX.

[DOI: 10.61882/jhgg.8.1.5]

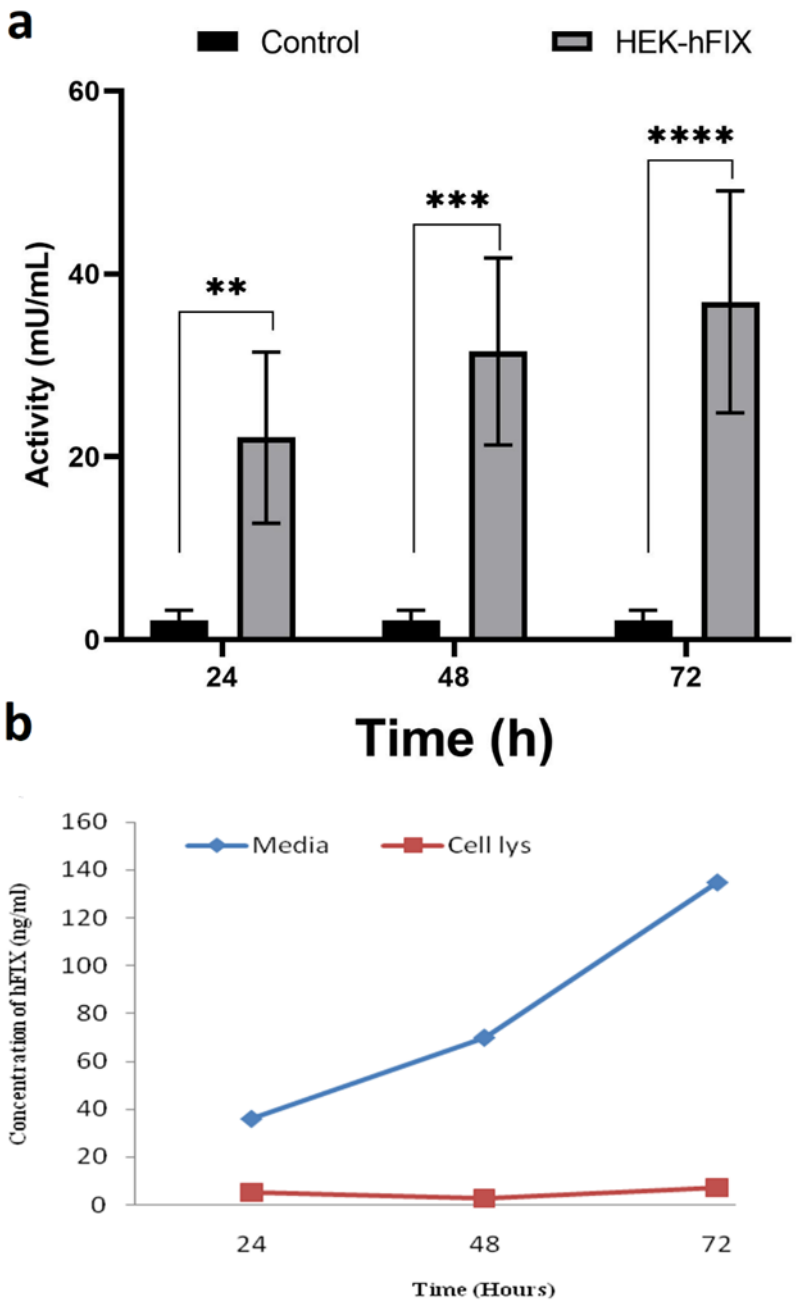


Figure 2. Coagulant activity of HEK cells transfected with *FIX* (a) and measurement of rFIX expression in culture medium and lysate of HEK cells (b) during 24, 48, and 72 hours after transfection (n=3). ****, *** and ** show significant difference at probability level of P<0.0001, P<0.001 and P<0.01.

3.3. S2 cells vs. HEK cells

3.3.1. rFIX expression

As can be seen in Figure 3, the expression of rFIX in transfected S2 cells was much higher than in HEK-hFIX cells, reaching about 6.5-fold. The average amount of rFIX expressed per 10^6 cells in HEK-hFIX cells at 24, 48, and 72 hours after the addition of vitamin K1 was 48, 54, and 63 ng/ml/10^6 cells, respectively, and in S2-hFIX cells on the first, second, and third days after induction by $CuSO_4$ was 118, 356, and 410 ng/ml/10^6 cells, respectively (Figure 3). This increase in expression indicates a better efficiency of S2-hFIX cells in expressing rFIX.

