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REVIEW

Hormones as doping in sports

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Abstract Though we may still sing today, as did Pindar in his eighth Olympian Victory Ode, "… of no contest greater than Olympia, Mother of Games, gold-wreathed Olympia…", we must sadly admit that today, besides blatant over-commercialization, there is no more ominous threat to the Olympic games than doping. Drug-use methods are steadily becoming more sophisticated and ever harder to detect, increasingly demanding the use of complex analytical procedures of biotechnology and molecular medicine. Special emphasis is thus given to anabolic androgenic steroids, recombinant growth hormone and erythropoietin as well as to gene doping, the newly developed mode of hormones abuse which, for its detection, necessitates high-tech methodology but also multidisciplinary individual measures incorporating educational and psychological methods. In this Olympic year, the present review offers an update on the current technologically advanced endocrine methods of doping while outlining the latest procedures applied—including both the successes and pitfalls of proteomics and metabolomics—to detect doping while contributing to combating this scourge.

Keywords Doping · Anabolic androgenic steroids · Erythropoietin · Growth hormone · Olympic games

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Introduction

The Olympic games, founded in 776 B.C. (date of the earliest recorded Olympic competition) in Olympia as a tribute to the gods but also to celebrate the virtues of athletic competition, peaceful coexistence and the magnificence of athletics, constitute Olympia's perennial contribution to the world, this symbolized by the eternally burning Olympic flame. Today, however, the Olympic games are being severely undermined by excessive commercialization and, more seriously, by the scourge of doping, defined as the use of performance-enhancing drugs. Though doping is not exclusively restricted to the Olympics but is detected, in general, in big sports events, it is especially associated with the modern Olympic games, as a large number of athletes throughout the world, many of them raised to the status of national "heroes", have been found positive over the past few decades [1, 2]. It should also be mentioned in passing that doping is not limited to human sport competitions but is also practiced in animal sports, e.g. equestrian sports [3].

Underlying this distressing phenomenon is regrettably a worldwide pervasive attitude that represents the exact contrary of the original and declared aims of the event as promulgated by de Coubertin in 1903, namely, the aspiration "not to have conquered but to have fought well". In the last 20 years, this highly damaging phenomenon, which has been dubbed the toxic torch [4], has spread even to the Paralympics, systematic sports drug-testing having revealed a series of rule violations and adverse analytical findings in disabled athletes [5]. The present review thus discusses doping as it pertains to the Olympics.

World Anti-Doping Agency (WADA) and the International Olympic Committee release an annually updated list of substances and methods prohibited at all time in and out of competition. The purpose of the annual banned substance list

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is both to unequivocally support doping controls and to designate advanced methods dedicated to the detection of known and recently outlawed substances [6]. WADA has created several international standards whose purpose is to harmonize anti-doping organizations and to provide a common platform for laboratories carrying out tests based on the prohibited list [7]. It is important to note that there is frequently differentiation between the official methods employed for anti-doping detection in WADA/IOC laboratories and strategies applied experimentally in various other laboratories. However, laboratories that are not accredited by WADA may apply methods and develop strategies, such as WADA laboratories, in support of the Athlete Biological Passport Program, where all data produced by laboratories can be collated.

In this review, reference will be made only to the methods tested by WADA/IOC.

The aim of this review is thus twofold: (1) to update data on drug abuse, especially steroids, growth hormone (GH) and erythropoietin (EPO) in Olympic sports events (hormones such as insulin and glucocorticoids solely being used as part of drug cocktails), and (2) to evaluate the current methods of doping, including the novel method of gene doping, as well as means for its detection, this chiefly via molecular medicine, a field which is enabling more effective modalities for the needs of anti-doping control in general.

Anabolic androgenic steroids

Mechanisms of action

Normal testosterone secretion is essential for male health, sport participation and performance, while prohibited testosterone administration in athletes, depending on duration and dosage, causes iatrogenic andrological disease [8]. Furthermore, anabolic androgenic steroids (AAS), including testosterone and synthetic derivatives of testosterone, first introduced in the 1950s to enhance performance among weightlifters, represent the premier of doping agents, of which the most commonly used and mode of administration are displayed in Table 1. However, the route of administration has been found to have an effect on its detection since quantification of testosterone in urine samples is reliable only when the drug has been administered intramuscularly [9]. Oral administration, by contrast, is more problematic since it results in rapid pharmacokinetics, this requiring urine samples to be collected as soon as possible following administration to enable reliable quantification [9]. AAS bind with variably affinity in the cytoplasm to the androgen receptor (AR), a member of the steroid hormone receptor family, where they exert potent anabolic and endocrine activities [10]. The AR's binding to the androgen response element triggers its potential to act as a transcriptional modifier of various genes [11, 12].

