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Research Article

Increased Expression Level of Human Blood Clotting Factor VIII Using NS0 Cell Line as a Host Cells

Mahboobeh Zarei,[1, 2] Elaheh Ferdosi-Shahandashti,[2, 3*] Mohsen Badalzadeh,[4] Gholam Ali Kardar [4, 5]**

[1]Student Research Committee, Babol University of Medical Sciences, Babol, I.R. Iran.
[2]Biomedical and Microbial Advanced Technologies (BMAT) Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, I.R. Iran.
[3]Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, I.R. Iran.
[4]Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran.
[5]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.

[*]*Corresponding author*: Elaheh Ferdosi-Shahandashti, Biomedical and Microbial Advanced Technologies (BMAT) Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, IR. Iran. Tel/Fax: +98-1132199592, E-mail: e.ferdosi@mubabol.ac.ir

[**]*Co-Corresponding author*: Gholam Ali Kardar, Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, P.O. Box 14185-863, Tehran, IR. Iran. Tel: +98-2166907415, Fax: +98-2166428995, E-mail:gakardar@tums.ac.ir

Background: Coagulation factor VIII (FVIII) is applied for spontaneous hemorrhaging inhibition and excessive bleeding after trauma in patients with hemophilia A. High-quality human recombinant factor VIII (rFVIII) has been produced relatively in large quantities in cultured mammalian cells. NS0 is one of the most common mammalian cell lines for therapeutic protein production. Production of rFVIII has increased due to low FVIII expression levels and rising demand for hemophilia A prophylactic treatment. Several methods have been developed to prevent cell cycle progression in mammalian cells for increased recombinant protein yields.

Objective: The aim of the study was to investigate the level of recombinant BDD-FVIII expression in NS0 mouse myeloma cells. Additionally, the study aimed to determine the effects of chemical drugs, Mitomycin C, Lovastatin, and Metformin on the secretion of FVIII through cell cycle arrest.

Materials and Methods: We cultured NS0 cells and transfected them with the 2 µg pcDNA3-hBDDFVIII plasmid by Lipofectamine 3000. The cells were treated with 10 $\mu g.mL^{-1}$ Mitomycin C, 20 µM Lovastatin, and 20 mM Metformin separately. After 24 and 48 hours, the samples were collected and, protein expression was analyzed using RT-PCR, Dot blot, and ELISA.

Results: A higher protein expression level was observed in treated cells 24h and 48h after treatment with all three drugs. According to real-time PCR, Metformin treatment resulted in the highest expression level within 24 h (P=0.0026), followed by Mitomycin C treatment within 48 h (P=0.0030).

Conclusion: The NS0 cell line can be regarded as a suitable host for FVIII production. FVIII protein expression level was increased by using Lovastatin, Metformin, and Mitomycin C drugs. Further investigations are suggested, and the potential application of these drugs to increase recombinant protein yield can be used to produce therapeutic proteins in the industry.

Keywords: Lovastatin, Metformin, Mitomycin C, NS0 mouse myeloma cells, Recombinant factor VIII (rFVIII)

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1. Background

Hemophilia is the most common inherited hemorrhagic disorder. Factor VIII (FVIII) deficiency causes hemophilia A. This X-linked recessive disorder Affects 1 in every 5,000 live male births and is characterized by prolonged and excessive bleeding after minor trauma or sometimes even spontaneously (1-3). Providing the missing coagulation factor through replacement therapy has been the basis of both prophylactic and therapeutic treatment (4). The application of recombinant FVIII products accounts for safer and more effective approaches, accompanied by viral inactivation and improved screening technologies. Although plasma-derived FVIII concentrates are widely available, about 75% of global hemophilia patients use recombinant FVIII products due to their superior safety profile. (1).

Mammalian cells genetically manipulated in culture have been used to produce relatively large quantities of high-quality human rFVIII (5). Although there are many mammalian cells available, only a few are applicable for recombinant protein production on the industry scale due to 1- high transferability with standard methods, 2- ease of adaptability to suspension cultivation in serum-free conditions, and 3- cost-effective scale-up (6, 7). A high degree of variation in productivity is observed in mammalian cells, with liver-derived cells, such as HepG2, SKHep1, and SMMC-7721, expressing at greater levels than other cells. However, manufacturers have chosen only three non-liver origin cell lines, including CHO, BHK, and HEK293 (8).

