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Expression and Synthesis of Recombinant Human Coagulation Factor VIII Using a Cell-Free Expression System

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ABSTRACT

Background and Objective: Hemophilia A is the most common type of this disease. For the treatment of hemophiliacs, the injectable concentrate of human factor VIII, derived from plasma and recombinant, is available in the market. Due to the risk of contracting infectious diseases due to blood transfusions or donated blood products, including plasma-driven factor VIII, recombinant factor VIII can be used, which is safe and has a higher purity than the blood-derived sample, while showing a longer half-life in the blood of consuming patients. The aim of this study is to evaluate the expression and synthesis of recombinant human factor VIII using a cell-free eukaryotic expression system as a new strategy for the synthesis of recombinant drugs.

Methods: In this experimental study, pcDNA3 vector containing the gene fragment without B-domain of blood coagulation factor VIII was transformed into susceptible DH5α bacteria. After the multiplication of the bacteria and the increase of the target vector, the amplified vector was extracted at this stage and its quality and concentration were determined. Then CHO-K1 eukaryotic cells were cultured and cell extract was prepared from the density of more than ten million of their cells. The extracted vector was combined with cell extract and expression solution, and the expression process was carried out at 30°C and 37°C. The level of expression was evaluated by ELISA and dot blot techniques.

Findings: Based on the ELISA technique adopted in this study, the expression level of recombinant human blood coagulation factor VIII in the base sample at a temperature of 30°C, sample 2 at a temperature of 30°C, base sample at a temperature of 37°C, and sample 4 of negative control at a temperature of 30°C were respectively reported as 275, 276, 273 and 0 ng/ml in a period of 20 hours. In the dot blot technique, the mentioned quantitative results were qualitatively confirmed by the color intensity of the spots.

Conclusion: The results showed that the cell-free expression method can be used for the synthesis of recombinant factor VIII. Under the conditions used in this research for cell-free expression of recombinant factor VIII, volume ratio of 50/50 of CHO-K1 cell extract was suitable, and the basic expression solution at 30°C showed the highest concentration of the product. Due to the use of CHO-K1 eukaryotic cell extract, the synthesized recombinant human factor VIII has post-translational modifications, which are very important for the functionality of this protein in the human body.

Keywords: *Recombinant Human Factor VIII, CHO-K1 Cell Line, Cell Extract, Cell-Free Expression.*

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Introduction

Factor VIII (FVIII) is one of the most complex plasma glycoproteins which plays a key role in the intrinsic pathway of blood coagulation (1). Deficiency of circulating factor VIII results in hemophilia A, an X-linked bleeding disorder that occurs in homozygous males and females (2). Factor VIII gene is located on the X chromosome (Xq28). Mutations in the gene encoding factor VIII occur almost exclusively in male germ cells. The result of this mutation is the absence or reduction of the synthesis of factor VIII or the synthesis of an abnormal protein (3, 4). About 10,000 new cases of hemophilia A are reported worldwide each year, with a prevalence rate of 1 in 5,000 in males (5).

The basis of the treatment of hemophilia is to increase the activity of insufficient coagulation factor in order to prevent bleeding. Drugs used in prevention and treatment include: plasma-driven human factor VIII concentrate, recombinant factor VIII made by genetic engineering technology, desmopressin acetate (DDAVP) (derived from antidiuretic hormone), and other drugs such as antifibrinolytics and topical hemostatic drugs. In the conventional treatments of hemophilia A, except for the complications and diseases caused by the transmission of unknown infectious agents, it is possible to mention the creation of inhibitory antibodies against factor VIII injected in the patient's blood. The prevalence of inhibitor in patients with severe hemophilia A is approximately 15-35% (6-8).

