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Advances in Analytical Methods for Erythropoietin Identification: From Clinical Use to Anti-Doping Applications

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Erythropoietin (EPO) is an endogenous glycoprotein essential for erythropoiesis regulation and is produced mainly by the kidneys and to a lesser extent by the liver. Its synthesis is stimulated by hypoxia and regulated by a complex feedback system. Endogenous EPO has 165 amino acids and plays a critical role in the survival, proliferation, and differentiation of erythroid progenitor cells through intracellular signaling pathways such as JAK2/STAT5, MAPK/ERK, and PI3K/AKT. Recombinant erythropoietin (rhEPO), produced in CHO cells using recombinant DNA technology, share the structure and biological activity of endogenous EPO and is widely used to treat anemia associated with chronic kidney disease, chemotherapy, chronic inflammatory diseases, and HIV/AIDS. It is also administered in elective surgery to reduce transfusion needs. However, rhEPO is misused in sports doping because it increases red blood cells, enhances oxygen delivery and physical performance. This misuse is common in endurance sports and presents technical, ethical, and medical concerns. To address this, analytical methods—including isoelectric focusing, SDS-PAGE, and isoform profiling—have been developed to detect exogenous EPO. Nevertheless, these techniques still face challenges related to sensitivity, microheterogeneity, and overlapping between endogenous and recombinant forms. This work reviews the biochemical, clinical, and technological aspects of erythropoietin, emphasizing analytical strategies for its detection, therapeutic applications, and ongoing challenges in anti-doping contexts.

Keywords: Erythropoietin, doping, analytical methods.

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Introduction

Doping is defined as the use of prohibited substances or methods by athletes to improve training and results in sports. In order to prevent the use of these substances, an organization dedicated to creating rules and tests to control or punish athletes who use these substances, the World Anti-Doping Agency (WADA) was created in 1999. Since its creation, the agency has annually published lists of substances and methods prohibited for use throughout the year of competitions or prohibited only at the time of competition. According to WADA, in 2020, 149,758 blood or urine samples were collected from athletes, of which only 1,007 were found to have adverse analytical results, such as interfering substances in the samples, in addition to concentrations where it was not possible to analyze what was actually present, among these 672 samples tested positive for at least one prohibited substance in the result.

Nowadays, prizes for first place are attractive, and they also give athletes visibility in search of sponsorships. In order to reach the top spots, athletes submit themselves to the use of prohibited substances that improve their performance in competitions.

Erythropoietin, which stimulates the production of red blood cells, is on the list of prohibited substances and is widely used in high-performance sports, where athletes need a lot of endurance, such as road cycling, long-distance marathons, triathlons, among other athletics modalities. Its use in sports practice was introduced in 1970, through the conditioning of athletes in hypobaric chambers, responsible for inducing the production of erythrocytes, and blood transfusions. In 1990, the International Olympic Committee (IOC) decided to prohibit the use of EPO and its analogues (**table 1**), not only for sports ethics, but also to preserve the health of athletes, since its use can cause undesirable effects (1). With the advancement of medicine and biotechnology, recombinant erythropoietin (rhEPO) was developed in 1988, and is thus widely used in clinical medicine to improve the quality of life of patients who required large quantities of blood transfusions.

rhEPO is a glycoprotein composed of 165 amino acids that form a polypeptide chain with two intramolecular disulfide bridges at positions (Cys7-161 and Cys29-33). It has three N-linked glycosylation sites (Asn24, Asn38 and Asn83) that can form two to four sialylated chains, and one O-linked chain (Ser126). It can contain up to 14 sialic acids, which are necessary for the hormone to reach the target sites, preventing rapid metabolism by hepatic receptors that recognize the exposed galactose structures, with subsequent excretion. The molecular mass of the glycosylated structure, which contains approximately 40% carbohydrates, is 30–34 kDa, and that of the peptide chain alone is 18 kDa (2).

Because it is a molecule with a high molecular weight, is complex and has little presence in tissues - in addition to being identical to its endogenous form - it is very difficult to detect in biological samples. Some differences in glycosylation pattern of EPO and rhEPO are shown in figure 1. Therefore, this work aimed to discuss the use of EPO as doping, its analytical methods, including advantages and disadvantages, and the effects of prolonged use without medical indication.

Table 1. List of prohibited erythropoietins (EPO) and erythropoiesis-stimulating agents.