Cell lines such as: CHO, NS0, and Sp2/0 are most commonly administered for biopharmaceuticals component expression (9). Since the 1970s, myeloma cells have been used to produce antibodies. Myeloma cells offer several advantages over CHO cells for manufacturing approved biotherapeutics. These include suspension growth, ease of scaling up, the ability to proliferate well under serum-free conditions, and a natural inclination for antibody production. Among the most commonly used myeloma cell lines is NS0, which has been employed for production in combination with the glutamine synthetase (GS) selection system. NS0 cells express minimal endogenous GS, and non-transfected cells are glutamine auxotrophs, eliminating the need for genetic alteration in NS0 for GS-based selection (10).

Over the last thirty years, the production of biotherapeutic molecules in mammalian systems has grown significantly. However, growing economic pressures have driven efforts to improve yields and reduce the costs of biotherapeutic agent production (11). It should be noted that protein production depends on the cell cycle phase, and several genes involved in ribosome biogenesis and protein translation are highly expressed during the G1 phase. Arrested cells are metabolically more active and larger than non-arrested cells at the end of the G1 phase. So, to achieve the highest productivity yield, the cell cycle is being arrested at the G1 phase in numerous cell lines, such as hybridomas and CHO (12). Various strategies have been developed to increase the recombinant protein production in mammalian cell cultures by inhibiting the cell cycle progression. Cell cycle arrest can be induced by adding cytostatic chemicals such as NaBu or DMSO, overexpression of cell cycle inhibitory proteins like p21Cip1 or p27Kip1, or controlling cell culture conditions like mild hypothermic temperatures (13).

2. Objectives

In the current study, we chose the NS0 cell line as the host for expressing rFVIII based on its high potential for recombinant protein production. We have also applied Lovastatin, Metformin, and Mitomycin C to inhibit the growth and increase the rate of protein expression.

3. Materials and Methods

3.1. Cell Culture, Transfection and Optimization

The NS0 mammalian cells were purchased from (Pasteur Institute-Iran and were used as host for hFVIII expression. The NS0 cells were grown in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco-BRL Life Technology), 100 U. mL^{-1} penicillin G and 100 $\mu g.mL^{-1}$ streptomycin (Sigma-Aldrich) and cultured at 37 ºC in 5% CO_2 and humidified conditions. One day before transfection, cells were seeded at a density of 5×10^5 cells in 0.5 mL of medium in 24-well plates following replaced of medium with 250 µL Opti-MEM (Gibco-BRL Life Technology) after 24h culture. A plasmid, pcDNA3-hBDDFVIII, was available in our lab, based on previous studies (KARDAR *et al*., 2010) (14), and used as a source for the B-domain-less hFVIII cDNA and as an expressing plasmid. The cells were then transfected with 2 µg plasmid DNA by Lipofectamine 3000 (Thermo Fisher Scientific-USA). About 48h after transfection, cells

were treated with three chosen drugs; Mitomycin C (10 µg.mL^{-1} – 2.5 hours)(15, 16), Lovastatin (20 µM-24 & 48 hours) (17, 18), and Metformin (20 mM-24 & 48 h) (19, 20) (Sigma-Aldrich) in order to increase the transient expression, then 24 & 48 after treatment, supernatant and cells were collected for expression assays.

3.2. Real-Time PCR

Total RNA was extracted from NS0 cells using the SanPrep Column RNA Miniprep Kit (bio basic) according to the manufacturer's instructions. cDNA was synthesized using by a cDNA reverse transcription Kit (rojetechnologies-iran). The synthesized BDD-FVIII-cDNA was subsequently analyzed by amplification of two fragments, using two specific primer pairs, namely F8-F (5′- GTGCCTTTTGCGATTCTGCT -3′), F8-R (5′-GCAGCTCACCGAGATCACTT -3′). A volume of 5 µL 2x SYBR Green master mix (Ampiliqon, Denmark), 5 pmol of each specific primer, and 0.5 µL of cDNA in a total volume of 10 µL, was carried out using StepOnePlus™ Real-Time PCR System (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to standardize the amount of applied RNA. Relative expression levels of rFVIII were measured by applying the 2^{-DDCT} equation.

3.3. Dot Blot

Proteins secreted in the supernatant of transfected cells were analyzed using the dot blot method. Briefly, 3 µL of supernatant samples were spotted on the PVDF membrane and allowed to dry for 10 min at room temperature. The membrane was then blocked with 3% skim milk in PBS-T for 1 h in a conditional shaker at RT. The membrane was washed three times using PBS+ 0.05% Tween-20 (PBS-T) as a washing solution. Then, the membrane was incubated with biotinylated Anti-hFVIII monoclonal antibody for 1 h with a shaker at RT and repeating the washing step. In the next step, the membrane was incubated with PBS-T solution containing HRP-Conjugated Streptavidin and repeated the washing step. At the end, the surface of the membrane was developed with DAB, and the expression was observed after the spots appeared. Various dilutions of commercially lyophilized human rFVIII (ReFacto AF 500 IU) as a positive control and the supernatant of non-transfected NS0 cells as a negative control were used. The density of dots was quantified with Image J software, and GraphPad Prism 9 was used to draw the graphs from the resulting data.