Table 1 A list of commonly used anabolic androgenic steroids

Drug	Manner of administration
1-Androstenediol	Oral
Boldenone	Parenteral
DHEA	Oral
Mesterolone	Oral
Metenolone acetate ester	Oral
Metenolone enanthate ester	Parenteral
Metribolone (methyltrienolone)	Parenteral
Nadrolone decanoate	Parenteral
Stanozol	Oral/parenteral
Testosterone undecanoate	Oral/parenteral
Testosterone enanthate	Parenteral

The enzyme 5-alpha-reductase seems to possess an essential role by converting AAS into dihydrotestosterone (androstanolone), which acts in the cell nucleus of target organs, while the enzyme aromatase converts AAS into female sex hormones (estradiol and estrone) [13]. By displacing cortisol from its receptors, they antagonize the catabolic effects of glucocorticoids. AAS increase strength (by about 5–20 %) and body weight (by about 2–5 kg) due to an increase of the lean body mass without reduction of fat mass, although no effects have been observed on endurance performance [14].

Stanozolol is an anabolic steroid compound particulary favoured among athletes and body builders since it boosts strength without weight gain, while it is not converted to estradiol [15]. Metribolone (methyltrienolone) is a potent anabolic steroid, a non-aromatizable androgen, the 17-methylated derivative of trenbolone, which is characterized by high potential for hepatotoxicity [16].

Physiology and therapeutic use

By stimulating protein synthesis, AAS contribute to build-up of the skeletal muscles. AAS increase strength (by about 5–20 %) and body weight (by about 2–5 kg) due to an increase of the lean body mass without reduction of fat mass, although no effects have been observed on endurance performance [17]. AAS are therapeutically applied to induce male puberty as well as to stimulate appetite in chronic wasting conditions (AIDS, cancer).

Adverse effects

Depending on duration and dosage of administration, AAS can cause various adverse effects marked by virilization and hirsutism in women, deepening of voice (permanent) as well as testicular atrophy (reversible) and gynaecomastia

Table 2 The endocrine side-effects of anabolic androgenic steroids, recombinant growth hormone and erythropoietin preparations

Anabolic androgenic steroids	
Secondary hypogonadism	Amenorrhoea
Ovarian cysts	Virilization in females
Clitoral hypertrophy	Testicular atrophy
Gynaecomastia (severe) in males	Hoarse voice (women)
Hirsutism, acne	Hair loss
Type II diabetes mellitus	
Recombinant growth hormone	
Increase in left ventricular mass	Increase cardiac output
Arthralgias, swelling (retention of fluids)	Glucose intolerance
Insulin resistance and type II diabetes mellitus	
Arterial hypertension, sweating,	Carpal-tunnel syndrome
Recombinant erythropoietin	
Increases red cell mass	Reduces blood flow due to increased viscosity
Increases the likelihood of thrombosis and stroke	

in men [18–20]. Furthermore, ASS abuse in adolescence may induce premature epiphyseal fusion resulting in stunted growth [21]. The endocrine adverse effects of AAS abuse are summarized in Table 2. Finally, via both genomic and non-genomic pathways, AAS can trigger aggressive behaviour and hostility as well as mood disturbances such as depression and hypomania [22, 23]. However, the development of side-effects induced by supraphysiological doses of AAS correlate, at least as regards the psychiatric symptoms, to the dose and duration of abuse [23].

Considerable changes of lipid profile result in decrease in HDL and increase in LDL concentration, as well as diabetes mellitus, arterial hypertension and cardiac morbidity have been associated with AAS abuse [18, 19].