[DOI: 10.61882/jhgg.8.1.5]

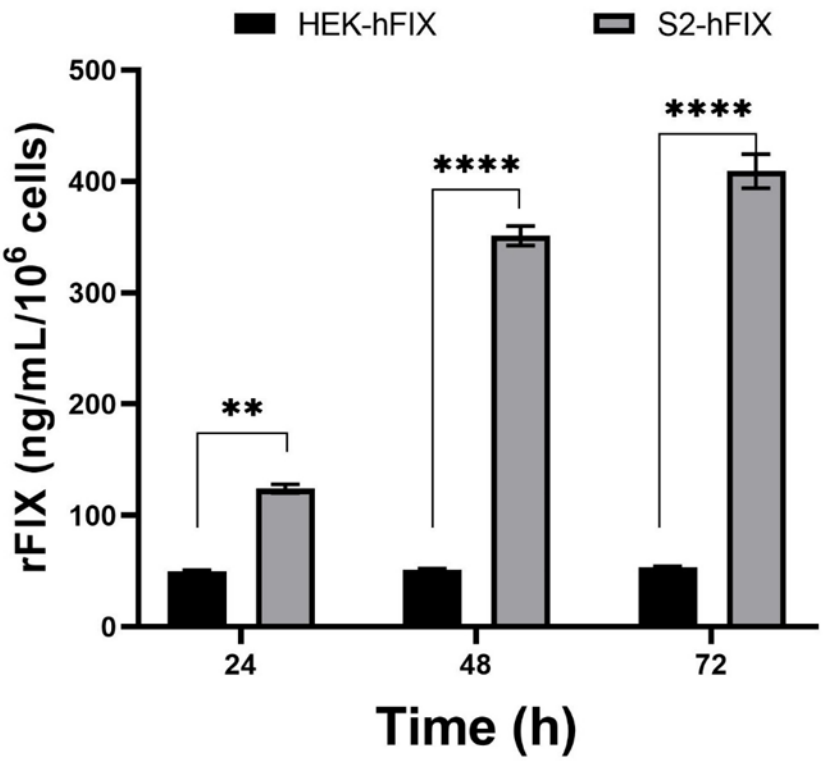
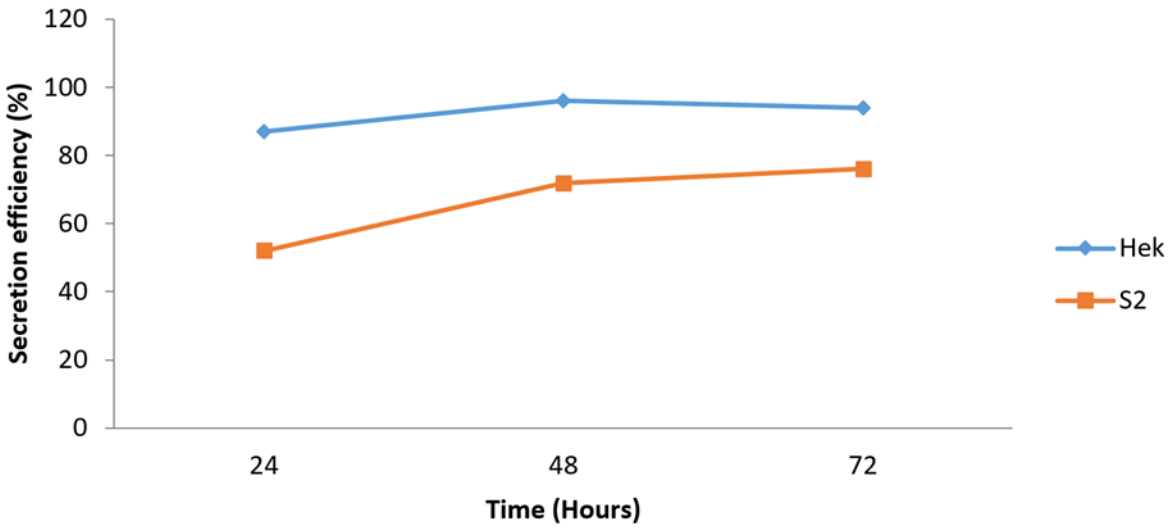


Figure 3. The comparison of rFIX expressions in S2 and HEK cell lines (n=3). **** and ** show significant difference at probability level of P<0.0001 and P<0.01.

3.3.2. Secretion efficacy

The secretion efficiency of a protein is defined as the ratio of the secreted fraction of protein to the total amount of that expressed protein (Kim et al. 2005). Based on this definition, the secretion efficiency of rFIX in S2 cells was calculated to be 52, 72, and 76% on the first, second, and third days after induction, respectively. However, the secretion efficiency of rFIX in HEK cells was measured to be 87, 96, and 94% on the first, second, and third days after induction, respectively (Figure 4). This means that the secretion efficiency of HEK cells is better than that of S2 cells.



