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A simple method for measuring long-term integrated testosterone levels in men

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Abstract

Steroids play multiple roles in the regulation of development, physiology, reproduction, and behavior. Measuring circulating steroids is especially challenging since concentrations are extremely labile, responding to stressors within minutes. Matrices that integrate long-term steroid levels are therefore valuable as biomarkers of baseline, as well as chronic steroid exposures. Here we report on a simple method to extract and measure accumulated testosterone from human fingernails using commercial EIA kits. Further, we demonstrate known human testosterone sex and age trends. Thus, this method is a potential tool for biomonitoring endogenous as well as exogenous steroid exposure.

Key words: biomonitoring, endogenous steroid levels, integrated matrices, non-invasive, fingernails.

Introduction

Steroid hormones influence and are influenced by development, physiology, and behavior. Circulating and integrated steroid levels can provide information on growth and reproduction, and offer prospective biomarkers for well-being and survival (Koren et al. 2012). Measuring steroids in integrated matrices, such as hair, feathers, and nails, is a progressively developing field. Unlike traditional blood sampling, hair and nail collection is relatively non-invasive, pain and infection-free. Hair and nails may be cut and collected quickly and efficiently, without the need for patient privacy or researcher health precautions. They are also easy to transport and store, without needing electricity and cooling. Moreover, while circulating steroid levels represent the momentary total (i.e., protein-bound and free) state, hair and nails provide a longer time-frame, presumably integrating free steroids over the time of their growth (i.e., weeks or months).

In mammals, nails develop by keratinocytes cell divisions in the proximal germinal regions (Baran 1981; De Berker et al. 2007). Nail growth is attributed primarily to the nail matrix and to a lesser

extent to the nail bed. The nail matrix is mainly composed of specific keratin proteins, cross-linked by keratin-associated proteins. Diverse factors influence nail growth, including numerous physiological and pathological conditions (reviewed in Zaiac & Walker (Zaiac & Walker 2013)). On average, human fingernails grow at a rate of 3 mm per month, while human toenails grow at a rate of 1 mm per month. During nail growth, multiple materials are transferred from the capillary blood vessels nourishing the nail to the growing nail (Palmeri et al. 2000). These are embedded in the nails, making this abundant matrix an attractive candidate for biomonitoring multiple blood borne substances. Indeed, poisonous materials (Barbosa et al. 2005; Button et al. 2009; Mehra & Juneja 2005; Suzuki et al. 1989), drugs (Shu et al. 2015) and endocrine-disruptors (Li et al. 2013) have been forensically detected in human nails. In addition, endogenous steroids have been analyzed in nail extracts. For example, infants that experienced in utero stress had higher DHEA (dehydroepiandrosterone) compared to infants from non-stressed mothers (Tegethoff et al. 2011). In another study, nail cortisol-to-DHEA ratio was found to be related to perceived exam stress in students (Warnock et al. 2010). Exogenous testosterone and its derivatives were measured in nails using LC-MS (Brown & Perrett 2011). However, the authors concluded that detection of these substances was not sensitive enough. GC-MS was used in a different study, yet this method requires derivatization (Choi et al. 2001). Here, we aimed to develop a simple and repeatable quantitative method for measuring endogenous testosterone in nails, and biologically validate it using known age-related trends.

Materials and methods

Nails collection

The study was approved by Bar Ilan University's Research on Human Subjects' Ethics Committee. In order to collect nail samples, we sent an electronic mail to all graduate students, staff, and faculty members in the Faculty of Life Sciences at Bar Ilan University, requesting fingernail donations from men, women and children. Samples were placed in an empty paper envelope marking only the sex and age of

the donator on it. Envelopes were deposited anonymously in our laboratory mailbox. Between November 2014 and May 2015 we collected 52 samples from men (ages 18.5-74), 6 samples from boys (ages 0.4-2), 11 samples from women (ages 18-53) and 11 samples from girls (ages 3-9).

Steroid extraction

We extracted steroids from nails using our published protocol for hair and claw-testing (Koren & Geffen 2009; Koren et al. 2006; Koren et al. 2008; Koren et al. 2002; Koren et al. 2012; Matas et al. 2016), with several modifications. Nails were weighed to the nearest 0.01 mg in safe-lock polypropylene tubes (Sarstedt, Germany). Samples were ground in mixer mill MM 400 (Retsch, Germany) for 10 minutes at 25 Hz. Methanol (UPLC grade, Sigma, Israel) was added, and the minced samples were sonicated for 30 minutes and then incubated overnight at 50°C, with gentle shaking. Next day, the supernatant was centrifuged at 17,000 g, transferred into a glass tube (Corning Inc., USA) and evaporated under a stream of nitrogen. Samples were reconstituted and testosterone was quantified in the assay buffer that was provided with the commercial enzyme-linked immunosorbent assays (ELISA) according to manufacturer's recommendations.