There are three generations of drugs for recombinant human factor VIII. The first generation of medicine includes recombinant factor VIII, which is similar to its natural type in terms of biochemical and pharmacokinetic properties. So far, no cases of infection with HBV, HCV and HIV have been reported regarding the use of these medicinal formulas. However, the risk of transmission of viral particles in the case of first-generation drugs has not yet been fully resolved. Human and animal proteins used in culture media, and human albumin used as a final product stabilizer, still have the potential for infection. In the second generation, human albumin has been replaced by carbohydrate compounds that play a stabilizing role. Since the culture medium contains human protein, and can serve as a source of infection, the second generation enters a viral inactivation process. The noteworthy point in the second generation is the omission of the B-domain. The expression of factor VIII mRNA without B-domain in CHO-K1 (Chinese hamster ovary) cells is 20 times higher than the expression of normal factor VIII mRNA. The culture medium is not enriched with human or animal proteins and instead of albumin, carbohydrate is used as a stabilizer. Nowadays, research is being done on the new generation of this product, which uses ligands produced by chemical processes instead of mouse monoclonal antibody in the purification process (9).

The third generation of recombinant coagulation factor VIII has provided a drug for hemophilia that virtually eliminates the risk of transmission of viral particles (10). Recombinant factor VIII production in human cell line has theoretical advantages that have been realized in practice. This product includes more complete post-translational modifications, such as tyrosine sulfation, which results in strengthening the binding to Von Willebrand factor and a relatively small increase in its lifespan. This action also leads to the absence of sugar structures Galα1-3Galβ1-GlcNAc-R (alpha-Gal) and N-glycolylneuraminic acid in factor VIII, which cause an immune response in certain conditions (11, 12).

The basis of the expression using a cell-free expression system was introduced by Eduard Buchner, not for protein synthesis, but for the conversion of sugar into ethanol and carbon dioxide. More than 60 years later, Nirenberg and Matthaei developed a cell-free expression system based on *E. coli* bacteria to study translation-related processes. This system paved the way for many of the sophisticated expression systems available today (13). Protein expression in vivo requires significant amounts of time and resources. In contrast, the cell-free expression system provides faster access to the desired protein. In addition, this method can be used in the expression of hard-to-express proteins (such as toxic proteins and transmembrane

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proteins) and proteins with improved characteristics using non-standard amino acids (14). The inherent ability of cells to produce recombinant proteins is limited due to the consumption of a significant portion of cellular resources for cell reproduction and survival (15). Today, with the invention of the cell-free expression system, due to the variety and portability of these systems, the potential of using them even outside the laboratories has increased (16). Moreover, by adding reporter genes and using them in this system, this system can be used in virus detection, metabolic modeling, toxin detection, biosensors, and genetic circuit validation (17, 18). Furthermore, in the production of biological materials, including vaccines, due to the fact that current vaccine production strategies are time-consuming and there is a risk of viral contamination, immunogenicity and low stability, it is recommended to use cell-free expression systems, which have extracellular transcription and translation processes, faster protein expression and synthesis, and easier optimization (19). The cell-free expression system can also be lyophilized for long-term use (20).

Among the cell-free expression systems, the cell-free expression system based on *E. coli* bacteria has the most usage and the highest efficiency in protein production (21). However, as our target protein is a eukaryotic protein and post-translational changes do not happen in the prokaryotic expression system, a eukaryotic host must be used. In eukaryotic systems, wheat germ, yeast, rabbit reticulocyte, insect, HeLa and CHO cells are used, and among these cells, CHO and HeLa cells show better post-translational modifications (21). Among CHO and HeLa cells, CHO cells need less facilities for growth and reproduction and are more economical. So far, recombinant factor VIII has not been produced using a cell-free expression system. As the production of this factor using this method is much simpler and safer, we decided to investigate the possibility of producing this protein by cell-free expression in this research.

Methods

In this experimental study, the eukaryotic cell line CHO-K1 was used after being approved by the Ethics Committee of Babol University of Medical Sciences with the code IR.MUBABOL.REC.1399.023. Different concentrations of some substances and factors effective in expression were used in a group including 4 samples of expression and synthesis of recombinant factor VIII.

