Updates on Novel Erythropoiesis-Stimulating Agents: Clinical and Molecular Approach

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Abstract Erythropoietin (EPO) is an important hormone responsible for the stimulation of hematopoiesis which is impaired in a variety of diseases, such as chronic kidney disease, cancer chemotherapy, and the use of some anti-HIV drugs. Difficulties in the purification of endogenous EPO due to problems such as technical limitations, heterogeneity of target cells, inadequate amount and immunogenicity of the resultant product, had limited the entry of endogenous EPO in the clinical applications. The integration of medical biotechnology and hematology has introduced novel procedures for the production of human recombinant erythropoietin (rHuEPO), and other erythropoiesis-stimulating agents (ESAs). To investigate and produce rHuEPO, the first step is to recognize the molecular biology and functional pathways, structure, metabolism, and basic physiology of EPO. In this review, all clinical indications, side effects, challenges and notable points regarding EPO, rHuEPO, and other ESAs have also been addressed along with its molecular

characterization, such as the modifications needed to optimize their rHuEPO biosynthesis.

Keywords Erythropoietin · Human recombinant erythropoietin · Erythropoiesis-stimulating agents

Introduction

Molecular Biology of Erythropoietin

EPO is a 30.4 kDa glycoprotein hormone with a protein portion consisting of 165 amino acids in the form of four antiparallel alpha helices, two beta sheet bonds, and two intrachain di-sulfide bridges in the Cys7–Cys161 and Cys29–Cys33 positions. The carbohydrate portion of the molecule accounts for 40% of the molecular weight; The carbohydrate structure forms the interaction of protein-carbohydrate network through the *N*- and *O*-linked glycosidic bonds. The *N*-glycoside bond is located at the asparagine 24, 38 and 83 residues. This type of bond protects EPO from the cleavage of proteolytic enzymes, and modulates the affinity of the hormone and its receptors. The *O*-glycosidic bond is located at residue position 126 (Fig. 1) [1]. The Carbohydrate component and the degree of EPO sialylation plays an important role in signaling and signal transduction to target cells. Different levels of negative sialylation result in various isoforms of glycoprotein [2, 3]. Erythropoietin hormone is sialic acid rich, and the baseline level of glycosylated EPO in serum is 5–25 IU/L that reaches 1000 times in severe anemia. The plasma half-life of EPO is 7–8 h, while non-glycosylated EPO is rapidly cleared from the circulation [4]. The total energy of the glycosylated EPO protein is 1128.54 kcal/mol, whereas the total of energy of single EPO and three units of glycans

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is 1327.63 kcal/mol [2, 3]. This suggests that the glycosylated form of EPO is more stable compared to the individual protein molecules and its glycan components [5].

Biosynthesis and Regulation of EPO

The *peritubular* fibroblasts of the *renal cortex* are the main source of EPO synthesis. EPO expression in other parts of the kidney, such as renal tubular epithelial cells, is mainly suppressed by *GATA transcription factors*, in particular GATA2 and GATA3. In normoxic conditions, EPO mRNA levels is not detectable in the renal tissue probably due to the low levels of expression [6]. But during hypoxia, the transcription of the EPO gene increases rapidly, and may increase mRNA levels to 200 times above the baseline level in less than 30 min [4]. EPO is also expressed in the liver, spleen, bone marrow (BM), lung and brain tissues. Consequently, in an area where hemoglobin concentration decreases and oxygenation is reduced, the expression of EPO rapidly increases [7]. EPO expression in the renal cortex is more influenced by changes in bloodstream and oxygen supply compared with other organs. As a result, in anemia when the concentration of hemoglobin and the oxygen content is reduced, EPO expression increases rapidly. During the fetal period, EPO is synthesized and secreted by the liver, and after birth (until the end of life) kidneys are the principal sites of production. The liver requires more oxygen to release EPO compared to the kidney. Hence, hemorrhagic disease of the newborn is not accompanied by fast bone marrow compensation because the site of EPO secretion is still active in the liver [8].

