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·*Review*·

Hormone abuse in sports: the antidoping perspective

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Abstract

Since ancient times, unethical athletes have attempted to gain an unfair competitive advantage through the use of doping substances. A list of doping substances and methods banned in sports is published yearly by the World Anti-Doping Agency (WADA). A substance or method might be included in the List if it fulfills at least two of the following criteria: enhances sports performance; represents a risk to the athlete's health; or violates the spirit of sports. This list, constantly updated to reflect new developments in the pharmaceutical industry as well as doping trends, enumerates the drug types and methods prohibited in and out of competition. Among the substances included are steroidal and peptide hormones and their modulators, stimulants, glucocorticosteroids, β2-agonists, diuretics and masking agents, narcotics, and cannabinoids. Blood doping, tampering, infusions, and gene doping are examples of prohibited methods indicated on the List. From all these, hormones constitute by far the highest number of adverse analytical findings reported by antidoping laboratories. Although to date most are due to anabolic steroids, the advent of molecular biology techniques has made recombinant peptide hormones readily available. These substances are gradually changing the landscape of doping trends. Peptide hormones like erythropoietin (EPO), human growth hormone (hGH), insulin, and insulin-like growth factor I (IGF-I) are presumed to be widely abused for performance enhancement. Furthermore, as there is a paucity of techniques suitable for their detection, peptide hormones are all the more attractive to dishonest athletes. This article will overview the use of hormones as doping substances in sports, focusing mainly on peptide hormones as they represent a pressing challenge to the current fight against doping. Hormones and hormones modulators being developed by the pharmaceutical industry, which could emerge as new doping substances, are also discussed. *(Asian J Androl 2008 May; 10: 391–402)*

Keywords: World Anti-Doping Agency; doping; hormone; sport

1 Doping in sports and the World Anti-Doping Agency (WADA)

Doping is a problem that has plagued the world of competition and sport for ages. Even before the dawn of Olympic history in ancient Greece, competitors have looked for artificial means to improve athletic performance (from eating figs in ancient Greco-Roman times, to injecting with modern-era recombinant synthetic products). It has taken several decades for sports organizations to realise the magnitude of the menace that doping poses to fair play and the hazards it presents to the health and well-being of athletes, thereby triggering the recent establishment of the systematic fight against doping.

It was only in 1967 that the International Olympic Committee (IOC) created a Medical Commission that initiated the introduction of antidoping regulations, including the first official list of prohibited substances (listing exclusively stimulants). The first doping control tests were carried out during the 1972 Munich Olympic Games, systematic screening of urine samples was introduced at the 1983 Caracas Pan-American Games, and blood testing was first implemented at the 1994 Lillehammer Winter Olympics.

WADA was created in 1999 as a result of the IOC-convened World Conference on Doping in Sport where

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both the IOC and governments agreed to create an independent agency to promote, coordinate, and monitor the fight against doping in sport internationally. The creation and implementation of the World Anti-Doping Code and the related International Standards is one of WADA's main responsibilities. The Code constitutes the cornerstone for harmonization of antidoping regulations across all sports and all countries.

2 Prohibited substances in sport

Doping is defined in the World Anti-Doping Code (see the current version at http://www.wada-ama.org/rtecontent/document/code_v3.pdf) as the occurrence of an antidoping rule violation. Among the potential violations of antidoping rules, those referring to the use or attempted use of prohibited substances or methods occupy a central place. Identifying prohibited performance-enhancing substances used in sports is a growing global challenge, one made more difficult by the development of ever more sophisticated drugs and advanced methods to avoid detection.

According to the Code, for a substance to be considered for inclusion in the Prohibited List, it shall meet two of the following three criteria: it has the potential to enhance or enhances sport performance; it represents a health risk for the athlete; or it is contrary to the spirit of the sport. Importantly, the Code establishes the principle of "strict liability", according to which the presence of a prohibited substance (or its metabolites) in an athlete's bodily specimen is enough to constitute an antidoping rule violation, irrespective of the athlete's personal culpability (intention or negligence) for such a finding.

The performance-enhancing effects of any given substance are for the most part directly related to its ergogenic effects (enhanced strength, higher energy production, and better recovery), anabolic potential (increased protein synthesis, especially in muscles), and/or stimulating properties (increased attention and loss of fear), which confer a competitive advantage to athletes.

The prohibited substances could be of two different origins: exogenous, that is, not ordinarily capable of being produced by the body naturally; or endogenous, that is, substances naturally produced by the body. Synthetic anabolic androgenic steroids (AAS), such as methyltestosterone and nandrolone, are examples of the former category. Endogenous hormones, such as human growth hormone (hGH), erythropoietin (EPO), testosterone, dehydroepiandrosterone, and insulin, belong to the latter group. Notwithstanding the potent effects of many exogenous compounds, the endogenous and biosimilar agents (analogs of endogenous compounds containing structural modifications that improve their biological effects, e.g. insulin analogs) offer a clear advantage to the dopers, in that they are more difficult to detect. We will therefore concentrate on this group of substances in this review.