Bacterial strain, cell line and plasmid: DH5α strain of *Escherichia coli* bacteria (StrateGene, USA) was used as a prokaryotic host for cloning steps. CHO-K1 cell line (Pasteur Institute, Iran) was used as a eukaryotic host to express hFVIII. pcDNA3-hFVIII plasmid was prepared from a previous study (22) and was used as a source for hFVIII cDNA without B-domain and as an expression plasmid.

Media, enzymes, chemicals and kits: LB agar medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0 with NaOH; purchased from Merck, Germany) as bacterial culture medium was used and ampicillin antibiotic powder (100 mg/ml) was added if needed to maintain the selective pressure. KpnI enzyme was purchased from Roche, Germany. CHO-K1 cells were grown in RPMI-1640 with 10% Gibco heat-inactivated fetal bovine serum and Pen-Strep 1X (Sigma, Germany). Anti-Factor VIII antibody (clone GMA-012) (Sigma, Germany) was used to measure hFVIII antigen and plasma-derived factor concentrate (Biotest, Germany).

Cloning of pcDNA3-BDD-hFVIII in bacterial host DH5a and plasmid extraction: DNA manipulation techniques, such as plasmid DNA extraction, DNA digestion, and cloning, were performed according to standard procedures (23). Heat shock method was used to transfer the recombinant vector into *E. coli* bacteria. The recombinant plasmid (containing the factor VIII coding gene) was transferred to the *E. coli* DH5α strain. Then the transformed bacteria were isolated after culture on LB agar culture medium containing ampicillin to be used in the next steps. Recombinant plasmid was also confirmed using kpnI

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enzyme digestion method. Then, using the instructions of the GeneAll Exprep Plasmid SV mini kit, plasmid extraction was performed from the transformed colonies.

Cell culture: In this study, the eukaryotic cell line CHO-K1 was used, which has a pseudo-epithelial morphology and grows adherently in the culture medium. The suitable conditions for the cultivation of this cell line are RPMI medium with 10% FBS in a 37°C incubator with 5% CO2 and 95% humidity.

Preparation of cell extract: The cells obtained from the cell flasks were collected and centrifuged at 100 g for 10 minutes. In the next step, they were washed with Wash Solution (Shafamel Company, Iran). Then the cells were immersed in the extraction solution (Shafamel Company, Iran) which had already reached room temperature. After taking this step, the cell supernatant was collected. The supernatant was collected and stored as cell extract for further steps.

Expression stage: In this stage, the expression solution, which consists of 18 different substances, was used to express the factor VIII gene (Shafamel Company, Iran). The cell extract obtained with an equal ratio was placed next to the prepared expression solution. These microtubes were incubated for 20 hours at 30 and 37°C (23).

50% v/v …… Cell extract

50% v/v …… Expression solution (solution X)

Dot blot technique: PVDF paper (Sigma, Germany) was used for this purpose. Factor VIII sample derived from plasma (Biotest, Germany) was prepared commercially and used as a standard (Figure 1-A), and was then exposed to air at room temperature to dry completely. In this technique, biotinylated antibody against factor VIII (Sigma, Germany) was used. To prepare the membrane, it was immersed in methanol for 5 minutes and then placed in PBS solution. Then it was placed on a suitable surface and marked with a marker. Samples and controls were placed on the paper in the amount of 3.5 microliters as spots. Factor VIII sample derived from commercial plasma was prepared and used as a standard. Then they were exposed to air at room temperature to dry completely. Then the paper was placed on the shaker and in the vicinity of the blocking solution for one hour at room temperature. Then the paper was washed three times and each time for 30 seconds with PBS/T solution. In the next step, the membrane was incubated with biotinylated anti-factor VIII antibody diluted 1:400 in PBS/T in a shaker for 1 hour and washed three times with PBS/T solution for 30 seconds each time. Then, the membrane was treated for 1 hour with Streptavidin HRP conjugate with a dilution of 1:2000 in PBS/T on a shaker and washed three times with PBS/T for 30 seconds each time. Then the substrate solution containing 150 microliters of DAB and 150 microliters of Peroxidase buffer was poured on the membrane and washed with distilled water after 15 minutes and spots appeared.