The production of EPO is regulated through the Hypoxia Response Element (HRE) in the *EPO* 3'-untranslated region, as well as its main mediator, the hypoxia inducible factor (HIF, which is a transcription factor produced in the liver and kidneys). The human EPO gene is located on chromosome 7, and consists of 5 exons and 4 introns [7, 9]. HRE is activated by the HIF (a heterodimer with two

subunits α and β , and a molecular weight of 100–120 kDa). The major activator of EPO is HIF-2, consisting of HIF-1 β and HIF-2 α . All the three known subunits of HIF α (HIF-1 α , HIF-2 α , HIF-3 α) are targeted and degraded in normoxic conditions by the von Hippel-Lindau (VHL) tumor suppressor [10]. While HIF-1 α and HIF-2 α act as activators of transcription, HIF-3 α s are known as transcription inhibitors [11]. HIF-1 α and HIF-2 α have target genes in common, but also have unique and specific targets. For example, anaerobic glycolysis is likely to be regulated by HIF-1 α [12], and HIF-2 α is involved in the regulation of EPO production in adults [13, 14].

In the presence of oxygen, HIF-1 α is hydroxylated by prolyl hydroxylase, degraded by proteases, and thus, is prevented from binding to the EPO locus. In hypoxic condition, a reduction of prolyl hydroxylase activity leads to the HIF-1 α stability, and an increase in transcription of the EPO gene. The positive effects of HIF increase through two cofactors HNF4 and CBP/P300 Coactivator. The *VHL* gene is required for the ubiquitination and degradation of HIF, and mutation in the *VHL* causes Chuvash Polycythemia (CP). Arg200Trp mutation is the most frequent mutations in *VHL* [4].

There are several transcription factors involved in the expression of EPO. For instance, EPO promoter is inhibited by the GATA2 and NF-KB, which are probably responsible for defective EPO expression in inflammatory diseases [7]. The expression of EPO in the brain plays a crucial role in the production and differentiation of neural progenitors, and the normal development of the brain [15–17]. After birth, EPO content and EPORs (EPO receptor) decrease in normal adult brain due to the gradual reduction of EPO production over time [18–20]. In addition, anxiety, distress, inflammatory, and ischemic conditions increase the expression of EPO and EPOR in the brain [18]. Gene Knock-out of EOPR in the brain increases apoptosis and the vulnerability of brain cells to hypoxia [21]. Erythropoietin may be responsible for the protection of the nervous

Fig. 1 Biological structure of EPO. Erythropoietin has two intramolecular di-sulfide bonds at positions Cys7–Cys161 and Cys29–Cys33. Moreover, the *N*-glycosylated bonds at the asparagine 24, 38, and 83 residues and an *O*-glycosidic bond is located at amino acid position 126

system from the aging-related damages. In fact, it is also considered as a potential anti-aging drug for the nervous system. EPO supplements may also delay the pathogenesis and progression of neurodegenerative diseases [22]. Recent studies suggest that EPO is likely to be involved in the wound healing process. This effect is associated with the stimulation of angiogenesis, and an increase in the expression of iNOS in the skin. Further studies show that EPO is also involved in angiogenesis through linking endothelial vessels and VEGF. Therefore, the stimulation of the mitosis leads to the formation of vessels [23, 24].

Regulatory Role of EPO

The response to EPO differs in various cells. The expression of the *erythropoietin receptor* (EPOR) is the most important factor affecting the cellular responses to EPO. This glycoprotein receptor (484 amino acids and one *N* glycan) is a homodimer of 59 kDa molecule consisting of an extracellular motif with WSXWS, a single transmembrane hydrophobic domain, as well as a variable cytoplasmic domain [25, 26]. CFU-E and BFU-E have a broad expression of EPOR in in-vivo conditions, and are highly sensitive to EPO, so that they undergo apoptosis in the absence of EPO. In the presence of EPO, CFU-E and erythrocyte progenitors are divided 3–5 times, generating 8–64 erythroblasts within 7–8 days. At the end of the maturity process, the nucleus of the cells is removed and reticulocytes are formed. The resultant Mature Reticulocytes and erythrocytes lack EPOR [25]. The EPOR gene, located on chromosome number 19, is expressed not only in erythrocyte progenitors, but also in the majority of tissues, such as human endothelial cells, the heart, the brain and the kidney in which the EPOR mRNA level is 10–100 times lower than the progenitors of erythrocyte [27, 28]. EPO activates the target genes essential for the evolution and amplification of RBC via binding to the EPOR on the surface of erythrocyte progenitors and triggering the JAK2/STAT5 signaling pathway. The enzymatic activity of JAK (Janus kinase) enzyme causes the signal transduction through tyrosine phosphorylation and dephosphorylation, and the phosphorylation of STAT proteins [29]. In addition, activation of Jack2 after the binding of EPO triggers several other signaling cascades such as MAPK and PI3K/AKT.

