



REVIEW



Production of recombinant coagulation factors: Are humans the best host cells?

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ABSTRACT

The main treatment option for Hemophilia A/B patients involves the administration of recombinant coagulation factors on-demand or in a prophylactic approach. Despite the safety and efficacy of this replacement therapy, the development of antibodies against the coagulation factor infused, which neutralize the procoagulant activity, is a severe complication. The production of recombinant coagulation factors in human cell lines is an efficient approach to avoid such complication. Human cell lines can produce recombinant proteins with post translation modifications more similar to their natural counterpart, reducing potential immunogenic reactions. This review provides a brief overview of the most important characteristics of recombinant FVIII and FIX products available on the market and the improvements that have recently been achieved by the production using human cell lines.

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Introduction

Hemophilia A/B are X-linked bleeding disorders caused by coagulation factors VIII (FVIII)/IX (FIX) deficiencies or dysfunction, characterized by decreased and delayed generation of thrombin, impairing clot formation and leading to hemorrhagic diathesis. In the severe cases, another frequent manifestation is non-traumatic (spontaneous) intra-articular bleeding (haemarthrosis). Chronic arthropathy, with loss of joint movement, can occur after repeated hemorrhagic episodes.¹

In the 1950s and early 1960s, the treatment available for the hemophiliac patients was based on the transfusion of whole blood or fresh plasma. In 1964, the clotting factors were identified and named. An article in *Nature* described the clotting process in detail, naming it as coagulation cascade.² Unfortunately, this treatment was not effective because these blood products had not enough factors VIII or IX to stop serious internal bleeding. Most severe hemophiliacs and some moderate or mild hemophiliacs died in childhood or early adulthood. The most common causes of death were bleeding in vital organs, especially the brain and a small bleeding after surgery or

following injury. Those who reached adulthood had serious deformations in the joints due to frequent bleeding. Hemophilia was one of the most painful diseases due to massive bleeding in joints and muscles.

The first major breakthrough in the treatment of Hemophilia was cryoprecipitate, which was discovered by Dr. Judith Pool in 1964. Dr. Pool found that slow thawing of frozen plasma formed a sediment containing FVIII.³ This allowed intravenous administration of more factor VIII in a smaller volume. For the first time, it was possible to obtain a sufficient coagulation factor VIII for controlling severe bleeding, enabling even surgical interventions. Cryoprecipitate became widely available to patients with Hemophilia A and also for patients with von Willebrand's disease and deficiency of fibrinogen or factor XIII. Thousands of patients have benefited.

By the late 1960s, industries began to develop different methods (often including cryoprecipitation) to separate FVIII and FIX from pooled plasma, resulting in concentrated lyophilized preparations of FVIII or FIX.^{4,5} The availability of these products has completely changed the lives of hemophiliacs, that were able to perform the treatment at home; becoming

independent of hospitals and having a better quality of life. Tragically, these products, obtained from hundreds of donations combined to form a plasma pool, were responsible for the transmission of blood-borne viruses such as hepatitis and HIV.

In 1984, the FVIII cDNA was cloned, allowing the production of recombinant FVIII, to avoid possible viral infections. This was certainly the second major breakthrough in the treatment of hemophilia A. Clinical trials in humans began 3 y later and in 1992, when the first recombinant factor VIII product (Recombinate[®]) was licensed, being the treatment of hemophiliacs revolutionized bringing more safety and efficacy to the patients.

Currently, 12 recombinant FVIII (Table 1) and 5 recombinant FIX (Table 2) products are available on the market. Despite the knowledge acquired over more than 25 y of industrial production, recombinant coagulation factors can still be considered a challenge for the industry, which is continuing to improve the manufacturing processes to offer an optimized and cost-effective treatment of the patients. This review provides a brief overview of the most important characteristics of recombinant FVIII and FIX products available on the market and the impact of using human cell lines for their production on the efficacy of treatment.