[DOI: 10.61882/jhgg.8.1.5]

4. Discussion

In the present study, we successfully developed transfected HEK and S2 cells expressing hFIX, and the results indicated the expression of this coagulation factor from these cells. Although HEK cells had a higher rFIX secretion efficiency compared to S2 cells, hFIX-transfected S2 cells showed a higher rFIX expression level compared to HEK ones.

The use of mammalian cells such as human embryonic kidney (HEK) in the production of recombinant proteins has advantages such as the induction of post-translational modifications. For example, it was shown that HEK cells expressing FIX have a greater ability to γ-carboxylate FIX and secrete rFIX compared to BHK cells (15). In the present study, it was also shown that HEK cells have a greater efficiency of FIX secretion compared to S2 cells, which is similar to the findings of the aforementioned study. In addition, Vatandoost and Dolatabadi (2017) developed a transient HEK cell line and showed that these cells have the ability to produce more hFIX compared to CHO cells (16). Interestingly, HEK cells overexpressing VKORC1 showed augmented rFIX production with the high efficiency of γ-carboxylation of rFIX, making them superior for large-scale production of rFIX (17). In this study, it was observed that S2 cells have a greater ability to produce rFIX compared to HEK cells. The use of S2 cells in the production of recombinant proteins is associated with advantages such as the lack of the need for serum and growth in suspension, which makes it ideal for the production of rFIX on a commercial and industrial scale (18). Also, a comparison of rFIX production in S2 cells with CHO cells indicated that this coagulation factor was produced more by S2 cells, which is similar to the findings of the present study (19). Also, in a study, it was found that the expression of rFIX in S2 cells transformed by transient gene expression technology was higher than of stable type (20).

Mammalian cells used for recombinant protein production have disadvantages including the long time required (sometimes up to two months) for clonal selection, which is a major obstacle to large-scale production of recombinant protein to meet the needs of patients (21). However, it seems that S2 cells derived from *D. melanogaster* can overcome this problem by reducing clonal selection to 2 weeks (22). In addition, it is necessary to have high levels of expression of the recombinant protein (FIX), which is an important requirement for the large-scale production of these proteins. In the present study, it was confirmed that the production of rFIX by S2 cells is higher than HEK cells, which can be attributed to the high copy number of the vector insertion in the cells and the presence of a strong Mtn promoter (23). Furthermore, γ-carboxylase is present in *D. melanogaster* and its activity is higher than that of the human form of this enzyme (24). It is likely that the higher coagulation activity of rFIX produced by S2 cells compared to HEK cells in the present study can be attributed to the higher γ-carboxylation of rFIX in S2 cells. Therefore, it seems that transgenic S2 cells to express coagulation factor IX would be a suitable approach for the large-scale production of this coagulation factor, although the problem of secretion of this factor into the culture medium needs to be addressed.

5. Conclusion

Overall, it is concluded that S2 cells expressing rFIX have a higher expression level of this coagulation factor compared to HEK cells. However, the efficiency of rFIX secretion was higher in HEK cells compared to S2 cells. Future studies are strongly recommended to overcome the problem of low secretion efficiency of the recombinant protein by S2 cells. Recombining S2 cells to secrete more of this coagulation factor in culture could potentially overcome this problem and be recommended for future research.