2.1 Hormones as doping agents

2.1.1 AAS

AAS, a class of steroid hormones related to the male hormone testosterone, are classic examples of doping agents [1]. These compounds enhance athletic performance by augmenting muscle mass and strength. AAS are misused by athletes and non-athletes alike, including schoolchildren, and there appears to be a trend for giving potentially harmful high doses in combination with other performance-enhancing drugs, such as hGH [2–6].

AAS are associated with serious adverse effects, such as reduced fertility and gynecomastia in males, masculinization in women, cardiovascular complications, cancer, liver toxicity, behavioural disorders and, after chronic high-dose usage, irreversible organ damage [6]. Still, the strong and long-lasting benefits to performance offered by these agents, their wide availability, and the documented attempts to develop new generations of sophisticated "designer" AAS that are increasingly difficult to detect, make AAS an all too easy choice for athletes ready to risk their health to get an unfair competitive advantage.

Recent high-profile investigations, conducted in the USA, have shed light on the scale of this problem. The notorious Bay Area Laboratory Cooperative case revealed the extent of the involvement of elite athletes with a laboratory devoted to the development of new classes of anabolic steroids, such as tetrahydrogestrinone (THG) and desoxymethyltestosterone (DMT). The "Operation Which Doctor", recently conducted by the district attorney's office in Albany (NY, USA), has exposed a network created to distribute illegal AAS and other doping substances. Furthermore, in 2005, federal prosecutors successfully disrupted a network of Mexican manufacturers supplying steroids to the US black market. A few months ago, the US Drug Enforcement Agency, as part of "Operation Raw Deal", seized more than 10 million doses of steroids and hGH from 56 laboratories in the largest crackdown on illegal steroids in US history. This investigation was assisted by the governments of nine other countries, including China, which constitutes a major source for the raw material.

AAS are among the most frequently detected drugs in sport. Analytical methods based on the combination of chromatographic (liquid chromatography [LC] or gas chromatography [GC]) and mass-spectrometric (MS) as well as isotope ratio MS (IRMS) approaches have been developed and successfully implemented by WADA-accredited laboratories for the detection of many of these compounds, including designer steroids (such as THG

and DMT). IRMS is particularly important for its ability to discriminate between exogenous and endogenous AAS like testosterone. Nevertheless, the results of these investigations have illustrated the continued widespread use of prohibited substances by athletes, in spite of the risks of detection leading to an antidoping violation and tough penalties that could lead to the end of their sporting career.

The combination of the development of more sophisticated detection methods and the increased awareness of the antidoping authorities, led by WADA and backed by the growing support of law-enforcement agencies and the pharmaceutical industry, provides the basis for the successful identification and banning of newly developed performance-enhancing drugs, including designer steroids.

2.1.2 Peptide hormones

Endogenous peptide hormones with potential performance-enhancing properties are listed in WADA's List of Prohibited Substances (see the 2008 List at http://www.wada-ama.org/rtecontent/document/2008_List_En.pdf) under section S2, "Hormones and related substances". The following substances, and their releasing factors, are prohibited: EPO; hGH; insulin-like growth factors (IGF; e.g. IGF-I and mechano growth factors [MGF]); gonadotrophins (e.g. luteinising hormone [LH] and human chorionic gonadotrophin [hCG]); insulins; and corticotrophins. Any other substance with similar chemical structure or similar biological effect(s) also falls under this section.

Recently, the misuse of endogenous hormones appears to have increased dramatically as a result of several factors. First, the development of molecular biology techniques, especially recombinant DNA technology, has provided ample supplies of relatively cheap synthetic hormones. With these synthetic versions, some of the risks associated with the use of purified natural hormones, such as the transmission of Creutzfeldt–Jacob disease after using hGH isolated from cadaveric pituitary glands, are eliminated [7]. Also, these substances are to a significant degree structurally and biochemically identical to the hormones naturally produced by the body, thereby making their detection extremely challenging. They are usually rapidly degraded and cleared from the body, considerably narrowing the time window for their detection. Some of them, such as hGH, are excreted in such minute quantities in urine that urine analysis is not considered a viable option. So far, the highly sophisticated spectrometric methods used for detection of AAS have not been successfully applied to these large and complex biological proteins, despite recent advances in MS-based testing of peptide hormones such as insulin, IGF-I, and hCG [8–14]. In most cases, the current methodologies for their detection rely on assays based on the immunological recognition of each substance by specific antibodies, which require individual development and extensive validation.