Quantitation of steroids

High sensitivity salivary ELISA kits (Salimetrics; item no.1-2402; Ann Arbor, USA) were validated for nails by examining parallelism with kit standards and linearity. Serial dilutions of separate pools for men, women and children all showed parallelism with the provided kit standards (univariate analysis of variance in SPSS; P=0.5; P=0.46 and P=0.48 for men, women, and children respectively). Linearity was demonstrated for men between 1-20 mg of nail extract, corresponding to 6-230 pg/mL of kit standard. For women, linearity was observed in the 30-50 mg nail extract range, corresponding to 100-200 pg/mL standards. In children, serial dilutions were linear in the 10-50 mg nail extract range, which is equivalent to 38-210 pg/mL salivary standards. The lowest concentration that we detected from a single individual

using the assay was 0.7 pg/mL, corresponding to 3.95 mg of nail matrix. According to the manufacturer, antibody cross-reactivity was reported as 36.4% with dihydrotestosterone, 21.02% with 19-nortestosterone, 1.9% with 11-hydroxytestosterone, 1.157% with androstenedione and less than 0.489% for all other steroids. By measuring six samples of the pool on the same ELISA plate we determined intra-assay variability to be 2.7%. By quantifying four samples of the pool on four different days we calculated inter-assay variability to be 11.78%. Recovery was calculated to be 86% by the addition of a known amount of testosterone to the nail extract.

Results and Discussion

We developed a simple protocol for human fingernail testosterone extraction and quantitation. According to our validations, whereas 5 mg of nails were needed to quantify testosterone in men, ten-fold was necessary to quantify testosterone in women and girls (i.e., 50 mg). This is comparable to the documented association between circulating testosterone in men vs. women (Longcope, 1986). Since a large amount of nail matrix was needed for the female analysis, we created a pool from all donated female nails. Figure 1 shows nail testosterone in adult men (mean for N=52; 5.04 pg/mg nails), women (mean for N=11; pool; 2.3 pg/mg nails), boys (mean for N=6; 1.8 pg/mg nails), and girls (mean for N=11; pool; 1.07 pg/mg nails). In order to perform statistical comparisons, testosterone levels were transformed using the Johnson Su transformation for normal distribution. All analysis was performed in JMP 12 (SAS Institute Inc.). Analysis of variance showed significant differences in testosterone levels between sex and age groups ($F_{59,3}$=9.665; P<0.0001). Tukey-Kramer post-hoc statistics (q=2.648; α=0.05) showed significant differences in nail testosterone levels between men and boys (difference 1.758; P<0.0001). These results support previous findings reported in the literature where testosterone concentrations are higher in men and women than in boys and girls (Andersson et al. 1997; Mouritsen et al. 2014). In this analysis, we found a two-fold difference between men and women's testosterone levels. Established normal ranges of circulating testosterone concentrations indicate a five-fold difference between low levels in men (usually 300 ng/dL) and high levels in women (usually 60 ng/dL). This discrepancy may be attributed to sampling

size, age and seasonal fluctuations, as well as specific matrix effect. Whereas blood samples reflect a momentary concentration, nails show accumulated long-term levels, which integrate concentrations over time.

We found a significant association between age and testosterone in male nails (R^2=0.405; N=58; P<0.0001; Figure 2). As documented in the literature (Vermeulen et al. 1972), testosterone levels reached a maximum in men in their 20s and than gradually decreased. Although the variation in circulating testosterone is high, the pattern is profound. In this study, we use commercial EIA kits to quantify testosterone. Immunoassays are widely used to quantify steroids due to their high sensitivity and procedural simplicity. Although mass spectrometry is superior in terms of specificity and flexibility to measure multiple compounds simultaneously, a large amount of tissue is initially needed, due to loss during sample prep and cleanup. For example, Choi *et al*. (2001) used 100 mg of nail matrix to find differences between testosterone in men and women using GC-MS(Brown & Perrett 2011; Choi et al. 2001), and Brown and Perrett (2011) did not find differences between males and females or exogenous testosterone users and non-users (Brown & Perrett 2011). Here we present a simple and reliable antibody-based method for testosterone quantitation using a minimal amount of nail matrix, without the need of derivatisation or costly equipment.