Class	Examples
Erythropoietin receptor agonists	Darbepoetins (dEPO), Erythropoietins (EPO), substances synthesized from EPO (EPO-Fc, Methoxy polyethylene glycol-epoetin beta (CERA), EPO mimicking agents and similar substances (CNTO-530, peginesatide)
Agonists that activate hypoxia-inducing factors (HIF)	Cobalt, Daprodustat (GSK1278863), IOX2, Molidustat (BAY 85-3934), Roxadustat (FG-4592), Vadadustat (AKB-6548), Xenon
GATA inhibitors	K-11706
Inhibitors of transforming growth factor beta (TGF-β) signaling	Luspatercept, Sotatercept
Innate repair receptor agonists	Asialo-EPO, Carbamilated EPO (CEPO)

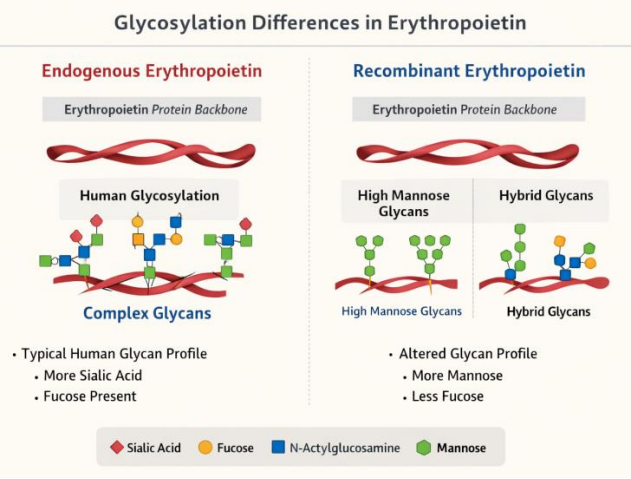

Figure 1. Differences in glycosylation pattern of endogenous and recombinant erythropoietin.

Methodology

This work was developed through a literature review with the objective of investigating EPO, covering its clinical applications, analytical methods and misuse in sports doping. The searches were carried out in the PubMed, Scielo, ScienceDirect, and Virtual Health Library (BVS) databases, in addition to institutional repositories.

The search strategy was based on the use of specific terms and combinations of keywords related to the topic. The descriptors "erythropoietin", "sports doping", "recombinant erythropoietin", "EPO detection methods", "hematological therapies" and "athletic performance" were used in combination with Boolean operators such as "AND" and "OR". This approach allowed for broad and specific research to cover different aspects related to EPO, including clinical applications, analytical methods and the impact of sports doping.

Inclusion criteria included articles, theses and reports published between 2004 and 2024, available in Portuguese, English or Spanish, with scientific and thematic relevance. Duplicate documents, articles with restricted access to the full text and publications that did not present relevant data or did not meet the objectives of the work were excluded.

The initial search resulted in approximately 450 documents. After analyzing titles and abstracts, 180 studies were selected for full reading. Of these, 65 fully met the inclusion criteria and were used in the construction of this work. The extracted data included information on the biological functions of EPO, methods of rhEPO detection, impact of sports doping and clinical regulations, which were organized and presented descriptively throughout the study.

Results and Discussion

Erythropoetin (EPO)

EPO is a glycoprotein produced in the kidneys (90%) and liver (10%), which the main function is to stimulate and regulate the production of red blood cells. It is produced endogenously in response to a lack of oxygen in the tissues (hypoxia), usually caused by inadequate oxygenation of the blood, stimulating erythropoiesis to normalize blood oxygen levels.

Erythropoiesis is highly controlled by a responsive feedback system, where there is a sensor in the kidneys. Although the exact location of this sensor is not known, it is believed to be located in the distal tubules (3).

Human EPO is initially produced in renal cells as a prohormone with a chain of 193 amino acids, of which the first 27 have no biological function and are only necessary for hormone secretion. Before being released, EPO is processed, removing these first 27 amino acids. In addition, the arginine, present at the carboxy-terminal end, is lost when the hormone enters the circulation. As a result, EPO has 165 amino acids organized in a single polypeptide chain, containing two intramolecular disulfide bonds and four polysaccharide chains, which are linked to specific amino acid residues (4).

During fetal life, EPO is produced mainly by the liver, but after birth, it begins to be produced by the juxta tubular cells located in the kidneys. EPO is encoded by a gene with five exons, located on the long arm of chromosome 7 (7q21). Analysis of the structure of the EPO gene revealed a series of sites that cooperate to regulate the expression of the gene, in particular its increased expression in hypoxia.