2.1.2.1 Gonadotrophins

hCG is a glycoprotein of approximately 37 kDa that belongs to a family of peptide hormones that also includes follicle-stimulating hormone (FSH), LH, and thyroid stimulating hormone [15, 16]. These are heterodimeric proteins made up of two polypeptide chains, a common α subunit and an individual β subunit, that determine the biological activity [16]. hCG, in particular, has an extended highly glycosylated β-carboxy terminus that facilitates its detection with specific antibodies or through MS [12–14, 17, 18].

hCG is produced abundantly by the placental trophoblastic cells during pregnancy, stimulating steroid hormone production in the ovaries [15]. Therefore, hCG is used as a marker for pregnancy tests [19]. However, due to its stimulation of endogenous steroid production, leading to increased testosterone levels without affecting the testosterone/epitestosterone ratio (used as a criteria for detecting doping with exogenous testosterone), hCG has also been used for doping purposes [20, 21]. Linked to this action, the side-effects associated with hCG misuse are similar to those induced by AAS. Although hCG is not considered to be widely abused, its use can not be ignored. Since its prohibition by the IOC back in 1987, several male athletes have been found to have taken hCG.

LH is synthesized and secreted by gonadotropes in the anterior lobe of the pituitary gland. LH mediates its biological activity through the same receptor as hCG [15]. In females, the LH mid-cycle surge triggers ovulation and induces the synthesis of progesterone and estrogens, whereas in men it stimulates the production of testosterone by Leydig cells [22, 23]. Human menopausal gonadotrophin is a combination of FSH and LH that also has the potential to be misused for performance-enhancing purposes.

The presence of hCG and LH is currently considered a doping violation if detected, above specific values, in male athletes. In females, establishing the origin of these peptide hormones (exogenous, or naturally produced) constitutes a challenging task. In particular, hGH levels could remain high for several weeks following early spontaneous miscarriage. In this regard, urinary hCG testing in female athletes might expose an unrecognized pregnancy, thus risking an invasion of privacy. Furthermore, in contrast to men, hCG is reported to have negligible effects on blood testosterone levels in women [24].

Currently, hCG and LH are detected in urine doping analysis by immunoassays using panels of hormone-specific antibodies. However, some of these techniques

present inherent limitations [25]. Therefore, specific new methods based on tandem mass spectrometry (MS/MS) and high-performance liquid chromatography combined with MS/MS have been developed through WADA-sponsored research [12–14, 26].

2.1.2.2 hGH and IGF-I

hGH is a species-specific, single chain polypeptide hormone expressed primarily in the somatotroph cells of the pituitary gland. Its secretion into the circulation follows a pulsatile pattern resulting in widely fluctuating blood levels that are influenced by multiple factors such as age, gender, sleep, physical activity, diet, stress, fever, steroids, and the environment [27, 28].

In serum, hGH exists as a complex combination of multiple isoforms including the major 22-kDa form (65%–80%) and minor isoforms resulting from alternative mRNA splicing (20 kDa and 17.5 kDa) or proteolytic cleavage of the mature protein ($GH_{1–43}$ and $GH_{44–191}$) [29–31]. hGH also exists as dimers and oligomers of up to 5 units, linked through covalent and non-covalent bonds, forming both homodimers and heterodimers [32]. Following secretion into the blood circulation, the unbound 22-kDa hGH has a short half-life of 10–20 min [27, 33, 34]. However, a proportion of circulating hGH forms complexes with hGH binding proteins that protect hGH from degradation and augment its half-life in circulation [35].

At this time, hGH is believed to be one of the most widely abused performance-enhancing agents. hGH is a pleiotropic hormone that stimulates many metabolic processes in cells. hGH affects protein, fat, carbohydrate, and mineral metabolism [27, 36]. Historically, some of the effects attributed to hGH, which might explain the attraction for its use as a doping agent, include the reduction of body fat and the increase in muscle mass (as well as strength if combined with moderate exercise), and its tissue-repairing effects on the musculoskeletal system. However, the translation of these biological effects into improvement in sports performance is based on anecdotal evidence, as the current clinical evidence of its anabolic effects in healthy adults is not well documented.

Recombinant hGH (rhGH), when given to GH-deficient individuals, has been found to increase exercise time and VO_{2max} after 6 months of treatment [37, 38]. Increased body muscle mass, decreased body fat, increased cardiac output, and improved wound healing has been described [28, 39]. In contrast, no significant effects on muscle strength or muscle protein synthesis have been reported in controlled clinical trials of healthy non-exercising young men or athletes given supraphysiological doses of rhGH [40, 41]. Similar results have been reported in studies conducted with elderly men [42].