The most typical cardiac abnormality is left ventricular hypertrophy with fibrosis, while rare cardiovascular substrate is eosinophilic myocarditis [24]. Nevertheless, the cause–effect relationship between AAS abusers and cardiac death has not been conclusively established, though ventricular arrhythmias and congestive heart failure have often been reported, and thrombotic complications (intracardial thrombosis, stroke, venous thromboembolism, cerebral venous sinus thrombosis) have been markedly associated with AAS abusers [25].

Detection of ASS abuse

AAS misuse can be conventionally detected by steroid profiling including precursors and metabolites as well the urinary testosterone/epitestosterone (T/E) ratio, of which normal levels are below or up to 4.0 [26, 27]. Needless to say, any sample displaying levels above this threshold should be quantitatively analyzed for confirmation by tandem gas chromatography/mass spectroscopy (GC/MS) [28]. Guidelines for confirmatory analysis by GC/MS and LC/MS/MS have recently been released by WADA and other organizations [29]. High-performance liquid chromatography–tandem mass spectrometry (LC/MS/MS), also employed in recent years in forensic toxicology, has been established as a powerful and reliable tool for quantitative confirmatory analysis of drugs used for doping [29]. Epitestosterone administration is prohibited because it lowers the urinary testosterone/epitestosterone ratio, a marker of testosterone administration [30]. However, use of a gas chromatography–combustion–isotope ratio mass spectrometry method resulted in quantification of the delta [13] C values for urinary epitestosterone as high in controls and lower in the epitestosterone group [30].

A two-step derivatization procedure has recently been introduced to enhance performance of electrospray ionization liquid chromatography–MS in detecting ASS, these being compounds that notably possess limited ionization efficiency [31]. Nevertheless, the newly initiated approach based on high resolution/high accuracy MS and ion mobility has the capacity to analyze the gas phase dissociation behaviour of several new drugs. The thus enabled cartography of fragmentation routes of new compounds may permit a more rapid identification of metabolites and "tailor-made" analogues developed for doping purposes [31].

Designer drugs

However, despite progress in the development of GC/MS and ion mobility based methodology and though it plays a key role in ASS abuse detection, it has failed to detect the so-called *designer drugs*, the prototype being tetrahydrogestrinone [32]. Because these emerging drugs are devised specifically to evade detection, they impose a considerable burden on anti-doping methods [33]. A recently launched paradigm is "1-androsterone", administration of which modifies the urinary steroid profile, and in particular the ratios of androsterone/etiocholanolone and 5α-/5β-androstane-3α,17β-diol and the concentration of 5α-dihydrotestosterone [34]. Meanwhile, 3α-hydroxy-5α-androst-1-en-17-one, a characteristic metabolite, is likely to play an important role by permitting a wider time frame of detection of steroid abuse, since screening performed as much as 9 days after a single administration of one capsule enabled its detection [34].

Boldenone, which was synthesized with the aim of producing a long-acting injectable methandrostenolone, is meanwhile one of the most frequently detected AAS, generally exposed via GC/MS, that allows identification of the active drug and its main metabolite, 5β-androst-1-en-17β-ol-

3-one (BM1) [35]. Boldenone and epiboldenone sulphates, which are markers for the exogenous origin of boldenone, may be employed to decrease the number of samples to be analyzed by isotope ratio mass spectrometry [35].

Other anabolic drugs

In addition, clenbuterol (administration of which to animals used as food for human consumption is today banned by the FDA) is a sympathomimetic and anabolic compound, detection of which has long been problematic. Recently, a highly sensitive and specific isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay was developed utilizing liquid/liquid re-extraction for clean-up with a limit of detection and quantification of 1 and 3 pg/ml, respectively [36].

Recently, it was demonstrated that genotype-based cut-off values may improve the sensitivity and specificity of the test, this demonstrating that genetic variation in androgen disposition is of importance in those instances when androgen urinary excretion profile should be tested [37]. However, in this case a deletion polymorphism in the UGT2B17 gene can lead to misinterpretation of T/E ratio, this accounting for a significant part of ethnic interindividual variability [38]. UGT2B17 genotype information is therefore crucial to the decision as to which initial cutoff ratio is to be employed for an individual as well as for enhancement of the sensitivity of the Bayesian analysis (a method of interpretation of probabilities) [39]. On the basis of these data, the proposition has been made that a Bayesian interpretation of consecutive tests in the same individual should be adopted to replace the epitestosterone ratio [40].