Testosterone has been implicated in various diseases, fertility issues, anxiety and stress-related disorders, as well as risk-taking, sexual, and aggressive behaviors. Nails may serve as an ideal matrix for long-term measurements of baseline or chronic testosterone levels related to stable physiological or psychological states, behavioral syndromes, and personality traits, as well as follow-up of exogenous testosterone exposure. Nail collection is relatively non-invasive, and samples are easy to store and safe to handle. Other advantages include their bountiful quantity and slow growth, reflecting long-term steroid concentrations. Our method offers the possibility for researchers to quantify steroids in nails in an easy, applicable way, and document stable trends. Utilizing nails for steroid analysis also enables periodic quantitation by repeated cutting, opening an exciting array of questions and comparative studies biomonitoring individual differences in endogenous steroid levels, as well as exposure to exogenous

steroids, endocrine disruptors and other environmental pollutants that may interfere with the hypothalamus-pituitary-gonadal pathways.

Conclusions

Measuring integrated steroid levels that reflect long-term baseline is highly relevant, since steroid concentrations are labile. Here we present a protocol for extraction and quantitation of testosterone in human fingernails. We biologically validated our methodology by demonstrating known changes in testosterone levels throughout men's lives, and expected sex differences. These allow the usage of this protocol for further comparative research and biomonitoring.

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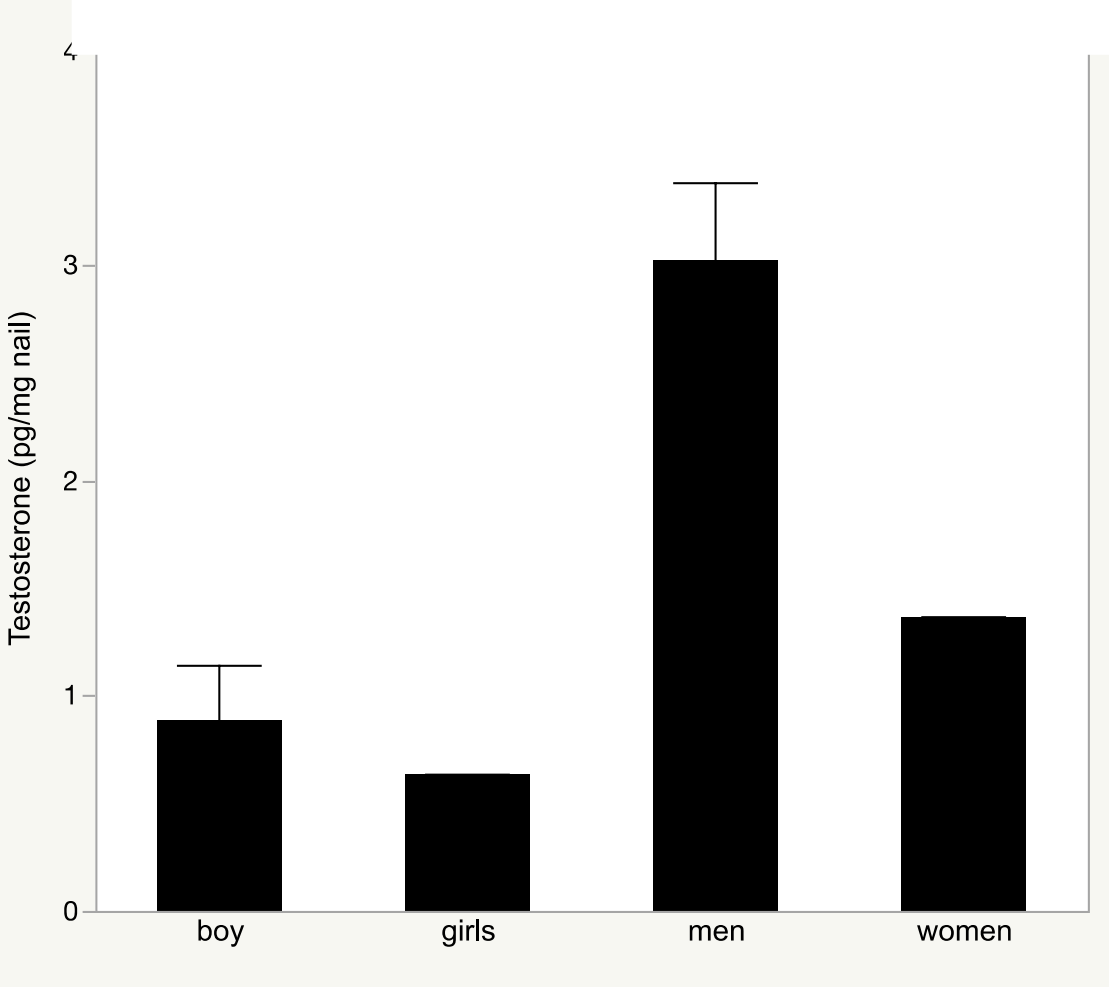
Figure legend