Commercially available recombinant coagulation factors

Recombinant factor VIII products

The first recombinant coagulation factor VIII, Recombinate[®], was launched in 1992 by Genetics Institute and Baxter Healthcare Corporation, Hyland Division. Kogenate[®], made by Bayer Healthcare Pharmaceuticals, was launched few months later, in 1993.⁶ Both products were developed using animal-derived proteins in the cell culture medium and human serum albumin in the final formulation, being considered as first-generation products.⁷ The risk of exposure to transmissible agents (nonenveloped viruses, hepatitis A, and parvovirus B19, Creutzfeldt–Jakob agent and its variant, or yet unknown agents) has led to the development of the second-generation products, in which the culture medium was supplemented with human-derived proteins instead of animal-derived proteins (fetal bovine serum) and no albumin was added to the final formulation. In this case, new

technologies were used to stabilize the FVIII molecule, with sucrose and trehalose, for example.⁸ Table 1 lists the recombinant FVIII-based products that have reached the market since 1992, and the main characteristics of each one.

Third-generation products involve those in which no animal or human proteins for cell cultivation and purification are used. Some studies have shown that patients treated with second-generation rFVIII products presented a higher risk of inhibitor development when compared with third-generation products.^{9–11} FVIII inhibitor is an immunoglobulin G (IgG) produced by patient that has high polyclonal affinity directed against the FVIII protein.¹² Inhibitory antibodies are directed usually against the A2, A3 and C2 FVIII domains.^{13,14} The binding of an inhibitor in these domains results in a steric blockade of the functional epitopes of FVIII.¹⁵

The mechanisms, involved in this different inhibitor incidence, remain unclear and have not been elucidated by the studies. However, it is possible that changes in the manufacturing process can lead to changes in the immunogenicity of the product.¹⁰ Despite this supposed lower risk of inhibitor development, some of the third-generation products use murine derived-mAbs during downstream processing (affinity chromatography). Although efficient, the use of murine mAb-derived affinity purification in the manufacturing process can raise the possibility of antigenicity when the product is infused into patients since residual mAb can be present in the effluent due to leaching from the support matrix used during chromatography. Besides there is a higher cost involved in mAb-based purification and issues related to stability since the antibody binding site may not be amenable to the rigorous conditions necessary to sanitize the column.¹⁶ The most recent rFVIII products approved (Eloctate[®], Nuwiq[®], Kovaltry[®]) use recombinant proteins produced in *S. cerevisiae* as affinity ligand or peptides to avoid the disadvantages listed above. The use of peptide instead of mAb for affinity chromatography can bring as advantages also a higher efficiency and selectivity.¹⁷

Despite the quality of rFVIII third-generation products in terms of efficacy and safety, the treatment improving these products is still limited because of the short half-life (8–12 h), being necessary injections 3 times per week or every other day for prophylaxis and treatment.¹⁸ This situation is associated with the

Table 1. Recombinant coagulation factor VIII products approved for Hemophilia A treatment.