The main structure responsive to hypoxia is an enhancer located 0.7 kb from the 3'-end of the gene. This enhancer contains three critical sites for the response to hypoxia, to which three intermediates called HIF-2α (hypoxia-

inducible factor), HNF-4 (hepatic nuclear factor) and ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator or HIF-β) bind. In the presence of these three factors, in association with p300, a transcriptional coactivator, a complex is formed that interacts with transcription factors, creating conditions for the activation of localized gene transcription, increasing the production of mRNA of the EPO gene. The process begins with the action of sirtuin-1 (whose production increases with hypoxic stress); this, in turn, produces deacetylation of the HIF-2α gene region, increasing the expression of this gene. The regulation of the EPO gene by hypoxia therefore depends fundamentally on the formation of the HIF complex; the HIF-β (or ARNT) subunit is constitutively expressed at levels that are not affected by oxygen tension, while the HIF-α subunit is not detected under normal conditions but increases in response to hypoxia (5).

When erythropoiesis is compromised due to some disease or deficiency, anemia occurs, as there is a reduction in the production of red blood cells, as in the case of patients with cancer, AIDS or chronic kidney disease. In these cases, when there is a deficiency in the production of erythropoiesis, exogenous EPO, also known as rhEPO (epoetin), is used. In addition to the clinical and legal use of rhEPO, it is also used illegally by athletes to obtain advantages in sports that require high physical endurance, such as long-distance cycling, swimming, marathons and other athletics.

Exogenous EPO is identical to endogenous EPO, which makes it difficult to identify it in analytical methods. It is included in the International Olympic Committee (IOC) list of prohibited substances, as are other substances that stimulate erythropoiesis. In the literature, there is not a reliable method for differentiating between endogenous and exogenous EPO.

After EPO is administered, it follows certain paths until it finds its receptor (EPOR), which is present in several tissues, but in very small quantities. After binding, a dimer is formed, which results in the activation of Janus kinase (JAK2), which has some functions, such as cell growth and proliferation (6). JAK2 autophosphorylates and, after this process, EPOR is phosphorylated into tyrosine residues that become sites for intracellular signaling proteins. These sites recruit other proteins and cells, initiating a signaling cascade process. The main signaling cascades activated by erythropoietin are:

- JAK2/STAT5 pathway: STAT5 is a protein recruited by the tyrosine residues of phosphorylated EPOR. JAK2 phosphorylates STAT5, which changes its structure and goes to the cell nucleus. After some conformational modifications, it plays its role in promoting survival, where it inhibits cell apoptosis, in addition to stimulating the proliferation and differentiation of erythroblast progenitor cells, increasing hemoglobin production and stimulating erythropoiesis (5).
- MAPK/ERK pathway: The activated EPO receptor recruits adapter proteins, such as Grb2 and SOS, which activate the Ras protein. This protein activates the MAPK cascade (Raf, ERK, MEK). Once the cascade is activated, it is responsible for stimulating cell proliferation, activating genes that promote cell division of progenitor cells. In addition, it promotes the regulation of factors that allow erythroblast maturation (5).
- PI3K/AKT pathway: EPOR activation also recruits and activates the PI3K protein (phosphatidylinositol-3-kinase), which phosphorylates molecules such as PIP2, forming PIP3. In turn, PIP3 activates AKT, known as protein kinase B, which plays important functions in the formation of new cells, such as inhibiting apoptotic pathways, stimulating cellular metabolism, providing energy for the differentiation and maturation of progenitor cells, and regulating the cell cycle, promoting cell division (5).

This signaling is controlled by negative feedback. The body itself controls these signaling cascades to prevent the overproduction of progenitor cells and, consequently, the excessive production of red blood cells.

Production of recombinant erythropoetin (rhEPO)

In 1977, EPO was extracted and purified from the urine of anemic patients using the recombinant DNA strategy. Based on the amino acid sequence obtained during purification, it was possible to clone EPO, enabling the development of rhEPO. This molecule is exactly the same as endogenous EPO and can be produced in Chinese hamster ovary (CHO) cells, in baby hamster kidney (BHK) cells, with omega-epoitin, and in human fibroblasts with delta-epoitin (6).

CHO cells are the most widely used expression system for the production of rhEPO. Their popularity is largely due to their high productivity and suitability for large-scale culture in the manufacture of biological products, including monoclonal antibodies, recombinant proteins, and vaccines. In addition, CHO cells are capable of performing post-translational modifications and generating complex glycosylation patterns similar to those found in human cells, which are essential for the biological activity and stability of many therapeutic proteins. These cells are also considered relatively resistant to viral contamination in biopharmaceutical production processes. Importantly, rhEPO produced in CHO cells has an amino acid sequence identical to that of endogenous human erythropoietin (6).