Human Recombinant Erythropoietin

In the late nineteenth century, a significant relationship between hypoxemia, anemia, and erythropoiesis was observed in experiments. In 1960, EPO was obtained from Anemic sheep plasma. In 1977, for the first time, Miyake

et al. [30] purified endogenous EPO in a very small amount from the urine of patients with Aplastic anemia, which was the first step in determining the role of this protein in the hematopoiesis process. Difficulties such as technical limitations, heterogeneity of target cells, inadequate, and immunogenicity of the resultant product had limited the entry of the endogenous EPO into clinical applications [31]. The advancements in recombinant protein production has also rapidly impacted EPO, so that the management of anemia became possible by the introduction of recombinant erythropoietin in the late twentieth century.

Prior to the availability of recombinant EPOs, blood transfusion was the only way to treat renal anemia. However, blood transfusion resulted in complications such as acute rejection, spread of infection and pathogenic microorganisms. Therefore, clinical studies have been conducted as soon as recombinant EPO was available. The early results showed that the use of rHuEPO could improve PCV [32, 33]. In 1987, the role of rHuEPO in increasing hemoglobin levels and eliminating the urgent need for permanent transfusion in adult hemodialysis patients was confirmed, and was approved to treat renal-related anemia [9, 34]. The recombinant erythropoietin is a man-made version of natural EPO produced by gene cloning. Recombinant erythropoietin drugs are also known as erythropoiesis-stimulating agents (ESAs) which are given by shot. ESAs lead to release the red blood cell from BM to the bloodstream [35]. The ESA are produced in the synthetic and recombinant forms, and their function is similar to endogenous EPO.

Types of rHuEPO

rHuEPO is divided into four different isoforms of alpha (α), beta (β), delta (δ), and omega (ω) based on the glycosylation pattern. The available forms include Epoetin [Epoetin alpha (Procrit/epogen), Epoetin beta (NeoRecormon and Methoxy polyethylene glycol epoetin (MPG-EPO)), zeta and Omega], and Darbepoetin alpha [36]. The half-life and various pharmacodynamic behaviors of these isoforms influence the final activity of rHuEPO. In addition to the glycosylation profile, the purification process and the culture medium of each isoform is also different. Thus, increasing the efficacy and the quality assurance of the drug requires a unique and optimized protocol [3, 5]. Epoetin beta is the first ESA that has been used. This drug was introduced in 1987 and approved in 1989 by the FDA for human consumption, and since then, other ESAs with similar function and different half-lives were introduced. It should be noted that the amino acid sequence of all rHuEPOs are similar to the native EPO [37]. Darbepoetin alpha is also a structural analogue of epoetin alpha while

MPG-EPO is an analogue chemically synthesized from EPO [38].

Epoetin is the international name for rHuEPO derived from eukaryotic cells, which is similar to human endogenous EPO in amino acid sequence. Epoetin alpha (Procrit and epogen) and Epoetin beta (NeoRecormon) have been used to treat anemia since 1993. In the United States, Epogen is used for the treatment of anemia caused by renal disease, and Procrit is used for other diseases. According to a study conducted on primates, it was suggested that Epoetin could be prescribed once every 2 weeks. Another human study also recommended the use of the drug once every 3 weeks [39]. Epoetin ω is also used in some parts of the world (but not extensively). The rHuEPO isoform is produced through the transfer of the EPO protein cDNA to the baby hamster kidney (BHK, from the Syrian hamster) cell line [40]. Epoetin zeta is a next-generation biosimilar with the same EPO-alpha amino acid sequence. This drug is used as an alternative in anemia and end-stage renal disease, and has been registered in accordance with European Medicines Agency (EMA) [41]. Eprex (erythropoietin made in Switzerland) and epocim (erythropoietin made in Cuba) are two commonly used types of epoetin outside of the United States of America. A significant increase in hemoglobin and hematocrits is observed in patients who receive Eprex. Side effects such as headache, pain at the injection site, and hypertension have been higher in epocim users [42]. Another EPOs includes EXI poietin and PD poietin. A study conducted in Iran to comparing these two EPOs in hemodialysis patients showed that PD poietin increased hemoglobin in higher levels in comparison to EXI poietin [43]. In Tunisia, there are two types of biosimilar rHuEPO alpha available under the name of EPOMAX produced to control the costs. During the experiments performed on hemodialysis patients, their side effects included a change in blood pressure, and headache and no death occurred during the study. Hemoglobin levels of the patients was stable, and no significant changes in biological parameters or blood pressure were observed until the completion of the study. Epomax[®] molecule is produced in vitro, in rodent cells, by recombinant DNA technology, and the Chinese hamster's ovary (CHO) cell line is transformed by the transfer of the plasmid containing the desired gene [44].