Product	Company	Generation	Cell line	Main characteristics	Approval year
Recombinate [®] (also market as Bioclate [®] by Aventis Behring)	Genetics Institute/Baxter (Currently Shire-Baxalta)	1	CHO	Full-length mAb (murine hybridome) –affinity chromatography Human albumin as stabilizer t _{1/2} =14.6h	1992, FDA
Kogenate [®] (also market as Helixate [®] by Aventis-Behring)	Bayer	1	BHK	Full-length mAb (murine hybridome) - affinity chromatography Human albumin as stabilizer t _{1/2} =12h	1993, FDA
Refacto [®]	Wyeth	2	CHO	B-domain deleted mAb (murine hybridome) - affinity chromatography Sucrose as stabilizer t _{1/2} =14.8h	1998, EMA 2000, FDA
Kogenate [®] FS (also market as Helixate [®] FS by Aventis-Behring)	Bayer	2	BHK	Full-length mAb (murine hybridome) - affinity chromatography Sucrose as stabilizer t _{1/2} =12h	2000, FDA
Advate [®]	Baxter	3	CHO	Full-length mAb (murine hybridome)-based affinity chromatography Trehalose and mannitol as stabilizer t _{1/2} =13h	2004, EMA 2007, FDA
Refacto [®] AF (also market as Xyntha outside EU)	Pfizer	3	CHO	B-domain deleted polypeptide ligand-based affinity chromatography Sucrose as stabilizer t _{1/2} =11-12h	2008, FDA
Novoeight [®]	Novo Nordisk	3	CHO	B-domain truncated mAb -based affinity chromatography Sucrose as stabilizer t _{1/2} =11-12h	2013, EMA
Adynovate [®]	Shire-Baxalta	3	CHO	Full-length, Pegylated mAb-based affinity chromatography Trehalose and mannitol as stabilizer t _{1/2} =13.4-14.7h	2015, FDA
Afstyla [®]	CSL Behring	3	CHO	B-domain truncated single chain protein mAb-based affinity chromatography Sucrose as stabilizer t _{1/2} =14.2-14.3h	2016, FDA
Eloctate [®]	Biogen Idec	4	Hek-293	B-deleted FVIII-Fc fusion protein (IgG1) Recombinant protein (produced in <i>S. cerevisiae</i>)-based affinity chromatography Sucrose as stabilizer t _{1/2} =19h	2014, FDA
Nuwiq [®]	Octapharma AG	4	Hek-293	B-domain deleted Recombinant Ab fragment (produced in <i>S. cerevisiae</i>)-based affinity chromatography Sucrose as stabilizer t _{1/2} =15h	2014, EMA 2015, FDA
Kovaltry [®]	Bayer	3	BHK expressing HSP 70	Full-length peptide ligand-based affinity chromatography Sucrose as stabilizer t _{1/2} =13-14h	2016, FDA

Abbreviations: CHO – Chinese Hamster Ovary cells; BHK – Baby Hamster Kidney cells; Hek-293; Human Embryonic Kidney cells; HSP 70 – heat shock protein 70.

lack of complete protection and higher treatment costs due to limited adherence of the patients to prophylaxis regimen. Prolonged half-life products, such as

Eloctate[®] and Adynovate[®], have the advantage of reducing the frequency of administration, thus improving the compliance of patients, and increasing

Table 2. Recombinant coagulation factor IX products approved for Hemophilia B treatment.

Product	Company	Generation	Cell line	Main characteristics	Approval year
BeneFIX [®]	Pfizer	3	CHO	Molecule with Ala-148 polymorphism Non mAb-based chromatography Sucrose as stabilizer $t_{1/2}=18.8\text{h}$	1997, FDA
Rixubis [®]	Baxter/Baxalta	3	CHO	Molecule with Ala-148 polymorphism Non mAb-based chromatography Mannitol/Sucrose as stabilizer $t_{1/2}=25.4\text{-}26.7\text{h}$	2013, FDA
Alprolix [®]	Biogen Idec	4	HEK-293	rFIX-IgG1 Fc fusion protein Protein A affinity chromatography Mannitol/Sucrose as stabilizer $t_{1/2}=82.1\text{h}$	2014, FDA
Ixinity [®]	Emergent Biosolutions	3	CHO	Molecule with Thr-148 polymorphism Mannitol/Trehalose as stabilizer $t_{1/2}=24\text{h}$	2015, FDA
Idelvion [®]	CSL Behring	3	CHO	rFIX-Albumin fusion protein Mannitol/Sucrose as stabilizer $t_{1/2}=92\text{-}94,8\text{h}$	2016, FDA

Abbreviations: CHO – Chinese Hamster Ovary cells; Hek-293; Human Embryonic Kidney cells.