Figure 1: Nail testosterone levels in men, women and children. Testosterone was extracted from 5 mg of nail clippings of individual men (N=52) and boys (N=6). Error bars were constructed using 1 standard error from the mean. For women (N=11) and girls (N=11) testosterone was extracted from 50mg of pooled nails. Significant differences were seen between sex and age classes (ANOVA: $F_{59,3}$=9.665; P<0.0001).

Figure 2: Transformed nail testosterone as a function of male age. Transformed testosterone = -0.151 + 0.0245*age - 0.0016*(age-28.06)^2. Johnson Su transformation was used to normalize the distribution of testosterone. Polynomial second degree fit R^2=0.405; N=58; P<0.0001

234 Figure 1



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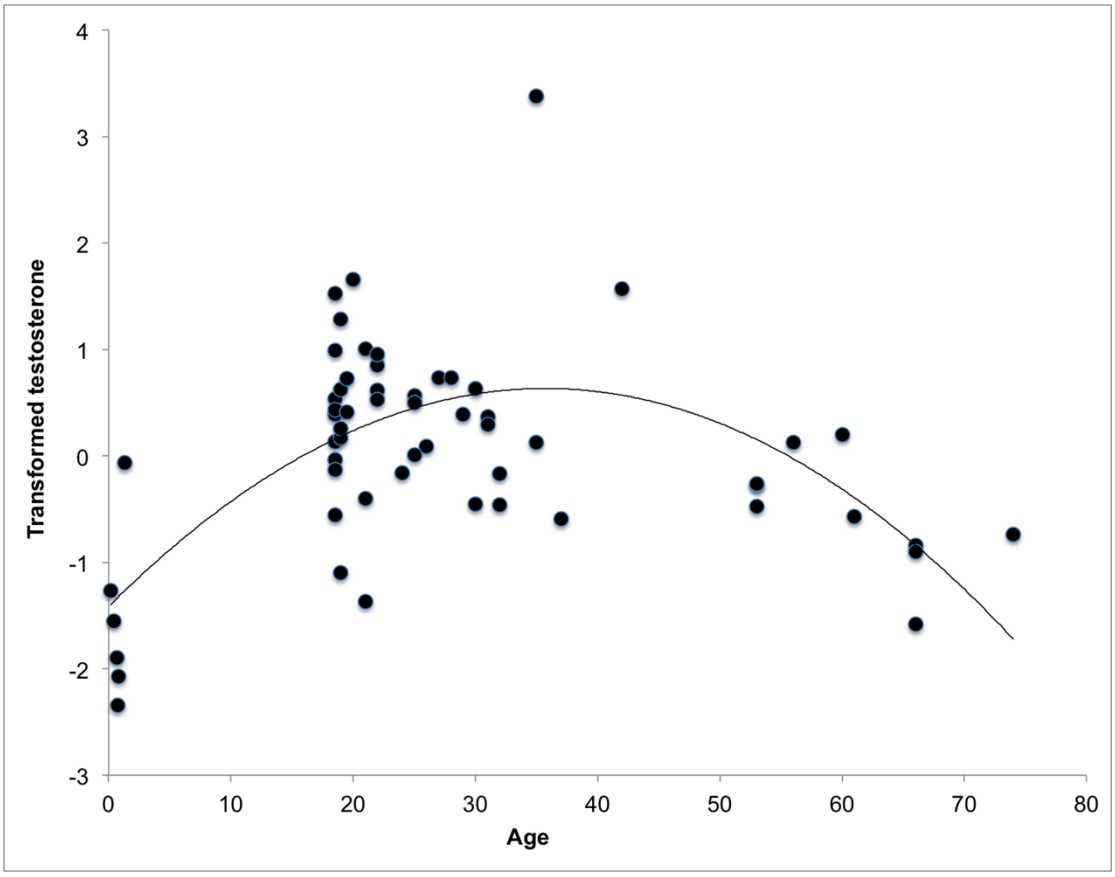
237 Figure 2



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