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Study of the endogenous steroid profile of male athletes from the Brazilian National Soccer Championship 2009

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Changes in the endogenous profile of androgenic anabolic steroids (AAS) may be interpreted as markers of doping. The objective of this study was to evaluate the endogenous profile of AAS in male athletes of the 2009 Brazilian National Soccer Championship, in normal conditions, particularly in the light of the revision of World Anti-Doping Agency's (WADA) Technical Document on the Interpretation of Endogenous AAS in athletes for doping control drafted in that year, as well as comparing these results to profiles already published in the literature. The upper limit of the 95% central reference interval of the following parameters for the studied population were estimated to be significantly higher than WADA's criteria, with a confidence of 90%: DHEA (about 2.3 times higher), Adiol (1.2 times higher), Bdiol (2.7 times higher), and Adiol/E (6 times higher). These findings seem to imply that WADA's criteria proposed in 2009 for DHEA, Adiol, Bdiol, and Adiol/E may not have been applicable to the studied population. Moreover, their comparison to previously published studies pointed to the need to evaluate in detail the appropriateness of adopting these criteria as universal, since there seems to be variations among different populations of athletes.

Keywords: endogenous profile; androgenic anabolic steroids; doping control; GC-MS; non-parametric statistics; reference values

Introduction

The interpretation of clinical results requires the comparison to either cross-sectional or longitudinal reference values. While cross-sectional comparisons use population-based reference intervals or critical limits and have diagnostic or investigational ends, longitudinal comparisons use the individual himself as a base of comparison and are employed for the long-term monitoring of health state.[1]

The establishment of population-based reference intervals and critical limits responds to demands of practical order, even though it is largely recognized that the ideal reference is the subject himself. The main objective of establishing these parameters is, therefore, much more to define what can be considered beyond expected, than to determine what is 'normal',[2] especially because this term can be misleading regarding not only the nature of the data, but also the state of the individual.[3]

When it comes to doping control, population-based reference values are extremely useful for monitoring the abuse of endogenous substances, like anabolic androgenic steroids (AAS). Similar to diagnostic medicine, which considers a result falling outside a reference interval not an unequivocal evidence of illness, but a warning to encourage further investigation,[4] an atypical analytical finding (ATF) concerning endogenous AAS compels the laboratory to ultimately confirm the results either by isotopic ratio analysis or by the longitudinal follow-up of the athlete.[5]

The establishment of universal population-based reference intervals is, however, a rather difficult task. First-of-all, strict standardization – by not only manufacturers of diagnostic kits looking for markets abroad, but also by clinical laboratories looking for accreditation – is an absolute requirement for the adoption of common reference intervals. Second-of-all, depending on the type of substance and on the existence of local population differences, stratification by age, gender, ethnicity, lifestyle, and other criteria may be required.[6,7]

It is safe to say, on one hand, that the network of laboratories accredited by the World Anti-Doping Agency (WADA) has already reached the proper level of standardization for the control of endogenous AAS and other prohibited substances – which have been demonstrated by its regular programme of proficiency tests. On the other hand, some parameters of the endogenous profile of AAS vary not only according to gender, age, and exogenous factors like the intake of alcohol and some medicines, but also among different populations. Consequently, many articles emphasizing the need for a more individualized approach have been published,[8] as well as some preliminary studies regarding the endogenous profile of local athletes.[9,10,11]

One important factor to be considered when studying the endogenous profile of AAS is the ethnical background of the population in focus. In the 1992 Olympic Games, in Barcelona, it was verified, for instance, that samples from athletes competing in sports where Asians predominated in the podium – like

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gymnastics and ping pong – presented distributions of urinary testosterone (T) concentration and T/epitestosterone (E) ratio dislocated to lower values.[12]

Recently it has been demonstrated that the bimodal profile of urinary T concentration was associated to the absence of the gene ugt2b17, in both alleles, in some individuals – a phenomenon that seems to be more common in Asians than in Caucasians. This gene codifies the enzyme UDP-Glucuronide Transferase, which is stereoselective for T and not for its inactive epimer, E, which is conjugated by another enzyme, codified by the gene ugt2b7: while the absence of ugt2b17 results in lower urinary concentration of T, thus in an also lower T/E ratio; the absence of ugt2b7results in lower urinary concentration of E, thus in a higher T/E ratio. As a result, the interindividual variation of the T/E ratio within a population may be very high.[13]