The assessment of an exclusive and non-specific threshold of T/E ratio to disclose testosterone abuse is therefore not an appropriate method. It has been proposed that an athlete's "endocrinological passport", including a longitudinal follow-up, ethnicity and genotype, may substantially improve detection of testosterone abuse [41]. Thus, the detection of doping is moving away from checking for quantified exposure to prohibited substance towards biologic assays detecting an effect of prohibited substances [12]. Cell-based biological assays comprise the future generation of assays which should be implemented by anti-doping laboratories to detect presence of androgenic anabolic steroids and other human AR ligands as well as assess the biological activity when the structure of the compound is not known [12].

Another method, the metabolomics-based approach that was introduced as a high-tech strategy to determine the anabolic steroid urine profile in livestock production, is an illicit use of natural steroids and is moreover hard to prove since the metabolites are unknown [42]. Despite the present lack of compelling data, metabolomics, involving study of the fingerprints of ASS metabolites, is likely to be added to the arsenal of anti-doping methods and control programs.

Recombinant human growth hormone

Recombinant human growth hormone (rhGH), which is favoured due to its anabolic, lipolytic and post-trauma healing properties, is commonly abused in sport with the aim of improving performance. Substantial efforts have been made to develop tests for detection of doping with rhGH, but the challenge is a difficult one.

Long-term efficacy of growth hormone in adults with growth hormone deficiency

It is well known that GH stimulates lipolysis and lipid oxidation thereby reducing fat mass, while GH and insulin-like growth factor-I (IGF-I) increase protein synthesis and thus increase protein mass. Meanwhile, it has been shown that long-term treatment of GH deficient adults with GH, normalizes body composition by increasing lean body mass and decreasing fat mass [43–45]. Exercise capacity is reduced in growth hormone deficiency (GHD) adults, while maximal oxygen consumption (VOmax), the aerobic capacity, is increased following GH replacement in subjects with adult GHD [46]. However, it has not been shown that GH increases muscle strength in GHD patients [47]. As primary impairment in the GH/IGF-I axis often leads to a high-risk cardiovascular profile which may partially be reversible during GH replacement [48].

Benefits and harm arising from GH use in healthy subjects

It has been reported that GH tends to increase whole body protein synthesis in some highly trained athletes, while in other athletes it does not [49, 50]. The use of rhGH in healthy adults causes a change in body composition with significant decrease in fat mass and significant increase in lean body mass [51]. However, the increase in lean body mass could be attributed to fluid retention caused by the anti-natriuretic properties of GH. There is also some evidence that combined administration of GH and testosterone could augment body cell mass [52]. A study in athletes, however, did not show an increase in muscle strength following the use of rhGH [53], and in distinction to GHD patients, the effects of GH on aerobic exercise capacity, VOmax, diverge considerably due to the differences in motivation, cardio-respiratory function and variability in exercise interventions [45, 54]. GH amplifies anaerobic exercise capacity (high power output of brief duration, e.g. sprint), hGH administration for 8 weeks having clearly

been shown to enhance sprint capacity [55]. Furthermore, it has been suggested that hGH may be beneficial in stimulating the supporting connective tissues (strengthening of the matrix by increased tendon collagen synthesis). This may then accelerate healing of soft tissue injury and bone fractures [56]. It is of note that GH administration improves cardiac function and, among the general public, acute and chronic GH treatment has been reported beneficial in patients with heart failure due to ischaemic or idiopathic cardiomyopathy [57].

GH in sports is seldom used in high doses (in other words, several times higher than the daily production rate of GH). However, when high doses are involved, the many side-effects of GH abuse in sports are actually signs of acromegaly, such as swelling and arthralgias, providing ample evidence of the harmful effects of excessive GH. More serious side-effects are diabetes and negative impact on heart function (increase in left ventricular mass and increased cardiac output).

The results of most of the controlled studies in which supraphysiological dosages of hGH have been used are less impressive than the claims of those who misuse hGH, a substance whose abuse is prevalent in top-level sport ([58] check).