References

1. Shen G, Gao M, Cao Q, Li W. The Molecular Basis of FIX Deficiency in Hemophilia B. International Journal of Molecular Sciences [Internet]. 2022; 23(5).
2. White GC, 2nd, Rosendaal F, Aledort LM, Lusher JM, Rothschild C, Ingerslev J. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost. 2001;85(3):560.
3. Wei X, Zhang H, Chen W, Zhang J, Dai J. A study of recurrent life-threatening thrombosis accompanied with the duplication of the factor IX gene. Thrombosis Journal. 2024;22(1):1.
4. B. Smolkin M, D. Shmookler A. Chapter 18 - Molecular diagnostics for coagulopathies. In: Coleman WB, Tsongalis GJ, editors. Diagnostic Molecular Pathology (Second Edition): Academic Press; 2024. p. 241-53.
5. Obeagu EI. Haemophilia B: A Review. Int J Curr Res Chem Pharm Sci. 2022;9(1):14-20.
6. Itzler R, Miller J, Robson R, Monahan P, Pipe S, editors. Improvements in health-related quality of life in adults with severe or moderately severe hemophilia B after receiving etranacogene dezaparvovec gene therapy. ISTH 2022 congress; 2022.
7. Franchini M, Frattini F, Crestani S, Bonfanti C. Haemophilia B: current pharmacotherapy and future directions. Expert Opin Pharmacother. 2012;13(14):2053-63.
8. Pipe Steven W, Leebeek Frank WG, Recht M, Key Nigel S, Castaman G, Miesbach W, et al. Gene Therapy with Etranacogene Dezaparvovec for Hemophilia B. New England Journal of Medicine. 2023;388(8):706-18.
9. Butt MH, Zaman M, Ahmad A, Khan R, Mallhi TH, Hasan MM, et al. Appraisal for the Potential of Viral and Nonviral Vectors in Gene Therapy: A Review. Genes [Internet]. 2022; 13(8).
10. Kesidis A, Depping P, Lodé A, Vaitsopoulou A, Bill RM, Goddard AD, et al. Expression of eukaryotic membrane proteins in eukaryotic and prokaryotic hosts. Methods. 2020;180:3-18.
11. Li W, Fan Z, Lin Y, Wang T-Y. Serum-Free Medium for Recombinant Protein Expression in Chinese Hamster Ovary Cells. Frontiers in Bioengineering and Biotechnology. 2021;9.

[DOI: 10.61882/jhgg.8.1.5]

12. Barone PW, Wiebe ME, Leung JC, Hussein ITM, Keumurian FJ, Bouressa J, et al. Viral contamination in biologic manufacture and implications for emerging therapies. Nature Biotechnology. 2020;38(5):563-72.
13. Kumar S. Recent advances in insect biotechnology. Introduction to Insect Biotechnology. 2023:65-80.
14. Jorge SAC, Santos AS, Spina Â, Pereira CA. Expression of the hepatitis B virus surface antigen in Drosophila S2 cells. Cytotechnology. 2008;57(1):51-9.
15. Hallgren KW, Hommema EL, McNally BA, Berkner KL. Carboxylase overexpression effects full carboxylation but poor release and secretion of factor IX: implications for the release of vitamin K-dependent proteins. Biochemistry. 2002;41(50):15045-55.
16. Vatandoost J, Dolatabadi B. Stable and Transient Expression of Human Coagulation Factor IX in Mammalian Expression Systems; CHO Versus HEK Cells. Gene Cell Tissue. 2017;4(2):e13096.
17. Pakdaman SF, Vatandoost J, Bos MHA. Enhanced functional recombinant factor IX production by human embryonic kidney cells engineered to overexpress VKORC1. Biotechnology Progress. 2020;36(2):e2938.
18. Adriaan de Jongh W, Salgueiro S, Dyring C. The use of Drosophila S2 cells in R&D and bioprocessing. Pharmaceutical Bioprocessing. 2013;1(2):197-213.
19. Vatandoost J, Bos MH. Efficient expression of functional human coagulation factor IX in stably-transfected Drosophila melanogaster S2 cells; comparison with the mammalian CHO system. Biotechnol Lett. 2016;38(10):1691-8.
20. Vatandoost J, Kafi Sani K. A Study of Recombinant Factor IX in Drosophila Insect S2 Cell Lines Through Transient Gene Expression Technology. Avicenna J Med Biotechnol. 2018;10(4):265-8.
21. O'Flaherty R, Bergin A, Flampouri E, Mota LM, Obaidi I, Quigley A, et al. Mammalian cell culture for production of recombinant proteins: A review of the critical steps in their biomanufacturing. Biotechnology Advances. 2020;43:107552.
22. Zitzmann J, Schreiber C, Eichmann J, Bilz RO, Salzig D, Weidner T, et al. Single-cell cloning enables the selection of more productive Drosophila melanogaster S2 cells for recombinant protein expression. Biotechnology Reports. 2018;19:e00272.
23. Vatandoost J, Zomorodipour A, Sadeghizadeh M, Aliyari R, Bos MHA, Ataei F. Expression of biologically active human clotting factor IX in Drosophila S2 cells: γ-carboxylation of a human vitamin K-dependent protein by the insect enzyme. Biotechnology Progress. 2012;28(1):45-51.
24. Bandyopadhyay PK, Clark K, Stevenson BJ, Rivier JE, Olivera BM, Golic KG, et al. Biochemical characterization of Drosophilaγ-glutamyl carboxylase and its role in fly development. Insect Molecular Biology. 2006;15(2):147-56.

[DOI: 10.61882/jhgg.8.1.5]