MPG-EPO (Mircera) has a long half-life (about 130 h), and different interactions with erythropoietin receptor compared to other ESAs [45] *Mircera* was approved for use in Europe in July 2007 by the swissmedic, and in November 2007 by the U.S. Food and Drug Administration (FDA) for use in the United States [46]. MPG-epoetin beta is a 60 KDa molecule in which erythropoietin is chemically bound to methoxy polyethylene glycol (PEG) butanoic acid via either

the N-terminal amino or the C-terminal group of the lysine [47].

Darbepoetin alpha is proposed as a hyperglycosylated human recombinant NESP (novel erythropoiesis stimulating protein) [48]. It requires less doses due to a higher molecular weight, more negative charge and a threefold longer half-life than epoetin [49]. Darbepoetin alpha increased hematocrit 3.6 times more than rHuEPO (at the same dose) when injected to the normal mice three times a week [50]. Administration of NESP (the final dosage in blood = 150–850 mu/mL) simulates the stress-induced hematopoiesis [29]. NESP has been purified in a liquid formulation (0.25% human serum albumin, 20 mM sodium phosphate and 140 mM sodium chloride), and a culture medium containing CHO [51, 52]. Due to the changes in the glycosylation pattern, Darbepoetin alpha has a longer half-life compared to endogenous EPO and epoetin alpha [53]. In 2002, it was approved by the FDA for the treatment of Chemotherapy-associated Anemia (CAA) [54]. Darbepoetin alpha (Aranesp, Amgen) is usually injected subcutaneously (SC), and its half-life in non-diabetic cancer patients is 74 h [21], but decreases in the intravenous injection for up to 21 h [55, 56].

Other Types of ESA

Peginesatide (Hematide) is a synthetic peptide conjugated to PEG, and was designed using the synthetic chemistry technique to emulate the pharmacological activity of EPO. The PEG units result in less immunological properties and more half-life in plasma. This peptide is composed of two chains containing 21 amino acids, covalently bonded via a linker derived from β -alanine and iminodiacetic acid. This molecule is not structurally homologous to rHuEPO and Naive EPO. Therefore, it can stimulate hematopoiesis even in the presence of an anti-EPO antibody [57]. Peginesatide was approved in the United States in March 2012 for the treatment of anemia caused by kidney diseases, but the use of the product stopped in February 2013 due to the side effects such as severe allergy and mortality [58]. EMA developed the first regulatory framework to evaluate biosimilars [4]. EMA-approved ESAs that are used to treat CAA include erythropoietin (Eprex, Aranesp, Neorecormon), EPO alpha biosimilar (Binocrit, Abseamed, EPO alpha hexal), epoetin zeta (silapo, Retacrit), and epoetin teta (Eporatio) [59].

There are some types of ESAs marketed in India. Epofit administrates to increase erythropoiesis in chronic kidney disease (CKD)-related anemia. Erykine administrates in CKD-related Anemia, HIV-related anemia (after zidovudine therapy, and chemotherapeutic anemia. Also, Erykine prescribe before surgeries that has a high risk of bleeding. However, it is contraindicated in cases with hypertension,

Allergy and pure red cell aplasia. As well, Epofer, Eposis, Epomine, Epotrust, Relipoiectin, Epotop, and Eporise are another conventional ESA marketed in India.

