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Research article

Salivary testosterone levels in preadolescent children

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Abstract

Background: Saliva reflects the plasma free fraction of testosterone which is biologically active, and available for uptake by tissues. Testosterone concentration in saliva, though differing slightly from the concentration of unbound testosterone in serum, is in good correlation with the latter, indicating that salivary testosterone provides a reliable method for determination of serum free testosterone. The study aimed to investigate salivary testosterone levels and their changes in preadolescent children and to study sexual dimorphism.

Methods: Testosterone levels were determined in 203 healthy preadolescent children (77 girls and 126 boys) from saliva samples by radioimmunoassay. Sampling was performed once a year with respect to circadian and seasonal fluctuations of testosterone. Data were statistically analyzed by Statgraphic software.

Results: Mean salivary testosterone concentrations (± SD) were 0.038 ± 0.012 nmol/L and 0.046 ± 0.026 nmol/L for girls and boys, with the medians 0.035 nmol/L and 0.041 nmol/L, respectively. Statistical analysis did not prove changes in salivary testosterone concentrations in the preadolescent period of life, with an exception of the insignificant fall at the age of 7 years, and an insignificant rise at the age of 9 years in girls.

Conclusions: Generally it can be concluded that salivary testosterone levels in our prepubertal subjects remained stable. There was no significant increase of salivary testosterone levels from the age of 6 until the age of 9 in both sexes. Sexual dimorphism in salivary testosterone levels was proved with significantly higher ($p = 0.009$) salivary testosterone levels in boys than in girls.

Background

Saliva sampling is advantageous to the patient, especially in children since it is a non-invasive, stress-free technique and enables multiple sampling. The primary entry of the steroid hormones as testosterone into saliva is via passive diffusion through the salivary gland epithelium. The concentration of the free hormone in plasma provides concentration gradient that drives diffusion of the steroid through the epithelial membrane into the primary secretory fluid within the acinar intercalated duct complex [1].

This holds true even under conditions of altered saliva flow rate, which may be reduced e.g. by anticholinergic medication [2] and increased by citric acid stimulation [3]. Thus, the concentration of unconjugated steroids in saliva does not depend on the rate of saliva production [4]. Testosterone concentration in saliva though differing slightly from the concentration of unbound testosterone in serum is in good correlation with the latter, indicating that salivary testosterone provides a reliable method for determination of serum free testosterone [5]. Radioimmunoassay techniques suitable for measuring the low concentrations of testosterone in saliva have become available later than in serum – the first RIA for determination of salivary testosterone in adults appeared as late as in 1976 [6] and the first reports on salivary testosterone in children followed more than a decade later; see [7] and the references therein. Therefore, not many reports relate to salivary testosterone levels in children so far, especially in preadolescents [7–14]; for survey of the literature see [14]. Besides establishing of the physiological concentrations in both sexes, especial attention was paid to prepubertal period and onset of puberty in boys [7–9,13] and also to some behavioral and psychosomatic aspects related to testosterone [10–12].

The aim of this work is to investigate the changes in salivary testosterone concentrations during preadolescent period, (6–9 years) in both sexes. We also have compared salivary testosterone levels in girls and boys in order to prove sex differences in young children.

Methods

Subjects and saliva sampling

Saliva samples were obtained from 203 prepubertal healthy children (126 boys and 77 girls) between 6 and 9 years of age. Saliva sampling was performed once a year within one week. With respect to circadian and seasonal fluctuations of testosterone in saliva [7,15], the sampling was carried out at a standardized time of the day, between 9.00 and 11.00 a.m., all during November. To avoid eventual diurnal fluctuations of testosterone, two samples of saliva were collected, the second sample an hour later after the first sampling, and the means from both collections were calculated.

The subjects were asked to clear their mouth and then a sugarless fruit-flavoured chewing gum was given to each of them as a salivation stimulant. Children let to accumulate saliva in the floor of their mouth and collected them directly into sterilized glass tubes. Contamination with food debris was avoided by rinsing the mouth with water and by delaying the collection for five minutes after rinsing to prevent sample dilution. Absence of blood contamination was checked by Salivary blood contamination kit (Salimetrics LLC, State College, PA, USA). Each child provided two 2 ml samples of saliva for assay. Samples of saliva were frozen at -left at -20°C until analyzed.