the bleed-free time-span of the patients. Eloctate[®] has been developed by fusing the dimeric CH2–CH3 domains of the human IgG1 Fc to the recombinant B domain-deleted FVIII. In clinical trials, Eloctate[®] presented 1.5-fold extended half-life as well as reduced annualized bleeding rates as compared with its non-fused counterpart, with no apparent immunogenicity.¹⁹ Adynovate[®] was developed by PEGylation technology from the recombinant full-length FVIII Advate[®].²⁰ The half-life was extended 1.8-fold in primates compared with the non-pegylated product (Advate[®]).²¹ In the phase I clinical trial, the half-life of Adynovate[®] was 1.4–1.5 times-higher when compared with Advate[®].²² No clear increase in the incidence of FVIII inhibitor has been reported up to date for the rFVIII pegylated products. Indeed, pegylation has been shown to obliterate the immunogenicity of recombinant proteins after administration into animals, as compared with the non-pegylated counterparts.¹⁹ Some authors are considering this extended half-life products as fourth generation products,²³ however in this paper, we will consider as fourth generation, FVIII products expressed in a human cell line as Casademunt and coworkers 2012.²⁴

The new therapeutic alternative for patients with inhibitor is a humanized bispecific antibody that binds to and bridges activated factor IX (factor IXa) and factor X, thereby acting as a factor VIII-mimetic agent.^{25,26} Emicizumab has a unique structure and it is not affected by factor VIII inhibitors. Moreover, clinical trials results showed that this product is safe and a single weekly subcutaneous administration has the

potential to reduce or prevent bleeding episodes in patients with severe hemophilia A with or without factor VIII inhibitors.²⁷

Recombinant factor IX products

Although smaller and less complex than FVIII (415 amino acids, single-chain molecule), as a vitamin-K dependent protein, recombinant FIX requires, besides the post translational modification (PTM) common to glycoproteins, a gamma-carboxylation and a proteolytic processing by PACE/Furin (Paired basic Amino acid Cleaving Enzyme) for efficient protein expression.²⁸ The first commercial product, BeneFIX[®], was launched few years after rFVIII products, in 1997 by Pfizer, and for many years, it was the only recombinant rFIX product available for hemophilia B treatment. Since the development, no animal or human-derived proteins were used during manufacture or formulation, being the earliest third-generation recombinant clotting factor.^{28,29} Rixubis[®] and Ixinity[®], approved in 2013 and 2015, respectively, are also third-generation recombinant products produced in CHO cell lines. Ixinity[®], different from the other recombinant FIX products carrying an Ala-148 polymorphism, has a primary amino acid sequence with the predominant Thr-148 polymorphism. In the plasma-derived FIX, both variants can be found.³⁰

Hemophilia B, similarly to hemophilia A, requires constant infusions for prophylaxis and treatment, also leading to the development of prolonged half-life

products. Alprolix[®] from Biogen, established by direct fusion (no linker region) of a monomeric Fc domain from human IgG1 to the carboxyl terminus of human FIX (rFIXFc), was the first developed in this category, being approved by FDA in 2014. Alprolix[®] presented 3 to 4-fold prolongation of terminal half-life in pre-clinical studies (mouse, rat, dog, and monkey) and 3-fold prolongation in phase I-II clinical trials.³¹ In a phase III study, it presented a half-life of 82.1 h, representing an administration every 1–2 weeks.³² Since Alprolix[®] is produced in a human Hek 293 cell line, it can be considered a fourth-generation product, like rFVIII products Elocate[®] and Nuwiq[®]. Idelvion[®] from CSL Behring is another product in this category recently approved by FDA. This product was developed by fusing rFIX with a recombinant albumin. During clinical studies, Idelvion[®] presented approximately 5-fold increase in half-life compared with plasma-derived and other recombinant FIX product ($t_{1/2} = 92\text{--}94,8\text{h}$) This increased half-life can result in an extension of dosing interval from every 3–4 d (conventional rFIX products) to up to every 14 d.³³

Human cell-based production can provide innovative recombinant coagulation factors

