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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

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Candida albicans in Urine Can Produce Testosterone: Impact on the Testosterone/Epitestosterone Sports Drug Test

Andrew T. Kicman,[1] John K. Fallon,[1] David A. Cowan,[1] Christopher Walker,[1] Sue Easmon,[2] and David Mackintosh[2]*

([1] Drug Control Centre, King's College London, Franklin-Wilkins Bldg., 150 Stamford St., London SE1 9NN, United Kingdom; [2] School of Life Sciences, Kingston University, Penrhyn Rd., Surrey KT1 2EE, United Kingdom; * author for correspondence: fax 44-20-7848-4980, e-mail andrew.kicman@kcl.ac.uk)

The International Olympic Committee-approved drug test to detect testosterone administration is based on the concentration ratio of urinary testosterone to epitestosterone (T/E). Usually the T/E is ~1, but after administration, the urinary excretion rate of testosterone increases and hence the ratio will be augmented; International Olympic Committee-accredited laboratories report T/E >6. Constant refrigeration of competitors' samples in transport is logistically difficult; hence, samples are usually transported at ambient temperature and may take several days to reach these laboratories. Preservative is not added because it is reasoned that adulteration with a foreign substance may lead to legal challenges. Concern has been raised about maintaining the integrity of the sample, particularly because possible urinary microbial production of testosterone may cause an adverse finding *(1)*, and several appeals have been made on that basis, e.g., the case of Mrs. Diane Modahl *(2, 3)*, a British athlete who had competed internationally.

A wide range of microorganisms can contaminate urine. Thus, testing the general hypothesis that microbial production of testosterone is possible simply by incubating a limited number of untreated urine samples at ambient temperature has not been fruitful. We decided that a better approach was to perform a relatively largescale study in which urine samples from females were

inoculated with a single potential candidate organism. To rationalize the choice of organism, a fresh approach was used that involved searching a comprehensive protein database (SWall; comprising >500 000 entries) to identify putative organisms that are most likely to produce enzymes able to convert steroid precursors to testosterone. Unlike prokaryotes (e.g., *Escherichia coli*), many eukaryotic microorganisms are capable of both de novo synthesis of steroids and transformation of steroid substrates. Essential enzymes in the conversion of the precursor steroids, androstenedione (androst-4-ene-3,17-dione) and androstenediol (androst-5-ene-3β,17β-diol), to testosterone in humans are 17β-hydroxysteroid dehydrogenase (17β-HSD) and 3β-hydroxysteroid dehydrogenase/4,5-isomerase (5-ene-3β-HSD), respectively. A search of the SWall protein sequence database revealed several yeasts and fungi that express proteins homologous with human 17β-HSD and 5-ene-3β-HSD; such proteins have been putatively identified as having these enzymatic activities. This, together with some direct experimental evidence, e.g., the results reported by Kastelicsuhadolc et al. *(4)* and Rizner et al. *(5)*, suggests that some eukaryotic microbes may synthesize testosterone. The choice of experimental organism was narrowed to *Candida albicans* because it is found in the typical vaginal flora. No clear cutoff for candiduria has been established *(6)*, and there is inadequate information regarding asymptomatic candiduria in healthy individuals. Urinary contamination in sports samples is unlikely to exceed 10 000 colony-forming units/mL (CFU/mL), but to account for contrary speculation, our experiment also incorporated inoculation of urine at 100 000 CFU/mL.

A total of 134 women (age range, 18–40 years; mean, 23 years) volunteered a urine specimen. Ethical permission and written informed consent were obtained in accordance with our institutions. A criterion for inclusion was good health and for exclusion was the use of antifungal preparations in the previous 2 weeks.

Each specimen was filtered (0.45 μm), and three aliquots (8 mL) of the filtrate were transferred into sterile tubes. The first aliquot was not inoculated (nontreated urines); the second and third aliquots, respectively, were inoculated with a strain of *C. albicans* at ~10 000 CFU/mL (10K-treated urines) and 100 000 CFU/mL (100K-treated urines). After incubation at 30 °C for ~90 h, all aliquots were analyzed for testosterone and epitestosterone (aglycone plus free fraction) as bis-trimethylsilyl ether derivatives *(7)*, essentially as described previously using gas chromatography–mass spectrometry (GC-MS) *(8, 9)*. The presence of *C. albicans* did not affect the extraction procedure; no significant differences in testosterone or epitestosterone

concentrations (10 μg/L) were found between calibrators in water to which the microorganism had been added (~6 × 10^6 CFU/mL) and untreated calibrators (*t*-test, $P > 0.5$). Between-assay precision was determined for three quality-control samples made from pooled urine collected from young women; the CV was <11.3% for testosterone concentrations between 1.3 and 37.8 μg/L and <13.4% for epitestosterone concentrations between 1.5 and 49.1 μg/L; the T/E ratio ranged from 7.2% to 11.2%. One-half of the treated urine samples underwent microbiological analysis, and all revealed multiplication of *C. albicans*.

Tests for normality (Kolmogorov–Smirnov test) revealed that nonparametric evaluation of the data was required. Comparison of the resulting steroid data (Friedman test) showed a significant difference between the nontreated and 10K- and 100K-treated urine for testosterone ($P < 0.0005$) and the T/E ratio ($P < 0.0005$), but not for epitestosterone ($P = 0.83$). Table 1 displays the number of positive and negative changes when comparing treated to nontreated urine samples and the results after further statistical analysis using the Wilcoxon signed-ranks test. Significant changes were observed for testosterone in 10K-treated ($P = 0.002$) and 100K-treated urines ($P < 0.0005$), although only 6 of the 134 samples showed an augmentation in testosterone ≥2 μg/L (maximum, 3.8 μg/L).