Radioimmunoassay (RIA)

Saliva including control samples or blank (bi-distilled water), 1.0 ml each, were spiked with [^{3}H]testosterone (Radiochemical Center, Amersham, UK, 1200 dpm/sample), and extracted in duplicate with diethyl ether (4 ml) in stoppered glass tubes. The aqueous phase was left frozen in solid carbon dioxide, organic phase was decanted and ether was evaporated to dryness. The extracts were dissolved in ethanol (500 µl), 100 µl of which were removed for determination of the losses during extraction, while the rest was evaporated again and taken for radioimmunoassay. A standard curve consisting of 0, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 nmol/l testosterone in duplicate, was prepared. Antiserum (rabbit-anti-testosterone-3-CMO: BSA working dilution 1:100 000) and the tracer ([^{125}I] iodohistaminyl-testosterone derivative, 15000 cpm), 100 µl each, were added, the volume was adjusted to 300 µl with working buffer (20 mmol/sodium phosphate-saline containing sodium azide and BSA, 0.1% each) and the tubes were equilibrated at room temperature for 1 hour or overnight at 4°C. After incubation dextran-coated charcoal suspension (0,025 and 0,25 g/100 ml, respectively, 1 ml) was added to each tube to separate the free fraction and the radioactivity of ^{125}I was measured in the supernatant using 12 channel gamma counter (Berthold, FRG). Results were calculated from the standard curve using a log-logit transformation, corrected for recovery and expressed as nmol testosterone per liter sample.

The analytical parameters of the method were as follows:Specificity: the only compounds that showed a significant cross-reaction were 5α-dihydrotestosterone (33.0%), 11β-hydroxytestosterone, estradiol and androstendione (0.1% each). Accuracy: the recovery of known amount (1–5 pg) of testosterone added to saliva (mean ± SD) was 101.4 ± 9.0 % (n = 24). Sensitivity: the lowest amount of the analyte detected with 95% probability was 1 pg. Precision: The between assay variation calculated from the results of a quality control run in each assay (N = 50) gave the value 0.220 ± 0.018 nmol/l (coefficient of variation 8.2%). After the assay had been in routine use the results calculated using the recovery measured for each sample were compared with those calculated using the mean overall recovery for all previous assays. No significant differences were observed (regression analysis r = 0.99, y = 0.996x + 0.02, n = 300).

Statistics

Following standard statistical procedures have been used for evaluation of the data: Kolmogorov-Smirnov two-samples test, chi-square goodness-of-fit test for testing of distribution, Mann-Whitney test for medians and t-test for

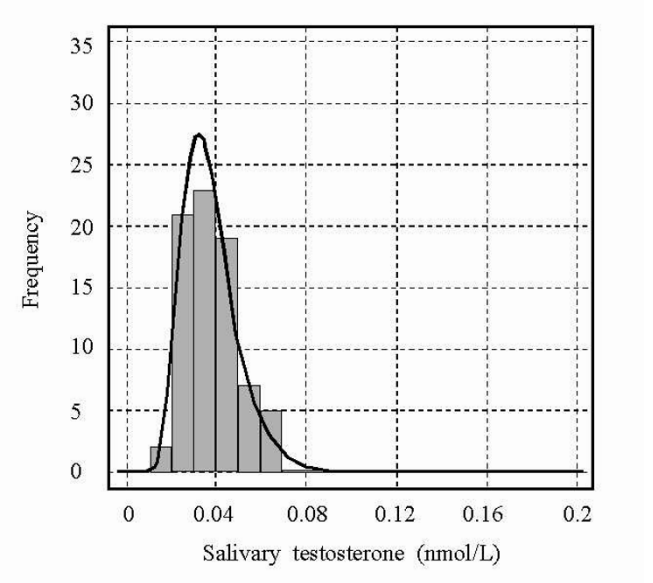

Figure 1
Lognormal distribution of salivary testosterone levels (nmol/L) in prepubertal girls.