Recombinant therapeutic proteins have been mainly produced in mammalian-based expression systems, being the Chinese Hamster Ovary cells (CHO), the predominant host used for commercial production.³⁴ This fact can be attributed to some key advantages: i) robust growth in chemically-defined and serum-free suspension culture, (ii) a reasonable safety profile regarding human pathogenic virus replication, (iii) ease generation of cell clones which are able to stably express the recombinant protein in sufficient yields and acceptable quality for human use, and (iv) ability to express recombinant proteins with human-like post-translational modifications.^{35,36} However, recombinant therapeutic proteins produced in non-human cells may contain glycan epitopes, mainly Gal α 1,3-gal (α -Gal) residues and N-glycolylneuraminic acid (Neu5Gc), that are antigenic to humans,^{34,37} and can potentially affect the efficacy of the recombinant product, since all humans tested have circulating antibodies against these epitopes.^{38,39} Although few adverse immunogenic reactions have been ascribed to CHO-derived recombinant proteins, this cell line possesses the enzymes (α 1,3 galactosyltransferase and CMP-

Neu5Ac hydroxylase) responsible for the formation of this non-human epitope.³⁴ Besides, it has been reported the presence of both Neu5Gc and α -Gal antigens in several commercially available FIX and FVIII products produced in CHO or BHK cells.⁴⁰

The formation of inhibitor antibodies against rFVIII/rFIX products is a severe complication, which is frequently observed in hemophilia therapy, and may render replacement therapy ineffective, especially in hemophilia A.^{24,41} Although the progress in the development of products and several immune tolerance induction (ITI) studies that have improved inhibitor management, it remains the major issue in the hemophilia treatment considering both the patient's perspective as well as clinician's and health-care provider's perspective.⁴² In a recent publication, Peyvandi et al. (2016) reported the results of a randomized trial to assess the incidence of FVIII inhibitors among patients treated with plasma-derived FVIII or recombinant FVIII (SIPPET, Survey of Inhibitors in Plasma-Product Exposed Toddlers).⁴¹ Only rFVIII products produced in CHO and BHK cells were used (Recombinate[®], Kogenate FS[®], Advate[®], and ReFacto AF[®]). The authors found that rFVIII products had nearly twice the rate of inhibitor development as to plasma-derived products. 37.3% of patients treated with rFVIII developed inhibitors versus 23.2% plasma-derived treated patients, corroborating previous related studies. Based on the results of this study, EMA has started a review of medicines containing factor VIII to evaluate the risk of developing inhibitor proteins in patients starting treatment of hemophilia A.

Aiming at a human-like glycosylation profile (similar to endogenous FVIII molecules), improving therefore function and reducing immunogenicity, Octapharma AG has developed the first rFVIII expressed in a human cell line (Hek293).^{24,43} Nuwiq[®] was approved in 2015 by the FDA, being the first fourth generation product. The cell cultivation is performed using a defined serum-free medium with no animal or human-derived supplementation; therefore, the risk for virus contamination in the final product is almost negligible. The purification process includes 5 chromatographic steps, including an affinity chromatography step with a recombinant protein as ligand, and 2 virus clearance steps with solvent/detergent treatment and nanofiltration to destroy/remove any theoretically occurring enveloped or non-enveloped

viruses.²⁴ Until now, no treatment related adverse effect or inhibitor formation was reported after Nuwiq[®] prophylaxis or on-demand treatment in children⁴⁴ and adults.^{45,46} with severe hemophilia A. Following the same trend, Biogen Idec also develop an rFVIII product (rFVIII Fc fusion protein, Eloctate[®]) expressed in Hek293 cells.⁴⁷

Although hemophilia B presents a lower risk of inhibitor development (1–5%) compared with hemophilia A (23–35%), the rFIX industry also considered the advantages of protein production in human cells. Besides the benefits of human PTM, Hek 293 cells were chosen for Alprolix[®] production due to greater capacity for γ -carboxylation and propeptide processing when compared with other mammalian cells.⁴⁸