Other endogenous parameters that may vary among different ethnical groups are phase I metabolites of T. A recent study from the Doping Control Laboratory of Switzerland implies, for example, that Asians may present a higher activity of 5α-reductase than Africans, Caucasians, and Hispanics, so the ratios between epimers like androsterone/etiocholanolone (A/Et) and 5α-androstane-3α-17β-diol/5β-androstane-3α-17β-diol (Adiol/Bdiol) are higher in the first ethnical group than in the other three groups.[14]

The objective of the present study is to evaluate the endogenous profile of AAS of male athletes from the Brazilian National Soccer Championship 2009, in normal conditions, assuming there are no individuals in this population using such substances, particularly in the light of WADA's Technical Document on the interpretation of results regarding this class of prohibited substances drafted in that year (TD 2009) but not implemented, as well as comparing these results to profiles already published in the literature.[5]

Experimental

All analytical and managerial procedures were accredited for the ISO/IEC 17025 standard by the Brazilian National Metrological Institute (INMETRO), and the laboratory is accredited by WADA.

Chemicals and steroids

All reagents used were analytical grade: *tert*-butyl methyl ether (TBME) and pyridine were purchased from Tedia (Fairfield, OH, USA); *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), from Chem Fabrik (Waldstetten, Germany); Ammonium iodide (NH_4I) and 2-mercaptoethanol, from Sigma (St Louis, MO, USA); Methanol, from Tedia (Fairfield, OH USA). β-glucuronidase from *E. Coli* (500.000 units) was acquired from Sigma (St Louis, MO, USA). The internal standard mixture containing 17α-methyltestosterone (500 ng/mL), 2,2,4,4-$^2H_4$-etiocholanolone (500 ng/mL), 16,16,17-$^2H_3$-testosterone (90 ng/mL), 16,16,17-$^2H_3$-epitestosterone (15 ng/mL) and 2,2,3,4,4-$^2H_5$-androsterone-glucuronide (500 ng/mL – used to monitor the deconjugation process) were a gift from the Cologne Laboratory (Institute of Biochemistry, Germany Sports University, Cologne, Germany).

Stock solutions of analytes and internal standard were prepared in methanol at a concentration of 1 mg/mL. These solutions were further diluted to yield appropriate working solutions. All solutions were sealed and kept at −14 °C until use.

Urine collection

For this study, 2417 urine samples from the Brazilian Soccer Championship of 2009 were evaluated. According to the Regulation for Doping Control of the Brazilian Confederation of Soccer (CBF), in each match two players from each team are randomly selected before the end of the game and sampled immediately afterwards. A sample of 75 mL of urine is collect from each individual, divided in flasks of 25 mL and 50 mL, then sealed and transported to the accredited laboratory (LABDOP-LADETEC/IQ/UFRJ), where the analysis takes place. Samples are rejected before analysis if they arrive with leakages, broken seals, or unreadable identifications. That was not the case of any of our samples.

Sample extraction and derivatization

The urine samples were prepared using the anabolic steroids method described by Schanzer and Donike[15] with some modifications,[16] and then, they were analyzed by GC-MS. The current procedure used by LABDOP-IQ/UFRJ[16] is briefly described below.

A urine volume of 2 mL was pipetted into a screw cap glass tube and 50 µL of internal standard were added. The pH was adjusted to 7 with 750 µL of a freshly prepared aqueous solution of 0.8 M sodium phosphate and mixed briefly on a vortex-mixer. Then, 50 µL of β-glucuronidase from *E. coli* were added, and hydrolysis was performed for 1 h at 50 °C. The mixture was alkalinized with 500 µL of 20% potassium carbonate solution (pH 10). The analytes were extracted with 5 mL of *tert*-butylmethylether (TBME), and the tube was capped and shaken vigorously for 5 min and centrifuged at 2000 rpm for 5 min (g = 536.5). The ethereal phase was transferred to another screw-cap glass tube and evaporated to dryness under nitrogen at 40 °C. The residue was dried in a desiccator for at least 40 min before derivatization. The residue was derivatized with 100 µL of MSTFA/NH_4I/2-mercaptoethanol (1000 : 2:6 v/w/v) and heated for 20 min at 60 °C. Three microlitres of the sample were injected into the GC-MS system. Concentrations were corrected for specific gravity.

Instrumentation

All urine samples were analyzed by GC-MS employing an Agilent gas chromatograph Model 6890 (Palo Alto, CA, USA), equipped with a 7673 auto sampler and coupled to an Agilent single quadrupole mass spectrometer MS 5973 Network (Palo Alto, CA, USA).