Modifications of rHuEPO

rHuEPO requires to undergo some modifications in order to have an increased half-life. Covalent modification of protein with PEG, or the use of the FC domain of antibodies, for instance, are the technologies utilized to increase the half-life of a protein [60–63]. Albumin has recently been identified as an ideal partner for rHuEPO due to its high half-life in the bloodstream [64]. A study that designed to increase the half-life of rHuEPO using albumin showed that the fusion of albumin domains and the intact albumin to the N-terminal of the EPO would lead to a long-lasting EPO-albumin fusion protein. In this study, EPO protein and albumin were fused by a GGSGG fusion linker [65, 66], and vectors including pZeoSV2(+), pIALE, pAD2LE and pAD1LE (Invitrogen, CA, USA) were used. The half-life of EPO-albumin fusion protein has been reported long, and similar to Darbepoetin alpha given by an IV injection [67]. According to the studies in the field of recombinant EPO production technology, overlapping oligonucleotides are required to produce rHuEPO. Thus, modifications such as eliminating palindromic sequences, minimizing reverse sequences (lengths less than 10 bp), optimizing the short-overlapping sites between each primer, and allowing the subsequent use of the primers for DNA sequencing are required to synthesize the oligonucleotide required for the rHuEPO gene synthesis. In some studies, pPICZ α -A vector and *pichia pastoris* have been used [68].

Clinical Applications of rHuEPO

Anemia is one of the common side effects of chronic kidney disease (CKD), and is mainly caused by the defects in the production of endogenous EPO. Most patients with advanced CKD suffer from anemia [69]. In a study consisted of hemodialysis patients, Ma et al. showed that reduction in hematocrit is correlated with an increased risk of mortality in CKD patients as an example, the risk of mortality in patients with hematocrit level of less than 27% is 1.33 times higher than the patients with hematocrit level of 30–33% [70]. In people with CKD, in addition to the reduction in the expression of EPO, decreased nutrient intake or absorption of iron, gastrointestinal bleeding, menstruation, frequent phlebotomy, and hemodialysis also causes anemia [71, 72]. Consequently, the impairment in EPO production along with the iron deficiency exacerbate anemia in these patients and reduce the response to ESA

[73]. Therefore, iron intake may optimize the response to treatments in patients with renal disease requiring dialysis, due to the need for lower doses of erythropoietin [74, 75]. Anemia is a common complication in children and adolescents with severe protein deficiency (kwashiorkor), and usually occurs as a soft fluid anemia. In 10–60% of cases of anemia in people with kwashiorkor, megaloblastic changes have also been reported. Protein deficiency does not seem to directly reduce hemoglobin synthesis, and animal study (in vivo) suggests that a reduction in the release of EPO is a major contributor to the anemia. This is probably due to a reduction in the metabolism of body tissues, and thereby decreasing the oxygen consumption and release of EPO. As a result, EPO is used to improve anemia in these patients [4].

ESA is also used in the treatment of CAA. The nephrotoxic impacts of some cytotoxic agents are one of the causes of anemia. Compounds containing platinum, such as Cisplatin, reduce the release of EPO from the kidneys. rHuEPO is used as a supportive care in HIV infection (due to the use of Zidovudine), malignancies (after chemotherapy and radiotherapy), and transplantation. Administration of ESAs is a good therapeutic alternative when blood transfusion is prohibited. A number of experiments have indicated that darbepoetin alpha is effective in the treatment of myeloma-related anemia. rHuEPO is also used in anemia related to autoimmune disease, congestive heart failure, hemoglobinopathy and acute hemolysis. rHuEPO abuse by athletes increases their physical tolerance and endurance due to increased oxygenation in the blood. For this reason, the International Olympic Committee has banned the use of rHuEPO for athletes. Further studies indicate that this recombinant glycoprotein, rHuEPO, increases the activity of the neurons and reduces apoptosis in these cells. In addition, the aging process is slow in these cells, and the mechanism is probably related to the increased activity of intracellular antioxidants [76].

In addition, treatment of immune system cells with rHuEPO and immunofluorescence detection showed that rHuEPO improved the morphology of the long-term cultured primary cells. Another case is the use of rHuEPO in Myelodysplastic Syndromes (MDS) [56]. Due to the lack of MDS treatment options, darbepoetin and ESAs are used to treat MDS-related anemia [77, 78]. According to Mizoguchi, Williams and Dukes (in separate studies), rHuEPO stimulated the formation of megakaryocytes colonies in a specific bone marrow culture medium. rHuEPO increases the production of acetylcholinesterase, a megakaryocyte marker in mice and some other species, in the BM culture medium [79]. According to a study, the size and ploidy level (which is a replication property) of megakaryocytes were measured before and after the addition of rHuEPO to confirm the proliferative properties of