3.4. Measurement of FVIII Antigen

The rhFVIII antigen in the conditioned cultured media was assayed by sandwich enzyme-linked immunosorbent assay (ELISA). Initially, the micro-plate was coated with a specific mouse Anti- hFVIII monoclonal antibody (clone GMA-012) (Sigma-Germany) and incubated overnight at 4 ºC. After washing three times with PBS-T buffer, the microplate was blocked and incubated for 1 h at room temperature (RT). After washing three times with PBS-T buffer, samples were applied to the wells along with standard concentrations, negative control (cultured media from non-transfected NS0 cell lines), and then incubated for 1.5 h at 37 ºC. After washing according to the previous procedure, a second biotinylated Anti- hFVIII monoclonal antibody was added to each well and incubated for 1.5 h at 37 ºC. After 3X washes with PBS-T buffer, HRP-Streptavidin was added to each well and incubated for 1 h at 37 ºC. After washing, 100 µL of tetramethylbenzidine (TMB) was applied to each well. 5 to 30 minutes later, when a suitable color change was made, 1 N H_2SO_4 was applied to stop the reaction. The resulting absorbance was measured at 450 nm. A standard curve was drawn from 2-fold serial dilutions of commercially lyophilized human rFVIII (ReFacto AF 500 IU), and the concentration of rFVIII in culture medium was expressed as IU. mL^{-1}.

3.5. Data Analysis

All experiments and assays were carried out in triplicates, and the generated data were presented as the means ± SD. Analysis of dot blot images was done using Image J. Excel (Microsoft), and GraphPad Prism 9 software (Graph-Pad Software) was used for all data drawing and analysis. Statistical significance was accepted at $p \leq 0.05$.

4. Results

4.1. Transient Transfection of the NS0 Cells

To study the expression of hBDD-FVIII, the mammalian cell line NS0 was transiently transfected by the recombinant plasmid. After transfection, they were treated with Mitomycin C, Lovastatin, and Metformin to optimize the transfected cells. Morphology of NS0

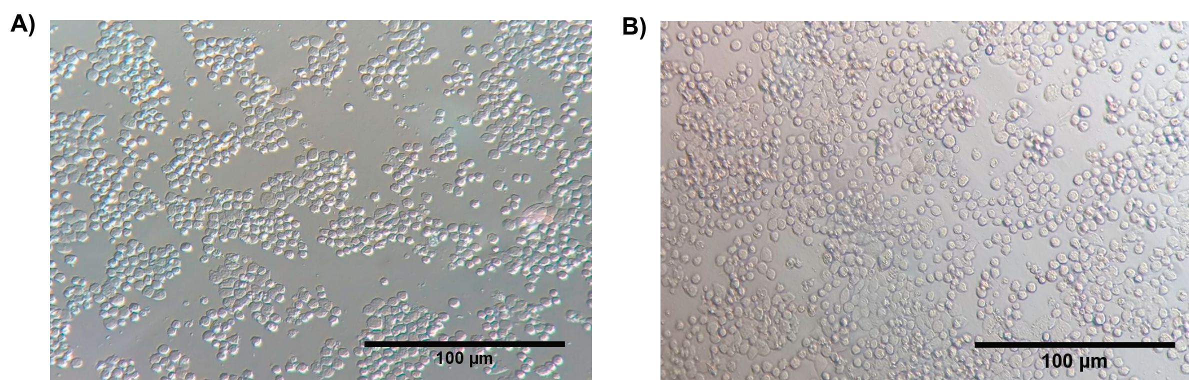

Figure 1. The morphological changes of the NS0 cells after transfection with the pcDNA3-BDD-FVIII. Cells were cultured at a density of 5×10^5 cells in 0.5 ml of medium in 24-well plates. **A)** Non-transfected NS0 cells, **B)** NS0 cells transfected with Lipofectamine 3000 and 2 µg plasmid DNA. The micrograph showed round cells with some variation in size and transparency.