Currently, few companies use Hek 293 cells in the commercial manufacture of their products, although widely used as expression system in scientific research. This cell line possesses good characteristics for commercial exploitation for recombinant protein production: ability to grow under serum-free suspension conditions, amenability to transfection, high yield protein production, apart from human-like posttranslational modifications.⁴⁹ Besides rFVIII and rFIX-based products, a Glucagon-1-like peptide (GLP-1) Fc fusion protein, Trulicity[®] (Eli Lilly), has been commercially produced in this cell line.^{48,49} Xigris[®], activated protein C, was the first recombinant therapeutic protein produced in Hek293 cells approved by the FDA (2001); however, it was voluntarily withdrawn from market in 2011 by its manufacturer (Eli Lilly).⁵⁰

In addition to Hek293 cells, novel human cell lines have been explored for improved recombinant FVIII production. Our research group showed that human hepatic cell lines, such as SK-HEP and HepG2 cells, have already been used for successful expression of rFVIII.⁵¹ The use of hepatic cell lines for rFVIII expression is motivated by the fact that FVIII hepatocyte and liver sinusoidal endothelial cells are generally considered the physiologic cellular origin for FVIII synthesis.⁵² When comparing FVIII secretion from a monkey kidney cell line (COS-7) to that of primary hepatocytes, it was found that in the COS-7 cell line rFVIII accumulated in the endoplasmic reticulum, what could be a consequence of insufficient glycosylation resulting in the misfolding of rFVIII. This result indicates that this cell line provides different glycosylation machinery from that in normal human hepatic cells.⁵³ SK-HEP-1 cells are particularly interesting due to their capacity to

express von Willebrand factor, which can help to stabilize the rFVIII produced. Indeed, the rFVIII produced by SK-HEP cells presented a higher *in vitro* and *in vivo* stability when compared with plasma-derived FVIII.⁵⁴ This cell line was already adapted to growth in serum-free chemically defined culture medium and showed high cell densities under scalable suspension cultures.⁵⁵ It has already been shown that SK-Hep cells could be an improved cell host for rFIX expression. This cell line did not express high amounts of VKORC1 and carboxylase, important enzymes for the production of gamma-carboxylated proteins (such as FIX), however this cell line secreted larger amounts of active protein when compared with Hek293T cells.⁵⁶ One possible drawback of this cell line for commercial manufacturing processes is its tumorigenic nature (adenocarcinoma). It is worth mentioning, nonetheless, that there are recombinant therapeutic proteins approved by regulatory agencies that are produced in tumorigenic cell lines, such as the human cell line HT-1080 (fibrosarcoma) and the murine NSO and Sp2/0 cell lines (myeloma).

The HKB-11 cell line is a hybrid of HEK293 and a human B cell line, 2B8 (a derivative of a Burkitt's lymphoma cell line) and was developed and patented by Bayer HealthCare.⁵⁷ This cell line grows well in serum-free suspension culture, shows high transfection efficiency, and secretes high levels of a variety of recombinant proteins with human-specific glycosylation profiles. Mei et al. (2006) showed that rFVIII expression in this cell line is 8 to 30-fold higher than the one obtained in other cell lines, such as HEK293 and BHK21 cells.⁵⁸ Bayer HealthCare is using this cell line for the production of Bay 94–9027 (damoctacog alfa pegol), a PEGylated recombinant factor VIII,^{40,58} which is currently in a phase III clinical trial (clinical-trials.gov).

Concluding remarks

The assessment of novel human cells lines for the production of recombinant coagulation factors can help to improve process productivity to make these products more affordable, as well as, to improve therapeutic efficacy, avoiding inhibitor development issues. It is worthy mentioning that in a not too distant future, gene therapy might be also an improved treatment modality; achieving a prolonged therapeutic effect with only one or a few treatments over a lifetime.

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

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