Table 1 Clinical indication of ESAs types

ESA	Indication	Administration		Dosage	References
		Injection			
Epoetin alfa (Epogen)	In adults	For CKD patients (hemodialysis and non-hemodialysis patients)	IV	Initial dose: 50–100 Units/kg body weight/3 times per week	[89]
			SC	For non-dialysis patients 150 Units/kg body weight/3 times per week For dialysis patients 200 Units/kg body weight/3 times per week	
		For HIV-treated patients with zidovudine (if the serum EPO level is $\leq$ 500 mUnits/mL, and zidovudine dose is $\leq$ 4.2 g/wk)	IV/SC	Initial dose: For 8 weeks, 100 Units/kg body weight/3 times per week	
Darbepoetin alfa	CKD	In chemotherapy patients with myelosuppressive malignancies	SC	Initial dose: for 8 weeks, 150 Units/kg body weight/3 times per week (if required, continue for 3 weeks, 300 Units/kg body weight/week)	[90]
		CKD 1 month (hemodialysis) $\geq$ 1 month	Not recommended		
		Chemotherapy patients $5 \leq$ years	IV	Three weeks, 50 Units/kg body weight/week Four weeks, 600 Units/kg body weight/week (900 Units/kg body weight/week after 4 weeks if needed)	
		With hemodialysis	IV/SC (IV is recommended)	0.45 mcg/kg body weight/week 0.75 mcg/kg body weight/2 weeks	
		Without hemodialysis	IV/SC	Four weeks, 0.45 μ g/kg body weight/week	
Peginesatide	CKD-related anemia in adolescent hemodialysis patients	Chemotherapy-induced anemia in non-myeloid malignancy	SC	One week, 2.25 μ g/kg body weight/week Three weeks, 500 μ g/week	[91, 92]
		No history of receiving other ESAs	SC/IV	0.04–0.08 mg/kg body weight/month	
		With the history of epoetin alpha use (at least 1 week after the last use of epoetin)	SC/IV	Previous epoetin dose $<$ 2500: 2 mg/month Previous epoetin dose 2500 to $<$ 4300: 3 mg/month Previous epoetin dose 4300 to $<$ 6500: 4 mg/month Previous epoetin dose 6500 to $<$ 8900: 5 mg/month Previous epoetin dose 8900 to $<$ 13,000: 6 mg/month Previous epoetin dose 13,000 to $<$ 19,000: 8 mg/month Previous epoetin dose 19,000 to $<$ 33,000: 10 mg/month Previous epoetin dose 33,000 to $<$ 68,000: 15 mg/month Previous epoetin dose 68,000 or greater: 20 mg/month	

Table 1 continued

ESA	Indication	Administration		References
		Injection	Dosage	
Methoxy polyethylene glycol/epoetin beta	With the history of darbepoetin alpha at least 1 week after the last dose of darbepoetin)	SC/IV	Previous darbepoetin dose < 12: 2 mg/month Previous darbepoetin dose 12 to < 18: 3 mg/month Previous darbepoetin dose 18 to < 25: 4 mg/month Previous darbepoetin dose 25 to < 35: 5 mg/month Previous darbepoetin dose 35 to < 45: 6 mg/month Previous darbepoetin dose 45 to < 60: 8 mg/month Previous darbepoetin dose 60 to < 95: 10 mg/month Previous darbepoetin dose 95 to < 175: 15 mg/month Previous darbepoetin dose 175 or greater: 20 mg/month	[93]
		IV/SC	Receiving epoetin < 8000 units/week or darbepoetin < 40 µg/week: 120 µg/month or 60 µg/2 week Receiving epoetin 8000–16,000 units/week or darbepoetin 40–80 µg/week: 200 µg/month or 100 µg/2 weeks Receiving epoetin > 16,000 units/week or darbepoetin > 80 µg/week: 360 µg/month or 180 µg/2 weeks	
		IV/SC	0.6 µg/kg body weight/2 weeks	
		SC	250 IU/kg body weight/3 times per week	
		SC	Stage 1: Three weeks, 20 IU/kg body weight/week (increase of the dose up to 40 IU/kg body weight/3 times per week if the response did not reach the desired level after 3 weeks) Stage 2: Dose reduction by half maintenance	
		IV	Ultimately for 4 weeks	
		SC	400–1600 IU/kg body weight/week	
		SC	Ultimately for 4 weeks	
		SC	300–1200 IU/kg body weight/week	
		SC	300–1200 IU/kg body weight/week	
Epoetin beta (NeoRecormon)	In premature newborns	SC		[94]
	Chemotherapy patients	SC		[95]
Epoetin beta (NeoRecormon)	In cases who need to homologous blood transfusion in their surgery	IV		
		SC		