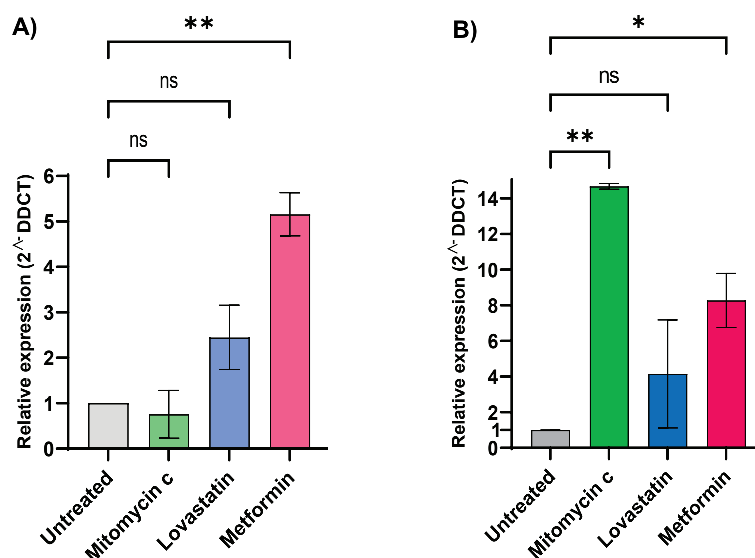

Figure 2. Expression assessment of rhFVIII, based on Real-Time PCR. A) Relative expression among untreated control cells and treated cells with Mitomycin C (10 µg.mL^{-1} - 2.5 hours), Lovastatin (20 µM-24 & 48 hours), and Metformin (20 mM-24 & 48 hours) after 24 h and **B)** after 48 h. Statistically significant differences between groups using one-way ANOVA was accepted at $p \leq 0.05$. ((ns) Not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ and (****) $p < 0.0001$).

cells was examined by an inverted microscope before and 24 h after transfection. (**Fig. 1**).

4.2. Expression Analysis

4.2.1. Analysis of the B-domain-less hFVIII Transcript

Quantitative comparative expression analysis was performed on NS0 cells transfected with plasmid pcDNA3-BDD-FVIII, optimized with three drugs separately. According to the results, a higher expression level of rhFVIII in the treated cells at 24 and 48 h after treatment with all three drugs was observed compared to the untreated NS0 cells (control sample). Furthermore, cells treated with Metformin expressed