The rhEPOs produced from CHO cells are the main ones commercially available and are known as alpha and beta. They have the smallest structural differences in terms of glycosylation pattern but have the same effect as endogenous EPO.

Clinical applications of EPO

The rhEPO has been shown to be effective in the treatment of different conditions related to red blood cell deficiency, some of the main clinical applications include:

- Anemia in chronic renal failure: in this disease there is a significant decrease in renal mass, which impairs the endogenous production of EPO, since this hormone is mainly produced in the kidneys. This leads to severe anemia, the main symptoms of which include fatigue and weakness. In this case, rhEPO is administered subcutaneously to restore red blood cell and hemoglobin levels, reducing the need for blood transfusions. Studies show that approximately 95% of patients experience an improvement in their quality of life. Strict monitoring of the dose is necessary during treatment, aiming to gradually increase hematocrit to levels between 33 and 36%. Overproduction of hematocrit (above 40%) can increase the risk of myocardial infarction (5).
- Anemia induced by chemotherapy: cancer patients who receive myelosuppressive chemotherapy often present bone marrow suppression, resulting from the action of chemotherapy drugs. In this scenario, the administration of rhEPO aims to stimulate the production of red blood cells, combating the anemia caused by the treatment (5).
- Anemia in chronic diseases: chronic inflammatory diseases, such as rheumatoid arthritis, lupus erythematosus, and inflammatory bowel diseases, can reduce or inhibit erythropoiesis, in addition to decreasing iron availability, favoring the development of anemia. When endogenous EPO levels are significantly below normal, the use of rhEPO is indicated to correct anemia (8).
- HIV/AIDS-related anemia: patients with HIV may develop anemia due to the suppressive effect of antiretrovirals, such as zidovudine, or due to the direct impact of HIV on the bone marrow. The rhEPO is used to treat symptomatic anemia in these patients (9).
- Preparation for elective surgery: in orthopedic or cardiac surgeries, where significant blood loss may occur, rhEPO is administered in a controlled manner to increase the patient's hemoglobin levels, reducing the need for intraoperative transfusions. Administration usually begins a few weeks before surgery (10, 11).
- Other uses in research: in addition to its established clinical uses, rhEPO has been studied to identify new therapeutic uses. A promising example is the use of rhEPO as a cardioprotector after myocardial infarction. Studies with canine models have shown that intravenous administration of rhEPO (at doses of 100 IU/kg and 1000 IU/kg) can induce angiogenesis in the ischemic myocardium, improving cardiac function and suggesting a potential mechanism of cardioprotection (12). In addition, rhEPO has demonstrated neuroprotective effects. In models of stroke in Sprague-Dawley rats, administration of rhEPO 24 hours before and up to 9 hours after occlusion of the circulation showed a significant reduction in infarct volume, indicating that rhEPO may have an important role in neuroprotection (13).

Therapeutic regimens generally involve doses of approximately 50–150 IU/kg administered subcutaneously or intravenously two to three times per week, with adjustments based on hemoglobin response, aiming to gradually increase hemoglobin levels while minimizing the risk of excessive erythrocytosis. Treatment is often maintained for weeks to months under strict medical monitoring (14).

Adverse effects related to the indiscriminate use of rhEPO

One of the main types of rhEPO marketed is epoetin alfa. Its adverse effects include cardiovascular complications, such as increased risk of myocardial infarction. Studies with rats and monkeys were performed to analyze possible adverse effects and, initially, it was observed that the adverse effects resulting from the administration of rhEPO are mild, including rash, headache, arthralgia, nausea and vomiting. A marked increase in hematocrit in monkeys (from 40% to approximately 80%) was also observed, which represents a serious risk to cardiovascular function, including difficulties in maintaining cardiac output, increased cardiac effort and risk of heart failure and impairment of tissue perfusion due to the substantial increase in blood viscosity (15-18). Increased production of red blood cells can lead to polycythemia, an excess of red blood cells, which can be responsible for causing blood hyperviscosity, hindering blood flow in small arteries and increasing the likelihood of ischemic events.