Detection of GH abuse

Robust tests are available to detect misuse of rhGH. In order to detect GH abuse there are two approaches. One, the analytical method, relies on the measurement of molecular isoforms of GH, while the second relies on measurement of sensitive markers of biological activity of rhGH [59]. Recently, a method was developed for detection of GH abuse (the GH-2000 project) based on the measurement of IGF-I and the amino-terminal pro-peptide of type III collagen (P-III-NP) by two commercial assays available for each analyte [60]. By using this method, decision limits were developed to measure IGF-I and P-III-NP in elite athletes, thus enabling the introduction of a test for GH abuse based on the detection of GH biomarkers [61, 62].

The GH isoform approach

Circulating GH consists of multiple isoforms all derived from one gene in pituitary somatotrophs. The main isoform is 22 kDa GH, but besides this there are others such as 20 kDA isoform and 17 kDa fragment. Some isoforms are not biologically inert. There are reports that acute exercise in trained adults changes the ratio of isoforms and that exogenous 22 kDa GH administration by athletes could alter the natural GH isoform ratio [63]. The same authors in another study conclude that a supraphysiological dose of rhGH in trained adult males suppresses exercise-induced endogenous isoforms of GH. This occurs via the classical negative feedback by IGF-I, which suppresses endogenous GH secretion, thereby suppressing endogenous molecular isoforms [64]. However, the suppressive effect on 20 kDa GH in males is difficult to assess because at baseline 20 kDa GH levels are already undetectable. Moreover, the high degree of identity in the amino acid sequence between recombinant and endogenous GH, the pulsatile nature of GH secretion combined with inter-individual variations, presents a challenge for determination of GH [65]. Recently, hGH isoform-specific antibodies, through the use of isoform differential immunoassays, have been successfully applied for better differential measurements of 22 and 20 kDa isoforms [66]. However, this approach may be limited by the short time window of detection (between 24 and 36 h after the last GH administration) and the abuse of agents that stimulate the GH/IGF-I axis, such as analogues of GH releasing hormone or GH secretagogues [67]. Therefore, particularly on account of the time frame of the isoform method, the most effective application of this would be an "out of competition" unannounced control.

The use of GH-dependent biomarkers

GH generates the expression of both circulating and peripheral IGF-I. The GH induced circulating IGF-I, IGF binding protein (IGFBP3) and acid labile subunit (ALS) are predominantly liver derived. GH also increases bone and collagen turnover markers, with type 3 procollagen (P-III-P) being the specific marker. The biological variability of IGF-I and P-III-P has been studied longitudinally in a placebo controlled double blind manner (project GH-2000), and subsequently in project GH 2004 in amateur athletes, and longitudinally in Italian elite athletes [68] The GH-2000 study, which was set up by the IOC and the European Union Research Program (EU), was implemented in the 2000 Olympic games, while the GH-2004, which included WADA and the US Anti-Doping Agency (USADA), was implemented in the 2004 Olympic games [69]. The results of these studies show that both markers, IGF-I and P-III-P, have small intra-individual variability irrespective of gender, age, training conditions and competition level [68]. Some have proposed the use of these biological markers of GH action as the athlete's "passport" whereby each athlete acts as his or her own control [70]. Thus, the cutoff for clean would lie within the mean + 3.7 SDs.

Another complementary GH-related biomarker is mannan-binding lectin (MBL), which is reported to increase, with a clear concentration of up to 700 %, following recombinant GH (rGH) administration while exhibiting a slow recovery time [71].

When adolescent (aged between 12 and 20 years) elite athletes are studied, the GH-2000 score rises and reaches a

peak in males aged 15 and females aged 13 and then falls. This incorporates the effects of puberty on serum IGF-I and P-III-P [72]. Thus, higher cutoff values for the GH-2000 score may be needed for adolescent athletes. The time window for detection of doping with rhGH based on the quantification of biomarkers is thus expanded to several days. The influence of co-administration of testosterone on the level of GH markers shows that IGF-I levels are not affected; however, the levels of P-III-P are increased [73]. Limitations in studying these GH-dependent biomarkers are the lack of a pure recombinant IGF-I standard, the need to extract IGFBPs and differences in antibody specificity in different assays for IGF-I and for P-III-P since no international reference standard is available. Research in the field of hGH doping is in progress in order to identify novel and more effective markers predictive of hGH abuse.