Measurement of FVIII antigen by ELISA technique: rhFVIII antigen in the culture medium was measured by ELISA method (Sandwich ELISA) on a microplate, covered with mouse monoclonal anti-hFVIII antibody. First, the expression solution containing FVIII was added to the wells and after one hour of incubation at 37 degrees, it was washed three times with washing solution (phosphate buffer containing 0.05% Tween 20). Then, monoclonal mouse anti-biotin antibody was added to the wells, and after incubation and washing, streptavidin enzyme was added to the wells, and after the same incubation as the previous steps and washing 5 times, TMB Stain was added and the reaction was stopped with 2N sulfuric acid. The obtained color was read at a wavelength of 450 nm. The plasmid-free expression solution was considered as negative control.

All the tests of this study were repeated three times, and in order to compare the mean quantitative results, Tukey's post hoc analysis of variance was used to examine the expression of recombinant human FVIII protein, and $p<0.05$ was considered significant.

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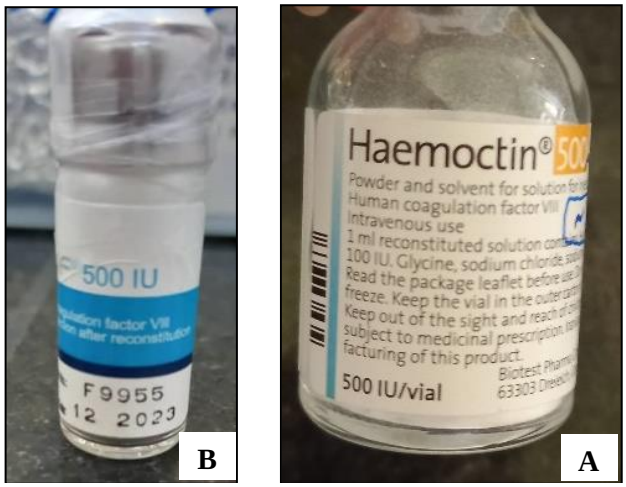


Figure 1. A: Plasma-derived commercial factor VIII, B: Anti-factor VIII antibody

Results

Plasmid cloning, extraction and digestion: The recombinant plasmid was propagated in the bacterial host. The presence of a 10 kb band in the electrophoresis results of the extracted plasmids confirmed the quality of the plasmids (Figure 2-A). The extracted plasmid, corresponding to the FVIII cDNA size (about 2 kb), was also confirmed by kpnI digestion of the recombinant plasmid. The selected plasmid was considered for expression analysis (Figure 2-B).

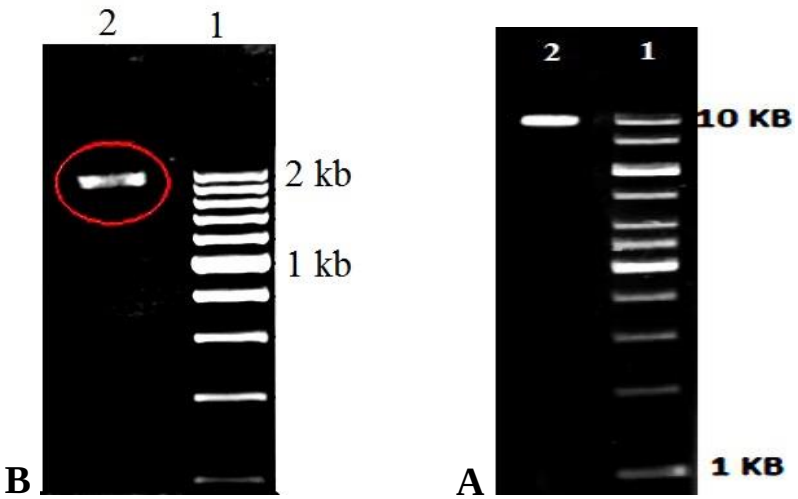


Figure 2. A: The band resulting from linearized plasmid electrophoresis, B: The band resulting from enzyme digestion by gel electrophoresis (column 1: ladder, column 2: sample)

CHO-K1 eukaryotic cell line culture: CHO-K1 cells were examined in terms of morphology and number, and after reaching 80% density, they were passaged to several flasks to reach a number of 50 million cells (Figure 3).