Furthermore, uncontrolled elevation of hemoglobin levels increases the risk of clot formation (thrombi), which can lead to stroke, deep vein thrombosis, myocardial infarction and pulmonary embolism. Patients with pre-existing conditions, such as a history of thromboembolism, are at greater risk with indiscriminate use. In athletes, increased oxygenation can be followed by thrombosis and sudden death due to extreme exertion (18).

EPO can also cause an increase in blood pressure, even in patients with previously normal levels, due to the increase in blood volume and the direct vascular effects of EPO. If left uncontrolled, hypertension can lead to complications such as left ventricular hypertrophy and accelerated atherosclerosis (19). There are reports of adverse immunological effects of EPO, such as the induction of an immunological response where antibodies against EPO neutralize the production of red blood cells, resulting in severe anemia and pure red cell aplasia (20).

Another important adverse effect occurs in cancer patients. EPO and its receptor have been recognized in some types of human cancer, such as breast, prostate, cervical, ovarian, glioblastoma, and head and neck squamous cell carcinoma. This is problematic because

stimulating the production of normal cells also stimulates the development and proliferation of cancer cells, in addition to inhibiting apoptosis (21). A study with 13,993 cancer patients from 56 clinical trials showed that the use of rhEPO is not ideal for this type of patient, with 1,530 patients dying during the study and the remainder presenting accelerated disease development. Although patients initially reported relief, soon the cancer cells showed partial resistance to drug chemotherapy (22).

An additional safety concern related to the misuse of EPO in doping involves the potential use of products obtained from unregulated or illicit sources, where manufacturing quality standards may not be adequately controlled. Recombinant biological products are highly sensitive to variations in production, purification, and storage conditions, and deviations in these processes may result in the presence of process-related impurities, host cell proteins, protein aggregates, degradation products, or structural variants. Such quality attributes can significantly influence the biological activity, pharmacokinetics, and immunogenicity of EPO-stimulating agents. In particular, protein aggregation and altered glycosylation profiles have been associated with increased immunogenic potential and may contribute to adverse immunological reactions, including the development of neutralizing antibodies and, in rare cases, pure red cell aplasia (23). Additionally, impurities and contaminants present in substandard or falsified biological products may increase the risk of systemic toxicity or organ damage. Recent regulatory discussions have emphasized the importance of rigorous impurity qualification and characterization in biological medicines to ensure product safety and consistency throughout the manufacturing process. These concerns are particularly relevant in the context of doping, where EPO may be obtained from non-regulated sources, potentially exposing users to products with unknown purity, stability, or structural integrity (24). Similar safety concerns have recently been highlighted in cases involving substandard or falsified peptide-based therapeutics, illustrating the broader toxicological risks associated with biological products of uncertain origin.

Pharmacokinetics of rhEPO

rhEPO is sold only as a powder for reconstitution, so it can be administered intravenously or subcutaneously, depending on the patient's needs. Its main route of administration is subcutaneous, where it has a slow and sustained absorption, with peak plasma levels being reached between 12-14 hours, with a bioavailability of 40-50%. The intravenous route, which is less commonly used, provides immediate absorption but has a shorter duration of action. Sialic acid is related to the pharmacokinetics of rhEPO; a study shows that when this acid is removed, the protein's half-life in the plasma becomes much shorter than the original, preventing the molecule from performing its biological role in maintaining erythrocytes (25).

After absorption, rhEPO is distributed in the target tissues, with EPORs located in the progenitor cells in the bone marrow responsible for initiating the differentiation of these cells. Its distribution volume is small, limited mainly to the vascular compartment, due to the large size of the molecule and its high specificity with the receptors (26).

rhEPO is metabolized mainly by target cells and the liver. After binding on cellular receptors, rhEPO is degraded into amino acids and glycans by the lysosomal pathway. The liver also participates in this biotransformation, but in a secondary way by the proteolytic pathway. The half-life of rhEPO is between 18-24 hours, depending on the type of rhEPO used; for example, darbapoietin has a longer half-life (27).

rhEPO is eliminated mainly by intracellular pathways, with catabolism in target tissues (bone marrow, liver and kidneys). It is also eliminated via the kidneys, although in a very small quantity, since rhEPO is very poorly filtered by the glomeruli due to its molecular size (26). One of the reasons for the difficulty in analyzing, characterizing and differentiating endogenous EPO from exogenous EPO is that there are some factors that modify the toxicokinetics of erythropoietin, such as genetic factors, where mutations occur in genes related to metabolism or EPO receptors; physiological conditions can also decrease metabolism and increase the half-life of erythropoietin (27).