Despite the apparent lack of performance-enhancing effects in short-term studies with normal subjects, rhGH is considered the doping drug of choice in a number of endurance and power sports. The recovery of great quantities of rhGH doses from "Operation Raw Deal" and the links established between illegal manufacturers and distributors and alleged high-profile athlete recipients show that hGH is being misused and abused in sports with the purpose of enhancing athletic performance.

Reportedly, it seems that athletes use hGH for long periods of time with supratherapeutic doses or in combination with other doping substances such as AAS. hGH appears to act synergistically when used in tandem with steroids, thus having an effect, albeit indirect, on muscle anabolism and athletic performance [34, 43, 44].

IGF-I is a small 7.5-kDa peptide that has shown anabolic effects on cytoskeletal muscles [45]. IGF-I associates in plasma to high-affinity IGF-I binding proteins (IGFBP), which increase its half-life in circulation. Under normal conditions, 75%–80% of circulating IGF-I remains bound to IGFBP-3 and the acid labile subunit (ALS) in a ternary 150-kDa protein complex [46, 47]. Therefore, many factors that influence hGH concentrations, such as circadian rhythm or the pulsatile nature of pituitary hGH release, have minimal or no effect on IGF-I [48].

hGH is one of the most important factors regulating IGF-I synthesis and its release into circulation. Other factors involved include nutrient intake, thyroid status, and estrogen and cortisol levels [48]. The association between hGH and IGF-I has been established by the low IGF-I concentrations observed in hGH-deficient pediatric patients and the elevated levels found in active acromegaly [49]. In active adults, serum IGF-I levels decline gradually with age, reflecting the concomitant drop in hGH secretion [50].

Arguably, most of the growth and metabolic effects of hGH are mediated by IGF-I, and include the increase in total body protein turnover and muscle synthesis [51]. In any event, IGF-I is the most important marker of hGH action in the liver [33]. hGH stimulates the liver to produce IGF-I, which is secreted into circulation and acts, in a paracrine manner, on other tissues of the body. IGF-I has an anabolic effect, particularly in muscle, by inducing protein synthesis through enhanced amino acid uptake [51–53]. Experiments in mice injected with a gene-delivery device that induces the myocyte overexpression of IGF-I have shown an IGF-I-mediated increase in muscle mass (by 15%) and strength (by 14%), without affecting IGF-I serum concentrations [54]. In addition, IGF-I stimulates muscle glucose transport activity and this effect is enhanced by acute bouts of intense exercise and training [55].

Mechano growth factor (MGF or IGF-IEc) derives

from an alternative splicing of the *IGF-I* gene and is expressed in a mechano-sensitive manner in skeletal muscles [56, 57]. The levels of MGF mRNA are low in resting muscle, however, resistance exercise and local tissue injury upregulate MGF expression, leading to the activation of muscle satellite cells and initiation of muscle hypertrophy [58, 59]. Due to these anabolic properties, alleged MGF preparations are advertised and illegally sold as anabolic agents over the Internet.

The long-term effects of rhGH on physical performance and athletes' health are not known. As a result of rhGH, levels of serum IGF-I are rapidly upregulated, and they remain elevated for several days following rhGH withdrawal [60]. The combination of hGH and exercise has also been shown to markedly enhance the expression levels of MGF mRNA in muscle [58, 59]. Therefore, the effects of this GH/IGF-I axis on muscle metabolism would have an additive effect, probably seen in the long-term after chronic hGH use.

hGH abuse by healthy persons could lead to serious side-effects. Based on the pathological changes reported in hGH-overproducing acromegalic patients, hGH abuse might increase the risk of diabetes, hypertension, cardiopathies, myopathy, osteoporosis, damage to joints and articulations, abnormal bone growth, and disturbed lipid patterns [28, 61].

The detection of doping with hGH constitutes a significant challenge for antidoping authorities and antidoping laboratories [34]. Despite the availability of multiple assays that have been used to determine hGH for clinical and research purposes, all are unsuitable to reliably assess hGH abuse in sports [62–65]. Indeed, because hGH is secreted in a pulsatile manner, an elevated measurement may just reflect peak pituitary release of hGH. In addition, exercise modifies the levels of hGH and the release could be influenced by variations in nutritional intake, stress, and other factors already mentioned, so it would be difficult to rely on only a single blood analysis that measures total hGH. To add to this complexity, exogenous rhGH is structurally and biochemically indistinguishable from the endogenous 22-kDa isoform, including a very short half-life with hGH levels returning to normal 8–20 h after being given.