Table 2 Side effects of some common types of ESA used in hematopoiesis. The most common side effect is a flu-like state with sneezing, coughing, and muscle pain. Other side effects for each of the ESA types are listed in the table below

ESA	Side effects	References
Epogen (Epoetin alfa)	Headache, body pain, diarrhea, cold symptoms (nasal congestion, sneezing, cough, sore throat), joint and bone pain, dizziness, depression, weight loss, insomnia, injection site reactions, vomiting	[97]
Darbepoetin alfa	Headache, abdominal pain, bodily pain, cough, skin rash and redness, diarrhea and injection site reactions (pain, bruising, inflammation, warmth, bleeding) can also cause serious complications such as high blood pressure and blood clots	[98]
Peginesatide	Shortness of breath, diarrhea, cough, nausea and arteriovenous fistula site complication	[99]
Methoxy polyethylene glycol-epoetin beta	Blurred vision, dizziness, weakness, body pain, coughing, chills, loss of sound, nasal congestion, slow or fast heartbeat, sweating, sore throat, nervousness, death	[100]
Epoetin beta (NeoRecormon)	Hypertension, headache, thromboembolism, multidose formulation of NeoRecormon contains benzyl alcohol and should not be prescribed to children under the age of three	[101]

rHuEPO on the megakaryocytic cell line. The results showed that rHuEPO, increased some of the proliferative aspects of megakaryocyte, in in vitro condition. As a result, the effect of erythropoietin is not limited to the erythroid cell line [80–82], and it is possible to incorporate EPO into the treatment of some thrombocytopenia and other megakaryocytic disorders (Table 1).

Challenges of rHuEPO Clinical Applications

The most frequent side effect of rHuEPO is mild-to-flu-like symptoms that subside over a 24-h period. Other complications of the treatment with rHuEPO include pain and erythema in the injection site, fever, hypertension (often occurring in patients with CKD treated with high doses), splenomegaly (in myeloproliferative disease, due to the stimulation of BM hematopoiesis), thrombosis, seizure, allergies, anaphylaxis and hypercalcemia [68, 83–85]. The patient should be monitored after receiving the drug due to the risk of thromboembolism.

If tumor cells express EPOR, treatment with rHuEPO may stimulate the growth of tumors [86]. According to studies, EPOR expression is elevated in several solid tumors, such as neuroblastoma (NB). EPO has also been reported to stimulate angiogenesis in NB. In a study on three NB cell lines expressing the EPO and EPOR mRNA, an increase in adhesion and chemotaxis was observed in response to the stimulation of EPO. Consequently, EPO supplementation may have an undesired effect on facilitating the process of tumor metastasis [87]. Development of red blood cell aplasia, which may be caused by anti-erythropoietin antibody is a rare but serious event. This antibody is probably produced due to the difference in the carbohydrate moieties of the erythropoietin and its recombinant biosimilars. As stated, rHuEPO can increase the blood pressure independent of the increase in blood

volume (in addition to the type of increased blood volume). The mechanism is that EPO induces Endothelin1 (ET-1), and produces Prostacyclin (PGI₂) (a vasodilator), and also increases the production of thromboxane₂ (TXB₂) (a vasoconstrictor) and disturbs the balance between these two compounds [88]. In the patients with no response to rHuEPO a slight increase of less than 10 g/dl in hemoglobin level is found after treatment with a 4-weeks standard dosing schedule [89]. High dose of IV ascorbic acid (vitamin C), high doses of IV carnitine, growth factors and cytokines (e.g., insulin-like growth factor and interleukin-3) are measures taken to optimize rHuEPO therapy in renal patients [74] (Table 2).

Discussion and Conclusion

The production of human recombinant erythropoietin has opened up a broad clinical approach to the treatment of anemic disorders (such as CKD, MDS, and various types of anemia caused by EPO deficiency). With respect to recent studies on the possibility of using rHuEPO in the treatment of coagulant and neurological disorders, an ever-increasing introduction of recombinant ESAs to the medical science is predictable. However, there are new clinical challenges in the use of these agents due to their confirmed proliferative properties in certain cancers. Technological advances in the field of medical biotechnology to produce recombinant proteins and protein engineering can be a key to solving the existing challenges in the clinical use of rHuEPO.

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Conflict of interest The authors declare that there is no conflict of interests.

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