Doping in Sport

Doping is defined as the use of prohibited substances or methods by athletes with the aim of improving their athletic performance. Some athletes resort to doping to accelerate recovery from injuries, allowing them to participate in competitions from which they would otherwise be excluded. Although anabolic steroids are most associated with doping, there are many other substances, drugs, molecules, and methods classified as prohibited (28).

Annually, WADA publishes an updated list of prohibited substances and methods, including those banned only during competitions and others prohibited throughout the year. This list aims to inform athletes who wish to compete regularly, helping them avoid illicit substances and methods. However, there are cases of athletes using the list to escape regulations and gain competitive advantage.

The WADA list includes several categories of prohibited substances and methods, such as anabolic agents; peptide hormones, growth factors, related substances and mimetics; beta-2 agonists; hormones and metabolic modulators; diuretics and masking agents; narcotics; cannabinoids; stimulants; glucocorticoids, as well as prohibited methods such as blood transfusions and genetic manipulation. There are also substances prohibited in specific modalities, such as beta-blockers and alcohol. Finally, WADA maintains a list of monitored substances, which are not prohibited, but whose use is monitored (28).

Doping practices typically involve lower but more frequent doses than therapeutics, intended to stimulate erythropoiesis while reducing the likelihood of detection.

Athletes have been reported to use microdosing strategies ranging from approximately 500 to 2,000 IU administered subcutaneously every 1–3 days, often in cycles lasting several weeks, particularly during altitude training or preparation phases before competitions. These regimens may be combined with periodic interruptions or tapering phases to avoid abnormal hematological parameters. Despite these strategies, such misuse can still lead to clinically significant increases in hematocrit and blood viscosity, thereby increasing the risk of hypertension, thromboembolic events, stroke, and other cardiovascular complications (29, 30).

Cases of use of RHEPO in sports

Historically, the technique of increasing red blood cell production began not with EPO, but with blood transfusions, which reduced the physiological sensation of exertion. In 1972, an alleged case involving athletes in sports such as cross-country skiing, biathlon, cycling, and long-distance running was reported, although no one admitted to using the technique. The confirmation occurred at the 1984 Olympic Games in Los Angeles, when cyclists from the United States confessed to using red blood cell concentrate transfusions before the competition. Following this confession, WADA included blood transfusions on its list of prohibited methods (28).

Doping cases involving rhEPO are difficult to quantify, as many became desperate due to the lack of reliable detection methods before the 2000s. However, at the 1984 Olympics, the deaths of cyclists were linked to EPO use. For the first time, rhEPO was included on WADA's list of prohibited substances. In 1994, blood tests were implemented, including a hematocrit limit of 50% for cyclists (28).

The Festina affair, which occurred during the 1998 Tour de France, is the best-known case involving rhEPO. During the event, a Festina team car was intercepted with prohibited substances, including rhEPO. Subsequently, tests revealed eight positive results for EPO and four for amphetamines. In 2013, the French Senate announced that at least 29 athletes tested positive for rhEPO in the 1998 Tour, including the top three finishers (31).

Another famous case was the North American cyclist Lance Armstrong, champion of the Tour de France between 1999 and 2005. In 2005, it was revealed that Armstrong used rhEPO in 1999, confirmed by analysis of frozen samples. Although initially acquired, new allegations surfaced in 2011. In 2012, samples from 2009 and 2010 confirmed the use of EPO, and all of Armstrong's titles were revoked. In 2013, he confessed to using banned substances (32).

After WADA banned EPO in 1984, some athletes began using darbepoetin, a more potent synthetic variant with a longer half-life. In 2002, the first case of its use was recorded at the Salt Lake City Winter Olympics. WADA then included EPO precursors and derivatives on its list of prohibited substances.

In Brazil, data on doping with rhEPO is scarce. In 2009, there were 12 cases of rhEPO use, mainly in endurance sports. Based on these cases, the Brazilian Code of Sports Justice was reformulated to align with the world anti-doping code, stipulating minimum penalties of four years for the use of prohibited substances. Among the notable cases is that of Simone Alves da Silva, who received a 30-year sentence after a repeat offense of rhEPO use.