A novel approach to detect doping with rhGH, described as the isoform differential immunoassay, has been proposed by Prof. Christian Strasburger and Drs Zida Wu and Martin Bidlingmaier [66, 67]. The isoform differential immunoassay was developed to detect hGH doping by exploiting the differences in the proportions of hGH isoforms under physiological conditions and following doping practice. The method is based on the essential principle that the normal composition of hGH in blood is a mixture of different isoforms, present at constant relative proportions. In contrast, rhGH is constituted only of the 22-KDa molecular form. Exogenous rhGH not only increases the concentration of the 22-KDa isoform but also suppresses the non-22-kDa concentrations for up to 4 days, thus altering the natural ratios established between these hGH isoforms [68]. By using two different immunoassays employing antibodies that recognize the monomeric 22-kDa hGH or a combination of pituitary-derived hGH isoforms, the elevated ratios of 22-kDa hGH to pituitary-derived hGH isoforms is used to indicate doping with rhGH. The first versions of these assays were implemented during the Olympic Games in Athens 2004 and Turin 2006. Currently, a commercial version of the assays, developed on a chemiluminescent platform, has passed the stage of testing validation and is due to be implemented across WADA-accredited laboratories.

This is the first step in tackling the abuse of hGH with a newly developed detection method capable of unequivocally identifying the use of exogenous rhGH. However, the method has a critical limitation in that it allows for detection in a short period of time, up to 36 h after rhGH administration [67]. Consequently, its effectiveness might be more suited for out-of-competition testing, in which testing is carried out on an athlete at any time without prior notice.

Other approaches are being developed in parallel to these differential immunoassays. Due to its pleiotropic nature, hGH affects the expression of many different proteins that could serve as pharmacodynamic markers of hGH activity. These include markers of hGH action in the liver such as IGF-I, IGFBP-2, IGFBP-3, and ALS, as well as markers of hGH action on bone formation and resorption and on soft tissue collagen turnover, including osteocalcin, N-terminal peptide of procollagen type III (P-III-P), C-terminal telopeptide of type I collagen, and C-terminal propeptide of type I collagen [34, 69]. The detection of such markers would uncover the manipulation of the hGH/IGF-I axis independently of the doping substance used, be it rhGH or other agents used to increase circulating hGH (e.g. analogs of GH-releasing hormone, hGH secretagogs, or even hGH gene doping). In studies conducted as part of the GH-2000 and GH-2004 projects [60], two particular markers, IGF-I and P-III-P, have been found to be particularly sensitive to the effects of exogenous hGH [70, 71]. Whereas the concentrations of circulating IGF-I rise rapidly after hGH use, the levels of P-III-P increase more gradually and stay upregulated for a longer period of time. Discriminatory formulae based on measured blood concentrations of these two proteins have been developed, allowing for optimal identification of rhGH administration samples for longer detection periods [70, 71]. The final aim is to combine both approaches, the differential isoform immunoassay and the marker detection, to in-

crease the chances of detecting doping with hGH both in and out of competition.

2.1.2.3 EPO

EPO is a 30.4-kDa glycoprotein hormone that is mainly produced by the kidney and is a key regulator of red blood cell production [72–74]. EPO stimulates the proliferation and differentiation of bone marrow erythroid precursors [75].

The introduction of recombinant EPO (rhEPO) has permitted the effective therapeutic treatment of anaemia associated with chronic kidney disease, HIV, myelodysplastic syndromes, bone marrow transplantation, hepatitis C, or following chemotherapy regimes against cancer [75–78]. First-generation rhEPO products include epoietin-α and -β, produced in transformed Chinese hamster ovary (CHO) cell cultures, and epoietin-ω, engineered in baby hamster kidney (BHK) cells [79].

EPO in blood circulation consists of isoforms that differ in their patterns of glycosylation and biological activity [80, 81]. The carbohydrate moiety of EPO consists of one O-linked and three N-linked sugar residues [82]. The sialic acid-ending *N*-glycans are essential for EPO's biological functions and duration in circulation [83]. A novel recombinant EPO (epoietin-δ or Dynepo) is produced in a human fibrosarcoma cell line (HT-1080), thus having a more human-like glycosylation pattern characterized by the absence of *N*-glycolylneuraminic acid [84]. The presence of extra *N*-glycans increases the half-life, and therefore the duration of biological effects, *in vivo*, of some recombinant EPO products such as darbepoietin-α (Aranesp, derived in CHO cells) [85, 86]. In contrast, de-sialylated EPO is rapidly removed from circulation. This apparent advantage of longer duration offered by the modified rhEPO might serve, conversely, to facilitate its detection by antidoping laboratories.