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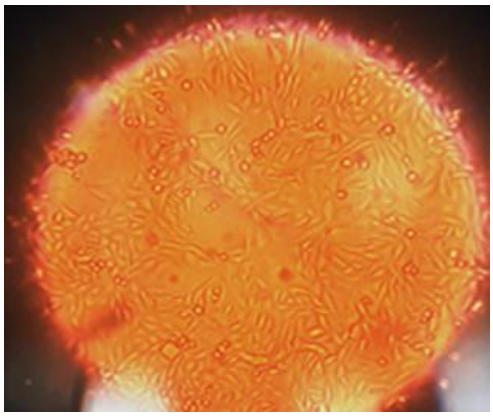


Figure 3. Microscopic and macroscopic examination of CHO-K1 cells one week after thawing

Analysis of the expression of recombinant human factor VIII: the expression solutions were removed from the incubator after 20 hours and then the dot blot technique was used to confirm the expression. The basic cell-free expression method left a rich stain on PVDF paper. Moreover, to compare the expression level in the samples, temperatures of 30 and 37 degrees Celsius were tested. The negative control sample (sample 1) has no stain, and the stain corresponding to the base sample at 30°C in the dot blot technique was slightly richer than the sample incubated at 37°C (Figure 4). In order to analyze the results of the dot blot technique, the obtained results were confirmed using image J software (Figure 5).

According to the results obtained from the ELISA technique, the highest expression level of rFVIII in the expression solution for 20 hours was estimated to be about 276 ng/ml (Figure 6). In order to quantitatively assess the samples and to optimize the expression, the following 3 types of reactions were used along with the control (sample 4) (Table 1).

Sample 1: basic sample including expression solution and recombinant plasmid; 20 hours at 30 degrees Celsius

Sample 2: increasing the concentration of cell extract by 1.4 times and phosphate by 10 times; 20 hours at 30 degrees Celsius

Sample 3: basic sample including expression solution and recombinant plasmid; 20 hours at 37°C

Sample 4: sample of expression solution without adding plasmid (negative control); 20 hours at 30 degrees Celsius.

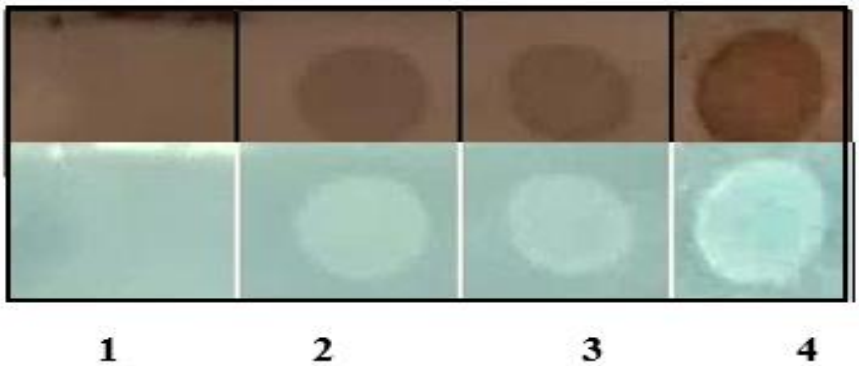


Figure 4. The results of the dot blot technique to evaluate the expression:
1. negative control, 2. sample incubated at 30°C, 3. sample incubated at 37°C, 4. positive control, commercial sample of plasma-derived factor VIII