RhEPO Doping Detection Methods

The analytical detection of EPO misuse in sports has evolved substantially over the past four decades, reflecting advances in biotechnology and anti-doping science. In the 1980s, detection strategies were limited because rhEPO had only recently been developed, and analytical methods were primarily based on radioimmunoassay (RIA) and early enzyme-linked immunosorbent assays (ELISA) designed for clinical quantification of circulating EPO levels. Although these assays offered adequate analytical sensitivity, they lacked the specificity required to differentiate endogenous EPO from recombinant forms, which share an identical amino acid sequence. During the 1990s, attempts were made to improve detection through electrophoretic approaches such as SDS-PAGE and immunoblotting, but these techniques were still insufficient to reliably distinguish endogenous and recombinant EPO molecules because of their very similar molecular weights. Therefore, WADA and the IOC sought qualified individuals to study and develop methods that could differentiate rhEPO from EPO. Furthermore, studies and cases of athlete deaths due to increased hematocrit and the possibility of heart attacks and strokes in healthy athletes using rhEPO led authorities to focus even more on detecting the misuse of this substance (29).

A major breakthrough occurred around 2000, when an urine analysis method was proposed, knowing that EPO is normally present at low concentrations. This method involved visualizing different isoelectric points, as they discovered that rhEPO has different acetylation and glycosylation levels than endogenous EPO. This method was the first official anti-doping control for EPO, used at the Sydney Olympics in 2000 (33). The isoelectric focusing (IEF) detection method for EPO is valid and reliable. It has undergone extensive scientific validation and has been successfully used for many years by accredited anti-doping laboratories worldwide. It is a well-established procedure widely accepted by the scientific community. The limit of detection of the method is 20 pg of protein applied to the IEF gel when combined with chemiluminescent detection (34). Furthermore, in all its decisions regarding EPO, the Court of Arbitration for Sport (CAS) has supported the validity of the EPO detection method. At a meeting in September 2005, the WADA Laboratory Committee reiterated its support for the method when applied correctly (35).

Due to the micro-heterogeneity in their structures, natural and recombinant EPO, which comprises several isoforms, some of which have charge differences, could be separated by isoelectric focusing. It was then found that the isoelectric patterns of the two recombinant forms of EPO, alpha and beta, are very similar. Both have an isoelectric point between 4.42 and 5.11, and both differ

from endogenous EPO, which has more acidic bands between 3.92 and 4.42. These differences allowed the excreted EPO to be attributed to either a natural or recombinant origin. To confirm the results of the urine patterns, a method based on IEF and double immunoblotting with a limit of detection of 1-5 pg/ml urine was also developed (36). This analytical procedure was applied to frozen urine samples from cyclists who participated in the 1998 Tour de France, marked by rhEPO doping scandals (37).

However, this method presents some challenges, such as obtaining reliable images of the isoelectric focus pattern of EPO in urine. Another difficulty is that the amount of EPO found in urine is extremely low, which prevents it from being replicated to actually prove that rhEPO levels are present. Furthermore, the urine needs to be concentrated, as dilution can lead to the loss of rhEPO samples. Also, this method can generate many false positives related to excessive proteinuria (37).

Another detection method is ELISA, which serves for the quantitative determination of EPO in human serum. This method is used for in vitro diagnostics or as an aid in the diagnosis of anemia and polycythemia. Two different rat monoclonal antibodies against human EPO are used, targeting well-defined regions on the endogenous EPO molecule. One of the antibodies is biotinylated, while the other is labeled for horseradish peroxidase for detection. The samples are simultaneously incubated with enzyme-labeled antibody and antibody-coupled biotin in a streptavidin-coated microplate. At the end of the incubation, the microwell is washed to remove unbound components, and the enzyme-bound solid phase is incubated with tetramethylbenzidine (TMB) substrate. An acidic stopping solution is added to stop the reaction and convert the color to yellow, the intensity of which is directly proportional to the EPO concentration in the sample. The limit of detection depends on the ELISA kit used but vary between 4-40 pg/ml urine. However, this detection method also has its limitations because the test may not be sensitive enough to discriminate against an abnormally low value. Furthermore, if plasma has high viscosity, it may also detect low EPO levels (38).

Efforts to directly detect rhEPO in urine led to parallel research seeking physiological markers of hormonal activity in this biological fluid. Based on this fact, it was proposed that the use of rhEPO may act not only by stimulating erythropoiesis, but also by promoting a process of fibrinolysis and/or fibrinogenolysis in the blood. This catabolic action of EPO would be associated with urinary concentration values of fibrin and fibrinogen degradation products, which are blood proteins involved in the coagulation cascade. A screening of 76 international-level athletes practicing sports potentially suitable for rhEPO use showed that more than 13% had elevated levels of these protein degradation products in their urine. As it was observed that physical exertion alone does not significantly promote this effect, it was concluded that the athletes had used rhEPO (35).