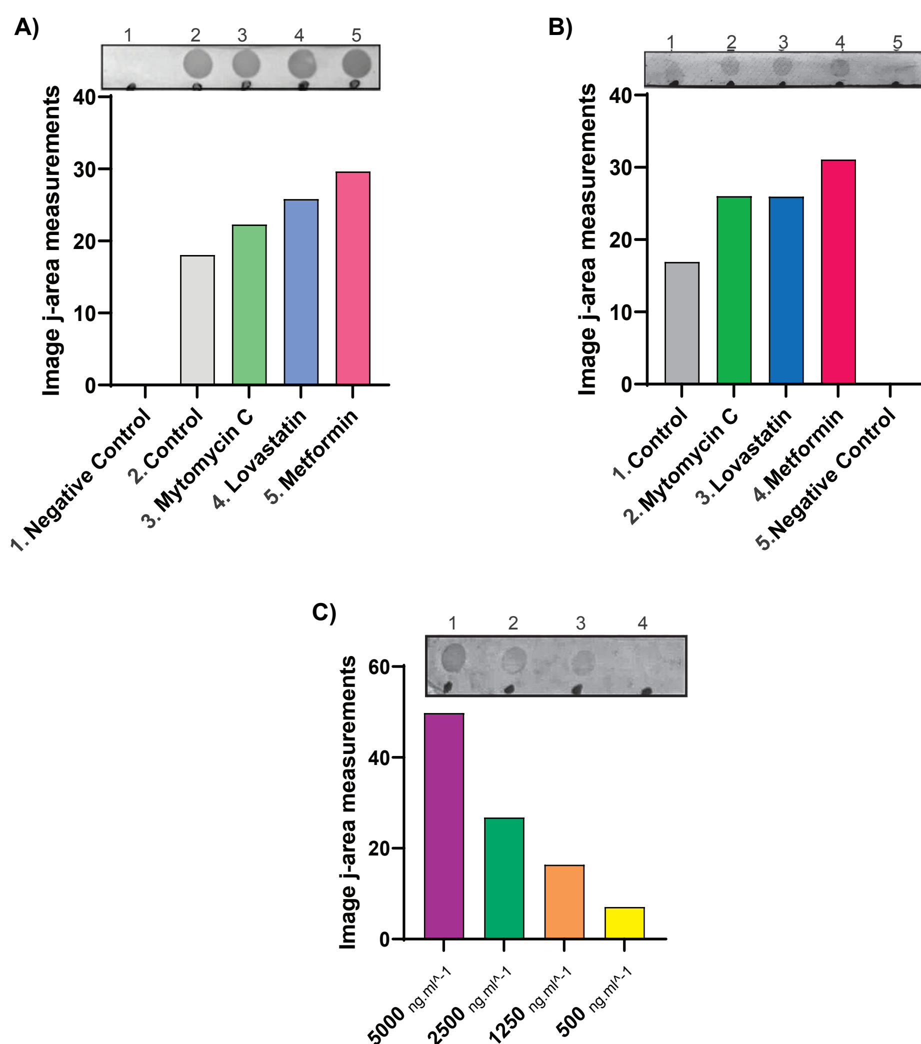

Figure 3. Expression evaluation of rhFVIII, utilizing Dot blot. The graphs below the Dot blot images illustrate data quantified with Image J and plotted using GraphPad Prism 9. **A)** comparison of non-transfected cells, untreated and treated cells: 1- negative control of non-transfected cells 2- transfected and untreated cells, and 3, 4, 5- cells treated with Mitomycin C, Lovastatin, and Metformin, respectively after 24 h. **B)** Supernatant samples after 48 h :1- transfected and untreated cells, 2, 3, and 4- cells treated with Mitomycin C, Lovastatin, and Metformin, respectively 5- negative control of non-transfected cells. **C)** Serial dilutions of commercially lyophilized human rFVIII as a standard.

higher levels of rhFVIII 24 h after treatment, and there was a significant difference compared to the control sample, p value=0.0026. Also, a higher level of rhFVIII expression was observed 48h after treatment of cells with Mitomycin C, p value=0.0030. (**Fig. 2**).

4.2.2. Supernatant Analysis with Dot Blot

An analysis of rhFVIII expression was carried out by dot blot assay using PVDF paper. This assay was done to qualitatively compare transfected versus non-transfected cells and the relative expression between treated and untreated cells. Additionally, semi-quantitative analysis was performed using an ImageJ processing program to obtain more reliable measurements. Examination of the blots showed that rhFVIII protein was successfully expressed in NS0 cells following transfection compared to the non-transfected cells. Also, an increase in expression of rhFVIII was observed in all treated cells with Mitomycin C, Lovastatin, and Metformin at 24 & 48 h compared to the untreated control cells (**Fig. 3**).

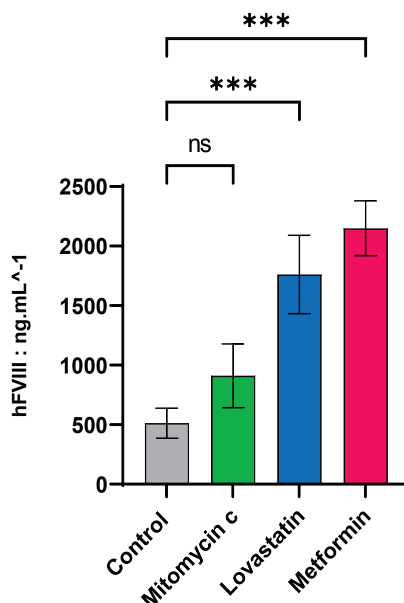

Figure 4. Measurement of the expressed recombinant hFVIII secreted in the cultured media of the transfected NS0 cells, by ELISA. The values are stated in ng.mL^{-1}. The concentration of rhFVIII in the culture media of treated NS0 cells with Mitomycin C (10 µg.mmL^{-1} - 2.5 hours), Lovastatin (20 µM-24 & 48 hours), and Metformin (20 mM-24 & 48 hours) after 48 h vs control (untreated-transfected NS0 cells). The error bar shows the standard deviation, and the results were obtained from 3 repetitions (Mean±SD). Statistically significant differences between groups using one-way ANOVA was accepted at $p \leq 0.05$. ((ns) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ and (****) $p < 0.0001$).

4.2.3. Investigating rhFVIII Protein Expression in the Culture Medium Using Enzyme-Linked Immunosorbent (ELISA)

ELISA assay was conducted on the supernatant of NS0 cells treated with three different drugs to examine the rise of rhFVIII expression. The concentration of rhFVIII in the culture media of NS0 cells treated with Mitomycin C (4.5 IU. mL^{-1}), Lovastatin (8.8 IU. mL^{-1}), and Metformin (10.7 IU. mL^{-1}) after 48 h was higher than that of the control sample, which had a concentration of 2.5 IU. mL^{-1}. Specifically, Lovastatin and Metformin-treated cells showed significantly higher levels of expression, with p-values of 0.0007 and 0.0001, respectively (**Fig. 4**).