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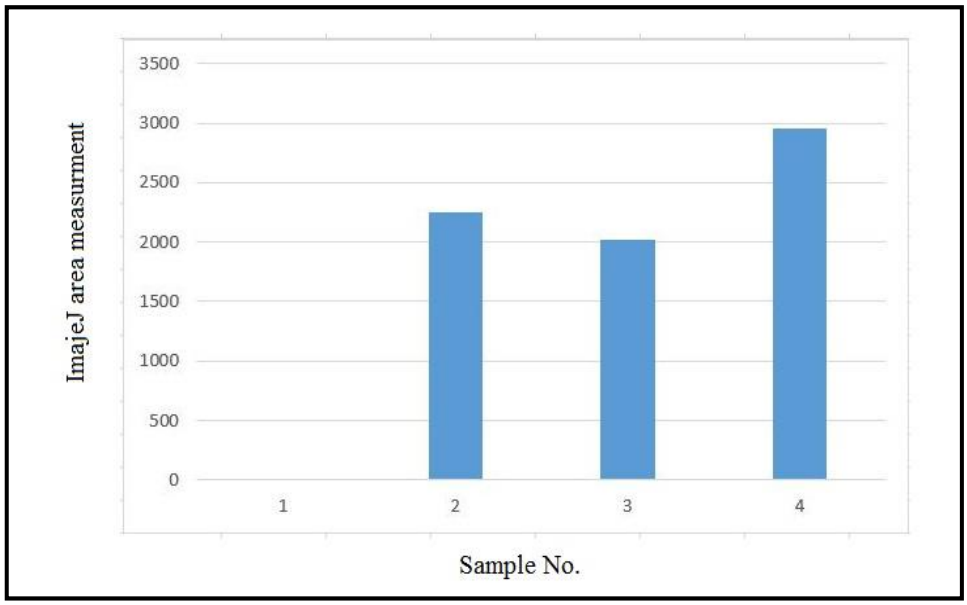


Figure 5. The result of dot blot technique analysis with image J software (1. negative control, 2. basic sample incubated at 30°C, 3. sample incubated at 37°C, 4. Plasma-derived positive control)

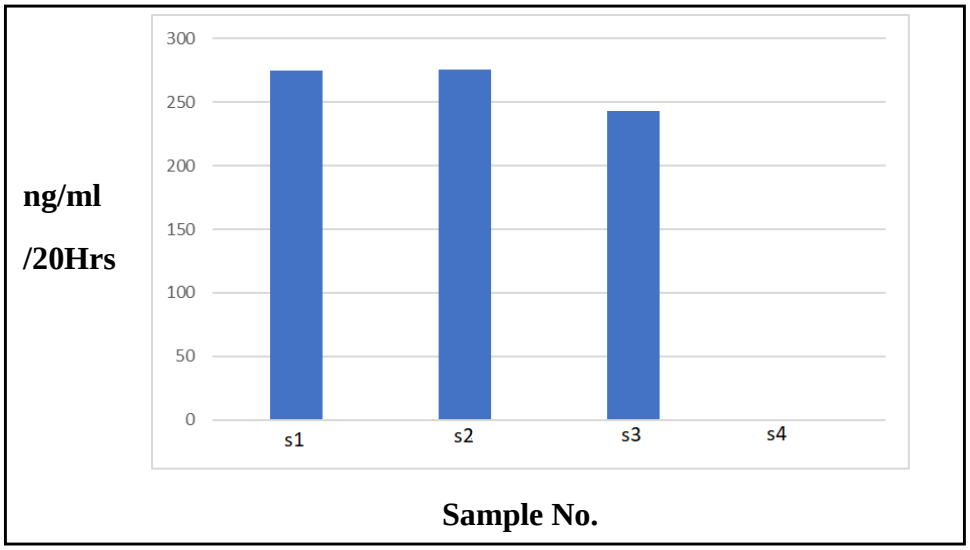


Figure 6. Quantitative investigation of FVIII expression in samples by ELISA technique (s1: sample 1, s2: sample 2, s3: sample 3, s4: sample 4)

Table 1. Comparison of factor VIII expression level in samples

Number of samples	The concentration of expressed recombinant factor VIII (ng/ml)
Sample 1	275
Sample 2	276
Sample 3	243
Sample 4	0