The recent expiration of patents protecting the intellectual property and marketing rights of the first-generation rhEPOs has further increased the menace of EPO abuse in sports due to the emergence of so-called "biosimilars" and generic copies of established EPO products [87]. Several other EPO analogs and derived molecules are also in different stages of pharmaceutical development, including continuous erythropoiesis receptor activator, a recently commercialized pegylated epoietin-α, a hyperglycosylated darbepoietin-α analog (AMG 114), the polymer-bound analog synthetic erythropoiesis protein (SEP), and EPO molecules fused to the Fc region of a human antibody or to another EPO molecule through flexible chemical linkers [88]. All of these preparations have been designed to increase the half-life in circulation and extend the biological effects of EPO *in vivo*. EPO mimetics (compounds structurally unrelated to EPO but with similar biological activity due to their ability to engage the EPO receptor) and hypoxia inducible factor-1 (HIF-1) agonists constitute additional efforts to mimic the actions of EPO in a clinical situation [87].

Due to its effect of increasing hemoglobin (Hgb)-bearing erythrocytes responsible for the oxygen-carrying capacity of the blood, EPO has been used extensively as a performance-enhancing aid in sports, particularly in endurance disciplines requiring an adequate supply of oxygen to the heart and the muscles. The first suspected cases of blood doping with recombinant EPO date back to the late 1980s, when a cluster of sudden deaths of European cyclists was associated with its market appearance [88]. Since then, numerous examples of doping with EPO have made the headlines. Most notoriously, the affair involving the Festina team during the 1998 Tour de France revealed a widespread abuse of EPO in cycling and led to a drastic change in the IOC's approach to doping in sports and ultimately to the creation of WADA. The withdrawal of six Chinese female track and field athletes from the Sydney 2000 Olympics coincided with the introduction of the first EPO blood tests, fuelling suspicions of EPO abuse as the cause for their previous exceptional performances. During the Salt Lake City 2002 Winter Olympics four athletes were stripped of their medals after being found positive for darbepoietin-α. The "Operación Puerto" investigation of a medical clinic in Spain, linking several elite cyclists with blood-doping methods, uncovered multiple doses of EPO and bags of frozen blood for reinfusion. Recently, a Canadian cyclist, Genevieve Jeanson, has admitted to having used EPO throughout her career after many years of denial. Many other examples of EPO abuse by elite athletes have been and are still being reported.

The excessive use of EPO is associated with serious adverse side-effects, including hypertension, headaches, and an increased rate of thrombotic events as a result of an EPO-induced rise in the hematocrit and thickening of the blood [89, 90]. In addition, EPO withdrawal could be implicated in neocytolysis, that is, the hemolysis of young red blood cells in the presence of increased hematocrit [91]. Ultimately, EPO abuse could cause death.

Methods for detection of doping with EPO include the combination of direct and indirect approaches [75]. The direct method currently used in WADA-accredited antidoping laboratories is based on differences in the pattern and extent of glycosylation of rhEPO as compared to the endogenously produced protein. The glycosylation pattern of rhEPO preparations is determined by several factors, including the cell line from which they are recombinantly expressed, the media employed for cell culturing, and the methods of protein purification [87]. The different arrangements of sugar residues found in rhEPOs result in differences in their isoelectric points that are detected by a method combining isoelectric fo-

cusing (IEF) and double immunoblotting [92–94]. The situation is more complicated for the detection of EPO variants produced in human cells, such as darbepoietin. Nevertheless, it is unlikely that darbepoietin behaves exactly as endogenous EPO using IEF, because the pattern of bound *N*-glycans is also tissue-specific [87].

The indirect methods incorporate changes of hematological parameters of erythropoiesis, such as Hgb, percentage of reticulocytes, and serum concentrations of EPO and soluble transferring receptors [75]. Some of these parameters could be disturbed for up to 4 weeks after rhEPO use, thereby increasing the chances of detection. In addition, such methods might offer the advantage of detecting other manipulations aside from the use of rhEPO. Algorithms have been developed that are capable of detecting the use of rhEPO either during the administration phase (ON-model: Hgb, EPO, and soluble transferring receptors) or during the wash-out period (OFF-model: Hgb and reticulocytes) [95]. The former model detects up to 100% of rhEPO-containing samples during the period of use, but has a short time window of detection (approximately 48 h) following injection of relatively low doses of EPO. In contrast, the OFF-model allows detection for up to 2 weeks after EPO withdrawal [96–98].

During the Sydney 2000 Olympics, a combination of the direct and indirect blood methods for detection of EPO was implemented. However, since 2004, only the urine-based IEF method has been used for the direct analysis of EPO use. Nevertheless, the indirect EPO methods will be integrated in the model foreseen for the creation of the hematological module of the athlete's passport (longitudinal follow-up over time of the athlete's biological parameters).