GC settings: carrier gas was helium at 1 mL/min, in constant flow mode, and the column used was an Agilent Ultra-1® capillary column (17 m × 0.2 mm × 0.11 µm; Palo Alto, CA, USA); injector temperature was 280 °C; injection mode, 3 µL, split ratio 1 : 10; pulse pressure, 345 kPa/0.80 min; the GC oven was programmed to raise the temperature from 140 °C to 180 °C (rate of 40 °C/min), from 180 °C to 230 °C (rate of 3 °C/min) and from 230 °C to 300 °C (rate of 40 °C/min), then finally to hold this last temperature for 3 min.

MS settings: ionization by electron impact; ionization voltage was set at 70 eV; ion source temperature, 220 °C; interface temperature, 280 °C; quadrupole temperature, 150 °C; transfer line temperature, 280 °C. All mass spectra were acquired in SIM mode.

Statistical analysis

The normality of the distribution of data was evaluated by the Kolmogorov-Smirnov test. The 95% central reference interval for each parameter, as well as the confidence interval of 90% for

Table 1. Comparison of the upper limits of the 95% central reference value for AAS endogenous parameters among different populations and WADA's reference values – urinary concentrations

	T (ng/mL)	E (ng/mL)	A (ng/mL)	Et (ng/mL)	DHEA (ng/mL)	Adiol (ng/mL)	Bdiol (ng/mL)
WADA's reference	200	200	10000	10000	100	200	200
Brazilian athletes	**165**	**162**	**6845**	**5374**	**228**	**245**	**546**
(n = 2400)	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$
	155–175	167–169	6480–7079	5245–5639	223–241	226–257	517–572
Argentine athletes[9]	**171**	**144**	**7413**	**6300**	**data not available**	**212**	**523**
(n = 3504)							
Belgian athletes[18]	**103**	**89**	**6700**	**4950**	**117**	**155**	**416**
(n = 2027)	$CI_{95\%}$	$CI_{95\%}$	$CI_{95\%}$	$CI_{95\%}$	$CI_{95\%}$	$CI_{95\%}$	$CI_{95\%}$
	96–114	80–97	6390–6860	4660–5280	108–123	143–169	394–445
Chinese athletes[11]	**12**	**35**	**3686**	**1928**	**data not available**	**35**	**71**
(n = 194)	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$		$CI_{90\%}$	$CI_{90\%}$
	9.9–17	29–39	2485–4749	1460–3076		32–40	52–136

CI, confidence interval; T, testosterone; E,epitestosterone; A, androsterone; Et, etiocholanolone; DHEA, dehydroepiandrosterone; Adiol, 5α-androstane-3α-17β-diol; Bdiol, 5β-androstane-3α-17β-diol.

each extremity was calculated using non-parametric statistics, as recommended by the International Federation of Clinical Chemistry.[17] Results were compared to other data found in the literature, regarding athletes from different nationalities.

Results and Discussion

The distribution of the studied parameters in the population of interest showed a non-normal pattern – confirmed by the Kolmogorov-Smirnov test at the level of confidence of 95% – skewed to the left (graphics not shown). These results are consistent with the distribution of biometrical indicators in general and with the distribution of hormone levels in particular, whose asymmetry can be explained by the existence of a minimum serum concentration necessary to hormonal action below which homeostasis would be in danger.

The 95% central reference intervals estimated for the concentrations of T, E, A and Et and for the ratios of T/E and A/Et were found to be within the acceptable range suggested by WADA in the TD2009 (Tables 1 and 2). However, the upper limit of the 95% central reference interval of the following parameters were estimated to be significantly higher than WADA's criteria, with a confidence of 90%: DHEA (about 2.3 times higher), Adiol (1.2 times higher), Bdiol (2.7 times higher), Adiol/E (6 times higher).

In order to make better sense of these results, three other similar studies were selected from the literature based on the same analytical method and statistical strategy, but focusing on athletes of various modalities from different countries – Belgium,[18] Argentina[9] and China,[11] respectively. Tables 1 and 2 compare the upper limits of the central reference intervals for each parameter estimated for each one these populations to our results.