Current investigations

Innovative bioanalytical approaches include application of microarray technology to detect differential gene expression profiles induced by rhGH [74], proteomics-based methods to identify more effective markers of rhGH biological activity [75], the use of liquid chromatography–mass spectrometry (LC–MS) based technology [76] and other procedures aimed at establishing fingerprints of GH abuse. Regarding LC–MS, a metabolomics study in equines has been introduced to analyze the metabolic biomarkers of GH administration, a model that can probably be applied in humans to assess the metabolic changes following rhGH intake [77].

Ever more sophisticated methods are being applied in order to improve the sensitivity and expand the time window for the detection of doping by means of rhGH, with research in this area being actively supported by WADA and numerous esteemed endocrinologists the world over, experts in the field of GH and IGF research, playing a pivotal role in providing this crucial information.

Recombinant human erythropoietin

Blood doping and erythropoietin

WADA describes blood doping as the use of drugs or any technique to increase red blood cell mass: this enables a greater O_2 transport to tissues thereby increasing aerobic capacity and endurance.

Clinical application

In clinical practice, patients suffering from chemotherapy-induced anaemia, with a haemoglobin concentration below 10 g/dl, benefit from epoetin therapy, which currently provides an alternative to red blood cell transfusion [78]. Since EPO regulates red blood cells production, it is increasingly being used in the form of DNA-recombinant human epoetin-alfa (rhEPO) to enhance aerobic performance among elite athletes [79]. Autologous blood transfusion is popular among sports people who engage in doping because its detection is also very difficult, in contrast to the homologous type which can be detected by flow cytometry [80, 81]. Darbepoetin, an analogue of rhEPO with a half-life of 18–24 h, as well as epoietin delta and continuous erythropoiesis receptor activator (CERA) with a half-life of about 6 days, are novel protein-based, stimulating erythropoiesis agents employed in blood doping [82, 83]. Both epoetin and darbepoetin bind to the EPO receptor inducing intracellular signalling via the same intracellular molecules as native EPO [84]. rhEPO abuse is, however, accompanied by high risks for thromboembolic events and myocardial infarction [85].

Detection of erythropoietin abuse

Recently, two different analytical techniques, isoelectric focusing (IEF) and chemiluminescent immunoassay, were applied for quantification of EPO [86]. Urine samples were treated with a stabilization mixture to chemically inactivate proteolytic enzymes and improve the electrophoretic signal of EPO, a method that might improve measurement of EPO [87]. Novel indirect methods based on haematological and/or molecular derivatives profiling also theoretically show promise as screening tools, followed by analytical testing of those athletes who are suspected of doping [87, 88]. However, an indirect technique that utilizes multiple markers of enhanced erythropoiesis, combined with a confirmatory test (isoelectric patterning), is likely to be the most feasible protocol for detection of rhEPO [79]. This protocol has been approved by the IOC Medical Commission as the "ON model" and has been applied since the 2000 Sydney Summer Olympics. Because doping with rhEPO has proved particularly challenging, WADA, as stated before, has launched the Athlete Biological Passport whereby evaluation of various indirect markers for blood doping is undertaken on an individual level [89]. Nevertheless, the efficacy of this passport has not been demonstrated.

An already established method in routine EPO anti-doping control is the sequential deglycosylation by exoglycosidase treatment (Reagent Array Analysis Method, RAAM) and subsequent sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), which has disclosed a structural difference between human endogenous and recombinant EPOs [90, 91]. A rapid protocol was introduced for screening purposes by enriching EPO-neonatal fetal receptor (Fc) from human serum and

subsequently achieving detection of EPO-Fc in the eluate with a commercial EPO ELISA kit [91]. rhEPO and the Fc-part of IgG can be transported after pulmonary administration via the Fc receptor and airway epithelium to the blood circulation. It was recently shown that a fast EPO immunopurification step combined with SDS-PAGE and Western double-blotting with chemiluminescence detection is capable of disclosing EPO-neonatal Fc receptor in serum together with all other recombinant EPOs [92]. These techniques may be particularly useful in the near future for detection of rhEPO abuse.

The development of monoclonal antibody immunoassays which combined with chromatographic separation of the glycosylated isoforms of EPO, can distinguish between endogenous human EPO and rhEPO as well as EPO analogues, such as MICERA, that upon evaluation can also be added to the armamentarium of anti-hEPO doping methods, is also in progress [93].