Currently, WADA is sponsoring several research projects aimed at improving the efficacy of this method as well as finding alternative approaches, such as the assay currently in development by the Swedish biotechnology company MAIIA (Uppsala, Sweden). Likewise, WADA has funded the development of a potent software tool, GasEPO, a support for the interpretation of IEF results [99].

An alternative indirect approach is the athlete's hematological passport, that is, the establishment of individual hematological profiles by the comparison of measured values of blood parameters against the individual's historical baseline, considered an effective tool for doping control. This approach would eliminate the inter-individual variability observed in the population-derived ranges currently used. Deviations from the normal longitudinal profile would result in a ban from participating in a particular competition, and in further investigations to establish an incidence of doping or possibly sanction for an antidoping rule violation. Recently, the Union Cycliste Internationale (UCI) became the first international sports federation to implement the athlete's hematological passport approach as part of its antidoping program. The application of longitudinal profiling might also help to eliminate reported cases of undetectable EPO profiles that have been observed in various situations and might have been caused by manipulation of the sample with EPO-degrading proteases [100].

2.1.2.4 Insulins

Insulin is produced by β cells in pancreatic islets through a complex proteolytic process involving the enzymatic cleavage of pro-insulin (9.6-kDa) into insulin and C-peptide. Human insulin is a 5.8-kDa polypeptide hormone consisting of two peptide chains of 21 (A) and 30 (B) amino acid residues connected by disulfide bonds. Insulin molecules are capable of self-association into hexameric aggregates, but only the monomers have biological activity [101].

Insulin is primarily used to treat insulin-deficient patients suffering from type 1 diabetes mellitus. However, due to its influence on many metabolic processes, insulin is also a potential performance-enhancing agent [51]. Insulin increases the rate of glucose uptake into adipose and muscle tissues and stimulates glycogenesis, thus increasing the intramuscular energy reserves. The combination of short-acting insulin and high carbohydrate diets has an anabolic effect on muscle mass through the inhibition of protein breakdown. The use of insulin could also improve post-competition recovery and stamina [33].

In addition to the availability of recombinant human insulins, such as neutral protamine hagedorn (NPH), several other insulin analogs have been developed, with altered pharmacokinetics compared with the naturally produced insulin. These compounds, produced by recombinant DNA technology, are classified into rapid-acting (e.g. aspart [NovoLog] and lispro [Humalog]) and long-acting (e.g. glargine [Lantus]) synthetic insulins that differ from human insulin only slightly in amino acid sequences [102–104].

The detection of insulin in biological fluids represents a complicated task. In blood, hemolysis and/or the presence of circulating anti-insulin antibodies can interfere with the results of the analyses. In urine, insulin is mainly excreted as a product of its metabolic degradation. Therefore, physiological urine concentration levels of native insulin are in the fentomolar range, requiring extremely sensitive assays for its detection. The detection of insulin in urine has, until recently, been done with the use of commercially available radioimmunoassays or enzyme-linked immunosorbent assays. Many of these assays, however, show cross-reactivity with human pro-insulin or products of degradation and are not discriminant enough to differentiate insulin from its synthetic

analogs. A sensitive radioimmunoassay test has been developed for the detection of insulin lispro and an enzyme-linked immunosorbent assay test, specific for insulin aspart, has also been reported [105, 106]. Recently, a new analytical method involving the combination of a sophisticated sample preparation procedure and LC-MS has been developed, with financial support from WADA, for the identification of endogenous and synthetic insulins in urine [8, 9].

2.1.2.5 Other hormone-related doping agents

One of the side-effects of using AAS includes the excessive production of estrogens that results in the development of gynecomastia and the suppression of endogenous testosterone [107]. In order to overcome these negative effects, athletes abusing AAS use anti-estrogenic drugs. In addition, a number of anti-estrogens increases testosterone blood concentrations in normal men through a rise in the pituitary release of LH [24]. Three main categories of anti-estrogens can be distinguished:

1. Selective estrogen receptor modulators (SERMs). These compounds, used for the treatment of breast cancer and osteoporosis, can act as agonists or antagonists of the estrogen receptor, depending on the cell type and tissue. For example, SERMs like tamoxifen and raloxifen are estrogen antagonists in breast tissue, but they act as estrogen agonists in bone [108, 109].

2. Aromatase inhibitors (AIs). These compounds block the synthesis of estrogen by inhibiting the activity of estrogen synthetase. They have also been developed for the treatment of postmenopausal breast cancer. Two types of AIs have been produced: type I inhibitors (e.g. 4-hydroxyandrostenedione), steroidal substrate analogs that inactivate the enzyme; and type II inhibitors (e.g. letrozole, anastrozole) that are non-steroidal competitive reversible inhibitors [110].