5. Discussion

Initially, the hFVIII was mainly obtained from human blood derivatives. However, Biotechnology innovations have made it possible to produce large quantities of hFVIII proteins from the culture medium of rodent or human cells contained with the hFVIII expression cassette (21). Turoctocog alfa pegol (N8-GP), a new extended half-life FVIII product expressed in the CHO cell line, was approved by the FDA in 2019 (22, 23), Efmoroctocog alfa (Eloctate®) is an FDA-approved BDD FVIII-fusion protein (rFVIIIFc) produced in the HEK cell line (23). One of the most common mammalian cell lines used for therapeutic protein production is NS0 (24), In the current study, we use this cell line for the first time as a host for hFVIII expression. As shown by Shabani *et al*. the lipofectamine is the best reagent for NS0 transfection (25), so we used this reagent and cells were transiently transfected.

In terms of applying heterologous expression systems, FVIII is one of the most challenging therapeutic proteins (26); also, the need for the production of recombinant FVIII has increased due to low expression levels of FVIII in other protein expression systems and a growing demand for prophylactic treatment of hemophilia A patients (27). Several methods have been developed to prevent cell cycle progression in mammalian cells to increase yields of recombinant proteins (13), the

individual and combined effects of low temperature, BLIMP1 expression, and NaBu treatment on CHO cells, reduced cell growth and arrested cell cycle phases in G1/G0, while increasing cell productivity (28). Sodium butyrate and sodium propionate increased rBDD-FVIII production more than sixfold (29). The effects of reducing the temperature to 31 ºC with valeric acid supplementation have been evaluated, and an increase in the specific cellular efficiency of FVIII expression levels up to 2.4-fold was obtained compared to the control group performed at 37 ºC (30). NS0 6A1/4–9F myeloma cell line, which expresses chimeric IgG4 antibodies and induces the expression of p21CIP1 cyclin-dependent kinase inhibitor, enhanced productivity of continuous mammalian cell cultures by arresting the cell cycle in G1 phase (31).

We decided to use Mitomycin C, Lovastatin, and Metformin in a novel way to increase rFVIII expression level in NS0 cell lines. Lovastatin has been shown to arrest various types of cells, including adherent and suspension cells, in the early G1 phase. As previous studies showed, Lovastatin, as an HMG-CoA reductase inhibitor, was used at a final concentration of 20 µM to Synchronize HeLa Cells and the Cell Cycle was arrested in the S phase by double thymidine blocking (32); furthermore, Metformin inhibits cell proliferation by inducing autophagy and arresting the cell cycle in the G0/G1 phase. Treated HEK293 cells with 20 mM Metformin reduced proliferation by 38% (19). Mitomycin C also arrests cells in the G1 phase of the cell cycle. Treating cells with 10 µg. mL^{-1} Mitomycin C for 30 minutes significantly reduced the central damage of Langerhans islet cells (16).

There could be several reasons that FVIII gene expression patterns obtained from RT-PCR, ELISA, and Dot blot do not match. One possible reason could be post-transcriptional or post-translational modifications that affect the stability or activity of the protein. The protein could be degraded or modified after translation, which could affect its abundance or activity. Additionally, there are some differences in the sensitivity or specificity of the three assays. Based on time line of transcription and then translation in cells, we assume that the FVIII expression pattern of RT-PCR at 24 h is almost similar to the expression pattern of ELISA at 48 h. The expression pattern of FVIII at 72 h with ELISA may be similar to that at 48 h with RT-PCR.

RT-PCR results demonstrated significant increased expression of Metformin at 24 & 48 h and Mitomycin C at 48 h, and Lovastatin has lower increased expression compared to the other drugs. According to the ELISA results at 48 h, a significantly increased expression level of Metformin (4.2-fold) and Lovastatin (3.5-fold) Was obtained, and Mitomycin C (2-fold) had lower increased expression. It can be concluded that the results of all three assays for Metformin showed higher increases in expression and confirmed each other's data. Nevertheless, more research and investigation will be necessary due to the lack of previous studies regarding the increased expression of recombinant proteins with these drugs and for investigating optimal treatment dosage and time and interpreting different functions of these drugs. We also suggest performing supplementary assays such as SDS_PAGE/Western.