Table 1. Number of positive and negative changes for testosterone (T), epitestosterone (E), and the T/E ratio.

	10K-treated vs nontreated urine			100K-treated vs nontreated urine		
Change	T	E	T/E	T	E	T/E
Positive,[a] n	74	62	92	86	62	99
Negative, n	30	63	37	23	62	33
Ties, n	30	9	5	25	10	2
P[b]	0.002	0.52	<0.0005	<0.0005	0.79	<0.0005

[a] Steroid concentrations or T/E ratio in treated urine greater than in nontreated urine.
[b] *P* values relate to the Wilcoxon signed-ranks test (based on the sum of ranks) and show asymptomatic significance (two-tailed).

For the T/E, compared with the 134 nontreated urines, positive changes were observed in 92 of the 10K-treated urines and 99 of the 100K-treated urines. The box plot in Fig. 1 summarizes the differences in the T/E ratios observed between the paired samples (treated minus nontreated). Compared with nontreated urine samples, the largest increases in the T/E observed for 10K-treated urine were 0.23, 0.27, and 0.42 and for 100K-treated urine were 0.44 and 0.51 (see extreme values in Fig. 1); further analysis by GC-tandem MS (GC-MS/MS) under identical chromatographic conditions showed no coeluting substance present that would interfere with testosterone quantification, as judged by peak height and area ratios of ion abundance.

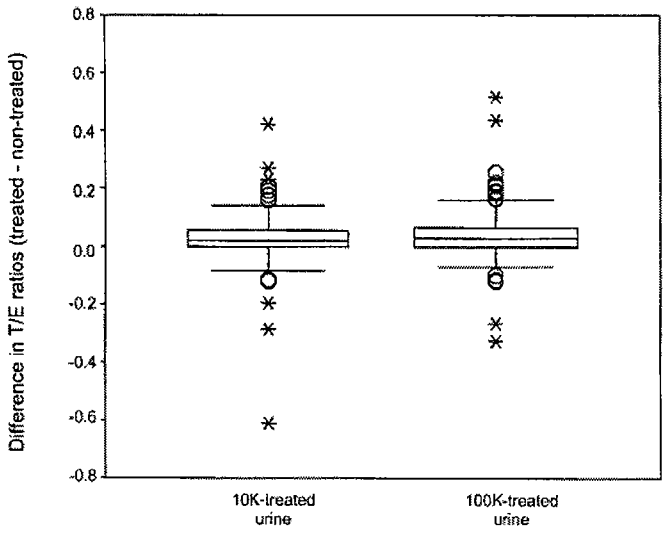

Fig. 1. Summary box plot of the difference in T/E ratios (treated minus nontreated).

The *box* represents the interquartile range, which contains 50% of the values; the *line inside the box* indicates the median. The *whiskers* represent the highest and lowest values, excluding outliers and extremes. The outliers (O) are values between 1.5 and 3 box lengths, and the extremes (*) are values >3 box lengths.

As an adjunct, incubation (30 °C for ~90 h) of purified androstenedione (containing no measurable testosterone) in 7 mL of sterile Ringer's solution (10 μg/L) with this strain of *C. albicans*, followed by extraction of steroids into diethyl ether and analysis of trimethylsilyl derivatives showed the presence of testosterone (~0.5 μg/L), this being confirmed as such by GC-MS/MS against an authentic standard (presence of precursor ion *m/z* 432 and the product ions *m/z* 417, 404, 327, and 209 in the spectrum). No testosterone was observed when androstenediol was added instead of androstenedione.

The data demonstrated that statistically significant amounts of testosterone can be produced in urine in the presence of *C. albicans*. However, because testosterone was increased but only to a minor extent, the resulting augmentation of the T/E ratios was also minor. Thus, in comparison with the laboratory reporting threshold (T/E = 6), these increases are of little evidential value for any individual case. The mechanism appears to be by conversion of urinary androstenedione rather than by synthesis de novo. If this is the case, augmentation of the T/E ratio will be limited by the microbial rate of formation of testosterone and the concentration of androstenedione originally present in a spot sample; we would expect the latter to be typically <10 μg/L based on

excretion rate data [Ref. *(10)* and references therein]. However, the maximum possible concentration of androstenedione that can be present in single urine specimens collected from healthy individuals is not known, nor are the number of strains of *C. albicans*. As a consequence, it is difficult to comment further as to the possibility of how much testosterone can be formed by different strains of this microbe.

de la Torre et al. *(11)* did not find testosterone production after inoculation of urine with selected organisms, but they considered that microbial contamination may hamper interpretation of results. Interpretation will be helped by isotope ratio MS ($^{13}C/^{12}C$) of urinary steroids to determine whether testosterone is of exogenous origin *(12, 13)*, as well as comparing the testosterone liberated by glucuronidase hydrolysis and/or that in the free steroid fraction to total testosterone. Notwithstanding, the addition of a suitable chemical preservative to reduce the possibility of microbial action appears to be a straightforward way of addressing this specific issue, and we recommend further consideration as to how the associated quasi-legal difficulties can be overcome.

In summary, these data do support the hypothesis, based on our model using a large challenge dose of a strain of *C. albicans*, that urinary testosterone can increase as a result of microbial action; however, the increases observed were small, and hence, the changes in the T/E ratio were minor. We thank Professors William Brumfitt (Royal Free and University College Medical School), Sally Bloomfield (Unilever Research), and David Gower (Drug Control Centre, King's College London). We also thank Michele Verroken (UK Sport) for helpful discussions.

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