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Discussion

In this research, the expression and synthesis of recombinant human factor VIII was done using the cell-free expression method, and the best temperature for the cell-free expression reaction of this recombinant drug was 30 degrees Celsius, and the highest expression level was obtained by multiplying the cell extract by 1.4. According to the different ratios considered in this research, it seems that for this expression reaction, the basic state, i.e. 50/50 ratio of cell extract and expression solution, is suitable. Recombinant factor VIIIs available in the market are all based on in vivo system and intracellular expression, which are designed, produced and sold using HEK, CHO and BHK cell lines (Table 2) (23). The reason for using these systems is the appropriate post-translational modifications or PTM and the simpler conditions for the cultivation of these cell lines, especially the CHO cell line.

In this research, for the first time in the country, a eukaryotic cell-free expression system was used, and according to other studies around the world, expression of recombinant factor VIII has not been evaluated using this method. The cell-free expression method of recombinant drugs, including recombinant factor VIII, allows us to eliminate the need for the purification process of drugs, which is the most important part of the production process and requires a high cost, or a very simple and brief process of product purification is needed. In other studies, based on the cell-free expression method, proteins such as membrane proteins have been synthesized, and the concentration of these proteins has been reported as 49 µg/ml and 980 µg/ml (14). In our study, the expression and synthesis of this recombinant drug with a cell-free expression system was reported to be 276 ng/ml; the reason for this concentration being lower compared to the mentioned studies can be due to the very high molecular weight of this protein (280 kDa) compared to the common proteins, including membrane proteins, which have a lighter molecular weight. They are likely to be more difficult to express and synthesize than other smaller recombinant proteins.

Table 2. Recombinant factor VIII available in the market (based on cellular expression)

Host cell line	Genetic changes on the factor VIII genomic fragment	The date of receiving approval from the Food and Drug Organization	Product name
BHK	Full length	1993	Kogenate FS
BHK	Full length	1993	Hleixate FS
CHO	Full length	2003	Advate
CHO	BDD	2008	Moroctocog alfa (Xyntha)
CHO	B-domain truncated	2013	Turoctocog alfa (NovoEight)
HEK	Full length	2015	Simoctocog alfa (Nuwiq)
BHK	Full length	2016	Octocog alfa (Kovaltry)
CHO	B-domain and four amino-acids of a3 domain deleted	2016	rVIII-single (Afstyla)
HEK	BDD rFVIII with fused Fc	2014	rFVIIIFc (Eloctae)
CHO	Full-length rFVIII	2015	rFVIII-pegylated (Adynovate)

In the study of Brödel et al., using cell lysate with a concentration of 500 million cells per milliliter, luciferase protein was synthesized during a reaction time of 4 hours at a reaction temperature of 33°C (14). The reaction temperature of this study was almost similar to our study, but the duration of its expression was much lower than our study, which could be the simpler expression and synthesis of this protein with a

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much lower molecular weight than factor VIII protein. In another study, Thoring et al. used a concentration of 500 million cells per milliliter for the synthesis of membrane proteins from lysate, for a period of 51 hours and a reaction temperature of 30°C. Moreover, in this study, in order to optimize and maximize expression, continuous-exchange cell-free systems were used, which is based on a reaction and a separate feeding chamber separated by a semi-permeable membrane. As a result, energy reaches the reaction chamber permanently, and waste materials from the reaction, which can also inhibit the continuation of the reaction, are removed from the reaction chamber (24). Due to the high cost of this method, it was not possible to use this method in this study.

As a result, since the product obtained from the cell-free expression and synthesis method, unlike cloning and cell expression, does not require expensive purification, it can be very important in terms of time and economy. In the future, more expression of recombinant drugs can be done using the cell-free expression method at a lower cost and without the time-consuming and expensive cloning technique. Of course, a handful of American and European biotechnological commercial companies have started their activities in this field and, they have produced some cell-free expression kits for some human proteins so far.

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