3. Other anti-estrogenic substances. These include estrogen receptor antagonists with no agonist effect, such as fulvestrant, prescribed for treatment of breast cancer, as well as clomiphene, used to treat infertility [111, 112].

For doping control purposes, anti-estrogens are detected by classical analytical chromatographic methods like LC-MS (e.g. exemestane, formestane, tamoxifen, and toremifene) [113–116] and GC-MS (e.g. aminoglutethimide) [117]. WADA has sponsored research projects to develop methods to detect anastrozole (by LC-MS/MS) and letrozole (by GC-MS) [113, 118] and is currently funding projects to synthesize certified reference materials for some of these anti-estrogenic compounds and their metabolites.

2.1.2.6 Hormones and hormone modulators in development

In addition to classical steroid and peptidic hormones, new classes of hormone agonists, antagonists, and modulators are constantly being developed by the pharmaceutical industry. WADA regularly monitors the clinical stages of investigational drugs as some of these could be used for sports performance enhancement. Once identified, projects aimed at detecting these new drugs before they are released into the market are funded by the Agency. Some examples are listed below.

2.1.2.6.1 Selective androgen receptor modulators (SARMs)

AAS are clinically prescribed to treat medical conditions such as male hypogonadism, muscle wasting disease, osteoporosis, and sarcopenia. However, due to their negative effects on high-density lipoprotein cholesterol levels and cardiovascular and prostate systems, other therapeutic alternatives are being sought. Recently, a novel class of investigational drugs, the SARMs, have been developed and some, like Ostarine (GTx, Memphis, TN, USA), are already undergoing phase II/III clinical trials [119]. These drugs appear to have the advantage of acting as full agonists of the androgen steroid receptor in target tissues such as muscle and bone, but they have minimal affect on organs such as the prostate and do not induce virilization [119].

As AAS are widely abused as doping substances, it is expected that SARMs, which retain all the advantages of enhancing performance while avoiding undesirable side-effects, will also be sought as doping substances. In anticipation of this trend, WADA is currently funding research projects to detect the misuse of SARMs.

2.1.2.6.2 Inhibitors of myostatin

Myostatin, also known as growth and differentiation factor 8, is a secreted protein and member of the transforming growth factor-β family that plays an essential role in skeletal muscle growth [120, 121]. Myostatin negatively modulates muscle satellite cell proliferation and inhibits muscle cell differentiation. Follistatin can interact with myostatin C-terminus, precluding its binding to the activin receptors and thus negatively modulating myostatin function [122]. Due to its properties, the naturally occurring mutations related to loss of function in the highly conserved myostatin gene produce muscle hypertrophy in both humans and animals [123, 124]. Mice carrying a targeted disruption of the myostatin gene show muscle fiber hypertrophy, hyperplasia, and a partial suppression of abnormal glucose metabolism and fat accumulation [125, 126]. Transgenic mice expressing a dominant negative activin II receptor or overexpressing follistatin also have dramatic increases in muscle mass [127].

In consideration of these properties, myostatin inhibitors have become attractive development drugs for the pharmaceutical industry. It is foreseen that such

compounds will be used as therapeutic treatments for muscle wasting diseases such as sarcopenia associated with aging, cancer cachexia, AIDS, amyotropic lateral sclerosis, and muscular dystrophy. Several myostatin inhibitors are under clinical development and their effects are based on the reduction of bioavailable myostatin. These include antibodies or fusion proteins directed against myostatin as well as soluble activin type II receptors [128–130].

Myostatin inhibitors have the potential to be a tempting doping option for cheating athletes. Although these compounds are still under clinical development, and in theory years away from commercialization, WADA has taken a proactive approach to include them on the Prohibited List and is currently funding research projects aimed at the detection of doping with myostatin inhibitors.

3 Conclusions

The sports world has made significant steps in the last few years to fight off the stigma of doping. However, it is envisaged that new doping threats will quickly emerge on the horizon. Therefore, the antidoping community, led by WADA and with the essential support of scientists and organizations committed to antidoping, will have to intensify current efforts. The wide availability of recombinant products similar to endogenous hormones, the generation of biosimilars and biological generics, analogs, and releasing factors of currently detectable substances, the potential threat of gene doping, and the appearance of new designer drugs, to name a few, can all lead to new doping practices against which appropriate doping control and detection methods must be developed. Complimentary to an effective testing program, a strong educational component is also needed to educate elite and occasional athletes on the serious health risks related to doping. A close cooperation between antidoping organizations and law-enforcing agencies for the identification and tackling of illegal drug manufacturers and supply chains also constitutes an essential element towards the success of the fight against doping in sports.

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