6. Conclusion

According to the obtained results, it can be concluded that the NS0 cell line can express rhFVIII and can be a suitable host for the production of this recombinant protein due to its unique properties. Mitomycin C, Metformin, and Lovastatin can be used for increased production of recombinant proteins and improve the efficiency of production systems.

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Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Statements and Declarations

Not applicable.

Conflict of Interest

The authors declare no conflict of interest

Author Contributions

M.Z, E.F-SH, and G.A.K; Formal analysis, Investigation, Data curation, visualization, and writing original draft preparation. E.F-SH, and G.A.K; Conceptualization,

Methodology, Validation, and Project administration. M.Z, E.F-SH, and G.A.K; writing review and editing. E.F-SH, and G.A.K; Supervision and Funding acquisition. All authors read and approved the final manuscript.

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Ethical Approval
This study was an MSc. thesis supported a part by Babol University of Medical Sciences [Ethic no. IR.MUBABOL.REC.1399.022] and a part by Tehran University of Medical Science [Ethic no. IR.TUMS. IAARI.REC.1399.003].

Consent to Participate
Not applicable.

Consent to Publish
Not applicable.

References

1. Mehta P, Reddivari AKR. Hemophilia. 2022 Sep 30. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023
2. Seaman CD, Xavier F, Ragni MV. Hemophilia A (factor VIII deficiency). *Hematol Oncol Clin North Am*. 2021;**35**(6):1117-1129. doi: 10.1016/j.hoc.2021.07.006.
3. Soucie JM, Evatt B, Jackson D, Investigators HSSP. Occurrence of hemophilia in the United States. *Am J Hematol*. 1998;**59**(4):288-294.
4. Weyand AC, Pipe SW. New therapies for hemophilia. *Blood*. 2019;**133**(5):389-398. doi: 10.1182/blood-2018-08-872291.
5. Jiang R, Monroe T, McRogers R, Larson P. Manufacturing challenges in the commercial production of recombinant coagulation factor VIII. *Haemophilia*. 2002;**8**:1-5. doi: 10.1046/j.1351-8216.2001.00115.x.
6. Tahmoorespur M. Evaluation of Different Expression Systems for the Production of Pharmaceutical Recombinant Proteins with Emphasis on Mammalian Cells. *J Biosafe*. 2016;**9**(1):51-65.
7. Baldi L, Hacker DL, Adam M, Wurm FM. Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. *Biotechnol Lett*. 2007;**29**:677-684. doi: 10.1007/s10529-006-9297-y
8. Kumar SR. Industrial production of clotting factors: Challenges of expression, and choice of host cells. *Biotechnol J*. 2015;**10**(7):995-1004. doi: 10.1002/biot.201400666.
9. Tripathi NK, Shrivastava A. Recent developments in bioprocessing of recombinant proteins: expression hosts and process development. *Front Bioeng Biotechnol*. 2019;**7**:420. doi: 10.3389/fbioe.2019.00420.
10. Lindskog EK, Fischer S, Wenger T, Schulz P. Host Cells. Biopharmaceutical Processing: *Elsevier*; 2018. **p**. 111-130. doi: 10.1016/b978-0-08-100623-8.00006-2.
11. Radhakrishnan D, Wells EA, Robinson AS. Strategies to enhance productivity and modify product quality in therapeutic proteins. *Curr Opin Chemeng*. 2018;**22**:81-88. doi: 10.1016/j.coche.2018.09.005.
12. Kumar N, Gammell P, Clynes M. Proliferation control strategies to improve productivity and survival during CHO based production culture: a summary of recent methods employed and the effects of proliferation control in product secreting CHO cell lines. *Cytotechnology*. 2007;**53**:33-46. doi: 10.1007/s10616-007-9047-6.
13. Sunley K, Butler M. Strategies for the enhancement of recombinant protein production from mammalian cells by growth arrest. *Biotechnol Adv*. 2010;**28**(3):385-394. doi: 10.1016/j.biotechadv.2010.02.003.
14. Kardar GA, Zomorodipour A, Moin M, Pourpak Z, Sadeghi M, Ataei F. The effects of novel mutations in A1 domain of human coagulation factor VIII on its secretion level in cultured mammalian cells. *Iran J Biotechnol*. 2010;**8**(3):139-149.
15. Barsov EV. Immortalization of Human and Rhesus Macaque Primary Antigen-Specific T Cells by Retrovirally Transduced Telomerase Reverse Transcriptase. *Curr Protoc Immunol*. 2011;**95**(1):7.21. doi: 10.1002/0471142735.im0721 bs95
16. Saito T, Saito T, Sato Y, Ise K, Anazawa T, Oshibe I, *et al*. Mitomycin C treatment significantly reduces central damage of islets in culture. *Pancreas*. 2012;**41**(2):245-252. doi: 10.1097/mpa.0b013e31822461c7
17. JavanMoghadam-Kamrani S, Keyomarsi K. Synchronization of the cell cycle using lovastatin. *Cell Cycle*. 2008;**7**(15):2434-2440. doi: 10.4161/cc.6364
18. Ma HT, Poon RY. Synchronization of HeLa cells. Cell Cycle Synchronization: *Methods Protoc*. 2011:151-161. doi: 10.1007/978-1-61779-182-6_10
19. Safari Z, Safaralizadeh R, Seyedzadeh MH, Orang AV, Zare A, Feizi MAH, *et al*. The induction of Metformin inhibitory effects on tumor cell growth in Hypoxic condition. *Iran J Allergy Asthm*. 2015:605-614.
20. Li B, Zhou P, Xu K, Chen T, Jiao J, Wei H, *et al*. Metformin induces cell cycle arrest, apoptosis and autophagy through ROS/JNK signaling pathway in human osteosarcoma. *Int J Biol Sci*. 2020;**16**(1):74. doi: 10.7150/ijbs.33787
21. Kingdon HS, Lundblad RL. An adventure in biotechnology: the development of haemophilia A therapeutics–from whole-blood transfusion to recombinant DNA to gene therapy. *Biotechnol Appl Bioc*. 2002;**35**(2):141-148. doi: 10.1042/ba20010082.
22. Giangrande P, Andreeva T, Chowdary P, Ehrenforth S, Hanabusa H, Leebeek FW, *et al*. Clinical evaluation of glycoPEGylated recombinant FVIII: efficacy and safety in severe haemophilia A. *Thromb Haemostasis*. 2017;**117**(02):252-261. doi: 10.1160/th16-06-0444.
23. Prezotti AN, Frade-Guanaes JO, Yamaguti-Hayakawa GG, Ozelo MC. Immunogenicity of Current and New Therapies for Hemophilia A. *Pharmaceuticals (Basel)*. 2022;**15**(8):911. doi: 10.3390/ph15080911.
24. Estes S, Melville M. Mammalian cell line developments in speed and efficiency. *ADV Biochem Eng Biot*. 2014:11-33. doi:

10.1007/10_2013_260.
25. Shabani M, Hemmati S, Hadavi R, Amirghofran Z, Jeddi-Tehrani M, Rabbani H, *et al*. Optimization of gene transfection in murine myeloma cell lines using different transfection reagents. *Avicenna J Med Biotechnol*. 2010;**2**(3):123.
26. Orlova NA, Kovnir SV, Gabibov AG, Vorobiev II. Stable high-level expression of factor VIII in Chinese hamster ovary cells in improved elongation factor-1 alpha-based system. *BMC Biotechnol*. 2017;**17**:1-15. doi: 10.1186/s12896-017-0353-6.
27. Hay J, Ernst R, Kessler C. Cost-effectiveness analysis of alternative factor VIII products in treatment of haemophilia A. Haemophilia: *World Federation of Hemophilia*. 1999;**5**(3):191-202. doi: 10.1046/j.1365-2516.1999.00308.x.
28. Torres M, Dickson AJ. Combined gene and environmental engineering offers a synergetic strategy to enhance r-protein production in Chinese hamster ovary cells. *Biotechnol Bioeng*. 2022;**119**(2):550-565. doi: 10.1002/bit.28000.
29. Chun B-H, Park S-Y, Chung N, Bang W-G. Enhanced production of recombinant B-domain deleted factor VIII from Chinese hamster ovary cells by propionic and butyric acids. *Biotechnol Lett*. 2003;**25**:315-319. doi: 10.1023/A:1022301528118.
30. Coronel J, Heinrich C, Klausing S, Noll T, Figueredo-Cardero A, Castilho LR. Perfusion process combining low temperature and valeric acid for enhanced recombinant factor VIII production. *Biotechnol Progr*. 2020;**36**(1):e2915. doi: 10.1002/btpr.2915.
31. Ibarra N, Watanabe S, Bi JX, Shuttleworth J, Al-Rubeai M. Modulation of cell cycle for enhancement of antibody productivity in perfusion culture of NS0 cells. *Biotechnol Progr*. 2003;**19**(1):224-228. doi: 10.1021/bp025589f.
32. Ma HT, Poon RY. Synchronization of HeLa cells. Cell Cycle Synchronization: *Methods Protoc*. 2017:189-201. doi: 10.1007/978-1-4939-6603-5_12.