Given the numerous uncertainties of the direct IEF method, another direct test for the detection of rhEPO and analogues using sodium dodecyl sulfate gel (SDS-PAGE), an anionic detergent that interacts with proteins, conferring negative charges to them, was proposed (39, 40). The distinction between recombinant endogenous EPOs and analogues in the SDS gel occurs due to the different molecular weights they present (38). Unlike IEF, which separates molecules according to their isoelectric points, in SDS gel electrophoresis, molecular mass determines the migration position of each of the applied proteins. Proteins with lower molecular weights migrate more rapidly through the gel towards the anode, while larger proteins migrate less. Twenty milliliters of urine to be tested are immunopurified to avoid overloading with proteins other than EPO. There is a protein reduction step with dithiothreitol, a strong reducing agent, and after electrophoresis on the SDS gel, double transfer and chemiluminescent detection are performed in the same way as in the IEF method (40). The SDS gel electrophoresis with chemiluminescent detection has a limit of detection around 5pg EPO (41). In ascending order, these molecules can be organized according to molecular weights: endogenous EPO (34 kDa), rhEPO α and β (36-38 kDa), NESP (38.5-40 kDa) and, lastly, exhibiting the highest molecular weight due to its attachment to a methoxy polyethylene glycol polymer, CERA (69-78 kDa) (29, 42).

Since 2009, SDS detection has been used to support the confirmation of positive IEF results (40). It is also a widely used method for the specific detection of rhEPO, since it generates a very characteristic band on the SDS gel and the detection window is increased when compared to IEF (39). Due to all these advantages, the use of SDS is listed as a recommended method for confirming a positive result in the WADA technical document TD2024EPO (43), which establishes parameters for interpreting the results obtained (29).

In summary, several analytical methods have been used for the detection of EPO in biological samples, each presenting distinct analytical advantages, limitations, and applicability in anti-doping testing. IEF separates EPO isoforms according to their isoelectric points and is widely used because endogenous and recombinant EPO exhibit different glycosylation patterns that result in distinct electrophoretic profiles. However, IEF alone may suffer from non-specific protein binding and interference from urinary proteins, which led to the development of IEF combined with double immunoblotting, currently considered the reference strategy in anti-doping laboratories because it improves specificity by reducing secondary antibody cross-reactivity and allows detection of subtle differences in EPO glycoforms. Nevertheless, this method is technically demanding, requires extensive sample preparation (including concentration and immunopurification), and involves relatively high operational costs and specialized equipment, limiting its scalability outside accredited laboratories. SDS-PAGE followed by immunoblotting with chemiluminescent detection provides complementary information by separating EPO molecules based on molecular weight and enabling the identification of certain EPO analogues or

biosimilars that may not be clearly resolved by IEF alone; however, this method also requires immunodetection steps and trained personnel. In contrast, ELISA assays are relatively inexpensive, rapid, and easily scalable for routine screening because they quantify total EPO concentrations with high sensitivity. However, ELISA cannot distinguish endogenous from recombinant EPO isoforms and therefore has limited applicability as a confirmatory anti-doping test. Analytical challenges common to these methods include the extremely low concentrations of EPO in biological matrices, the presence of structurally similar erythropoiesis-stimulating agents, potential interference from urinary proteins or contaminants, and the need for pre-analytical purification steps. Consequently, routine anti-doping workflows often rely on a combination of screening assays and confirmatory electrophoretic techniques to balance sensitivity, specificity, cost, and operational feasibility (44).

Conclusions

EPO is a substance of great relevance in both therapeutic and sporting contexts. Its role as a pharmacological agent in the treatment of diseases such as anemia, especially in chronic kidney disease patients, demonstrates its efficacy and clinical importance. In the sporting context, the use of EPO as a doping agent has brought ethical and health challenges to the sport, compromising both the safety of athletes and the integrity of competitions. The detection of rhEPO was an important milestone in the fight against doping, thanks to the development of reliable methods such as IEF, ELISA, and SDS-PAGE. These methods allow differentiation between natural and recombinant EPO, increasing the accuracy in identifying misuse. Despite challenges such as very low concentrations and the possibility of erroneous results, scientific advances and the work of institutions such as WADA ensure greater reliability in anti-doping control.

Author contributions

M.B.S.: Conceptualization, Methodology, Writing – original draft, B.P.S.: Writing – review and editing, M.D.A.: Conceptualization, Supervision, Writing – review and editing.

Conflict of interest

The authors declare no conflicts of interest.

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