Transcriptomics, proteomics and metabolomics have been introduced in the last decade for the discovery of biomarkers as an indirect method for doping detection [94]. However, the results, especially with regard to rhEPO, have not as yet led to a routine application in doping control, this mainly due to the complexity and inter-individual variability of human transcriptomes, proteomes and metabolomes [94].

Epigenetics and gene doping

It has been established that excessive training, by inducing stress and releasing neurotransmitters and cytokines, may naturally affect the expression of endogenous genes via DNA methylation. However, the abuse of certain physical performance-enhancing substances, such as AAS or GH, may also change the expression of genes via epigenetic mechanisms such as DNA methylation and histone modifications [95], this technique bearing the name of "gene doping". Gene or cell doping is defined by WADA as "the non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance" [96, 97]. The main targets of gene doping are the myostatin gene, EPO and IGF-1. For example, removal or decrease of the expression of the myostatin gene is capable of elevating hypertrophy and muscle power [98].

The discovery of "physical performance genes", which have led to novel techniques for gene transfer for the purposes of gene doping [99], is a particularly highly detrimental feature of modern day doping because of the extreme difficulty in detecting it [99]. Direct as well as indirect testing methods have been implemented by WADA to assist in the challenging task of detecting gene doping [100]. Direct methods search for recombinant proteins or gene insertion vectors, while indirect methods, by nature more subjective, are based on the clinical examination of the athlete in an attempt to register bodily changes or structural differences between endogenous and recombinant proteins [101]. This raises the urgent need for sophisticated molecular biology techniques, such as the use of lab-on-a-chip techniques and nanoparticles, to enable the distinction between the "normal" and "modified" genome [102], while complex methodology is required to trace gene doping. A real-time PCR assay targeting sequences within the transgene complementary DNA corresponding to exon/exon junctions—which, due to their interruption by introns, are absent in the endogenous gene—may allow detection of trace amounts of a transgene against a broad background of the endogenous gene [103]. This is an effective method to detect exogenous DNA. The incorporation of an internal threshold control (ITC) serves to avoid confounding false positive or false negative results, while simultaneously obtained fluorescence emission signals determine the cycle thresholds for amplification of the target and ITC sequences [104]. This method, according to the authors, may enhance the detection capability of gene doping.

Gene doping is a highly complex issue as there is a very fine line between legitimate medical intervention and non-legitimate exploitation of an individual's genetic make-up for competitive advantage.

Conclusions

Over the past few decades, doping has become ever more complex and widespread, increasingly involving exploitation of the fields of endocrine-pharmacology and molecular biology. It thus currently represents not only an individual health hazard but also a menace to society itself, undermining the principles and significance of all the great sports events, and in particular the Olympic games. Faced with this problem, the scientific world is today striving to confront the challenge, in particular in regard to the recent development of hormones abuse, ever more complex methods and new technology being deployed for the task. It is evident that anti-doping policy should proceed to implementation of newly developed analytical methodology and advanced instrumentation as part of a strategy to clearly distinguish between the use of legitimate medication and the use of illicit substances. Furthermore, a multidimensional strategy to counter the scourge needs to be developed combining strict "prohibitionist" measures with preventive-educational programs. Meanwhile, the effort needs also to entail an individualized approach, incorporating counselling on a personal basis, which takes strongly into account the social and psychological background of

each athlete [105]. Meanwhile, with regard to athletics, there must be full support of the effort exerted by the WADA to detect banned substances and compounds so as to eradicate doping, accompanied by legislative changes and longer disqualifications. By continuing to upgrade collaboration with national Anti-Doping Agencies, WADA will be enabled to considerably improve efficacy, thereby gaining ever better control of doping, consequently reducing fraud while, vitally, lessening the health risks incurred by athletes. The prevention of harm to the athlete and the guarantee of fair play should be the target [106].

With the 2012 Olympics now behind us, it is everyone's sincere hope that the testing program employed by London's King's College Drug Control Centre in collaboration with WADA, enlisting techniques of unprecedented sophistication, has made the 2012 London Olympics the cleanest games to date [89], thus ensuring adherence to the true spirit of the Olympic games.

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