It can be observed in Table 1 that urinary levels of all monitored steroids in the Chinese population are consistently lower than those in the other three populations, and they do not exceed WADA's criteria. In the specific case of T concentration and T/E ratio (Tables 1 and 2), such results were already expected as there are several reports in the literature on the higher incidence of the deletion of the ugt2b17 gene in the Asian population. As also expected from other reports,[14] the ratios of A/Et and Adiol/Bdiol seem to be higher in Chinese athletes (Table 2), which could indicate a higher activity of 5α-reductase.

Table 2. Comparison of the upper limits of the 95% central reference value for AAS endogenous parameters among different populations and WADA's reference values – steroid ratios

	T/E	A/Et	Adiol/E	Adiol/ Bdiol
WADA's reference	4	4	1	2
Brazilian athletes	**4.1**	**2.9**	**6.4**	**1.8**
(n = 2400)	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$	$CI_{90\%}$
	3.9–4.3	2.8–3.1	6.1–6.7	1.7–1.9
Argentine athletes[9]	**5.6**	**2.9**	**data not available**	**1.6**
(n = 3504)				
Belgian athletes[18]	**4.3**	**3.6**	**data not available**	**1.7**
(n = 2027)	$CI_{95\%}$	$CI_{95\%}$		$CI_{95\%}$
	3.9–4.5	3.4–3.7		1.5–1.9
Chinese athletes[11]	**2.54**	**3.8**	**data not available**	**1.6**
(n = 194)	$CI_{90\%}$	$CI_{90\%}$		$CI_{90\%}$
	1. 9–3.3	3.1–4.4		1.4–2.0

* CI, confidence interval; T, testosterone; E,epitestosterone; A, androsterone; Et, etiocholanolone; DHEA, dehydroepiandrosterone; Adiol, 5α-androstane-3α-17β-diol; Bdiol, 5β-androstane-3α-17β-diol.

Comparing urinary levels of endogenous AAS among Brazilian, Argentine, and Belgian athletes (Table 1), it seems that the upper limits of the 95% central reference intervals for T, E, Adiol, and Bdiol of Brazilian and Argentine athletes are higher than those estimated for Belgian athletes, but only Adiol and Bdiol's upper limits are high enough to exceed WADA's criteria. Differently, Brazilians and Belgians seem to present similar upper limits for A and Et, but their interpenetrating confidence intervals do not contain the upper limit estimated for Argentine athletes. In the case of DHEA, the upper limits of both Brazilian and Belgian athletes exceed WADA's criteria, although the latter seems to be lower than the first (there is no data on the Argentine population).

Comparing steroid ratios among Brazilian, Argentine, and Belgian athletes (Table 2), the data presented do not allow us

to conclude that there is a significant difference among the upper limits of the 95% central reference interval for Adiol/Bdiol in each population, since the confidence intervals of the Brazilian and the Belgian population interpenetrate, and the Argentine limit is included in the Belgian interval. Neither there seems to be a significant difference between the upper limits of the 95% reference intervals for T/E of Brazilian and Belgian athletes; for the Argentine population, however, this parameter assumes a greater value, exceeding even WADA's criteria (the absence of a confidence interval for this parameter does not allow for any further consideration). In the case of the A/Et ratio, the confidence interval of the upper limit of the Brazilian athletes includes the upper limit of Argentine athletes, but is slightly lower than the Belgians. These data are corroborated by the comparative study developed by Strahm,[14] which has not been able to detect a significant difference between T/E, Adiol/Bdiol and A/Et ratios of Caucasians and Hispanics either.

Conclusion

The results reported in the present study seem to imply that WADA's criteria for DHEA, Adiol, Bdiol, Adiol/E, and Adiol/Bdiol as proposed in 2009 may not have been applicable to male athletes competing in the Brazilian National Soccer Championship of that year. Moreover, the comparison of such results to previously published studies pointed to the need to evaluate in detail the appropriateness of adopting these criteria as universal, since there seems to be variations among different populations of athletes.

Finding more stable indicators of abuse of endogenous AAS can be envisaged as the best strategy in the long term for improving doping control on major international competitions, since steroid passports could represent a financial burden to individual athletes from developing and least-developed countries. In the short term, especially in what concerns local competitions, the estimation of specific reference limits may be a good alternative, since they can reduce the waste of resources and efforts on false ATFs, a situation that tends to be aggravated by the introduction of new criteria for the interpretation of results that may be proven not truly universal. Fortunately, both these aspects have been included in the latest draft of WADA's Technical Document for the interpretation of results regarding AAS, released in 2010,[19] but yet to come into force: on one hand, there was a change in criteria that should be evaluated in detail; on the other, the use of local references has become a possibility.

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