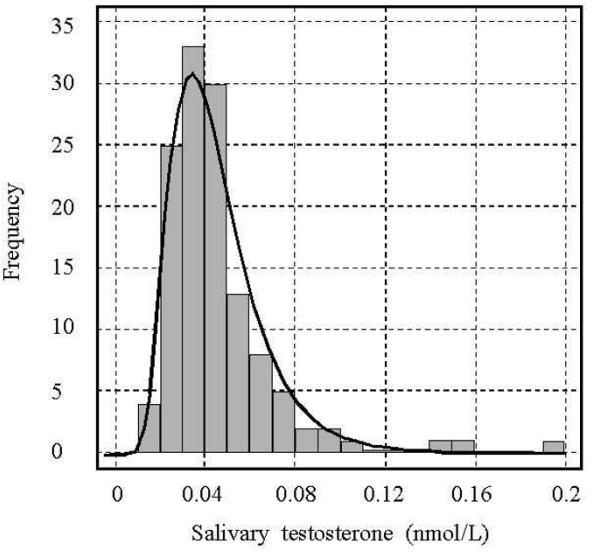

Figure 2
Lognormal distribution of salivary testosterone levels (nmol/L) in prepubertal boys.

means. The effects of age groups were tested by analysis of variance and Kruskal-Wallis non-parametric test. Statistical analysis was carried out with STATGRAPHICS Plus Version 7.0 software.

Results

Salivary testosterone concentrations were measured in healthy children aged 6–9 years within four subsequent years. Testosterone concentrations in girls were normally distributed, but it was not the case for testosterone levels in boys, because of several atypically high values. Samples for girls and boys were properly fitted with lognormal distribution LN (-3.337; 0.1084) and LN (-3.1855; 0.1875) respectively. The chi-square goodness-of-fit test gave $p = 0.36$ for girls and $p = 0.17$ for boys. Kolmogorov-Smirnov test gave $p = 0.80$ for girls and $p = 0.43$ for boys. Overall mean testosterone concentrations were 0.038 nmol/L (SD = 0.012) for girls and 0.046 nmol/L (SD = 0.026) for boys. Figure 1 shows lognormal distribution of salivary testosterone values for girls. Figure 2 shows lognormal distribution of salivary testosterone for boys. Salivary testosterone levels for all age groups and for both sexes separately are shown in Table 1 and on Figure 3.

Sexual dimorphism in salivary testosterone values was tested by Student's t-test for logarithmically transformed values. The results confirmed significant differences between both sexes ($p = 0.009$), boys having higher salivary testosterone levels than girls. The same results gave Mann-Whitney test for original data with $p = 0.032$. The difference in variances was also significant ($p < 0.003$). After transformation the 95% confidence intervals for mean values of testosterone were (0,035; 0.040) and (0.042; 0.049) for girls and boys, respectively. When the sample was tested for differences between individual age groups no significant differences in salivary testosterone levels were found between ages 6 and 9 ($p = 0.10$ and 0.92 respectively by Kruskall-Wallis test and $p = 0.14$ and 0.82 respectively by ANOVA for logarithmized values), with the exception of the modest but statistically insignificant decrease in 7-years old girls and increase in 9 years old girls. It can be concluded that salivary testosterone levels remained relatively stable during studied preadolescent period.

Discussion

The most of studies on salivary testosterone in children dealt with the onset of puberty in boys [7–9,13]. Only few reports are available as far as salivary testosterone in younger healthy preadolescent children are concerned, though the first reports on determination of testosterone in saliva appeared as early as in seventies [6]. This is mainly due to the fact that availability of assays with sufficient sensitivity is restricted. In Butler's study [7] salivary testosterone in 84 boys was determined, but only 11 subjects were younger then 10 years. In Albertson-Wikland's study [18] 12 prepubertal boys were examined. In the recent report of Granger et al. [14] 90 boys and 85 girls aging 8–12 years have been investigated for salivary testosterone by adapting a commercially available serum testosterone kit, reaching the detection limit below 1 pg/ml.

Table 1: Median and Mean values (with SD) of salivary testosterone levels (nmol/L) in different age and sex groups of preadolescent children with sample size of each group.

Age	Sex	N	Mean	Median	SD
6	Girls	34	0.039	0.039	0.011
	Boys	58	0.046	0.039	0.027
7	Girls	24	0.035	0.032	0.013
	Boys	30	0.043	0.037	0.018
8	Girls	14	0.036	0.036	0.013
	Boys	21	0.050	0.042	0.038
9	Girls	5	0.047	0.047	0.010
	Boys	17	0.046	0.046	0.015
All ages	Girls	77	0.037	0.035	0.012
	Boys	126	0.046	0.041	0.026

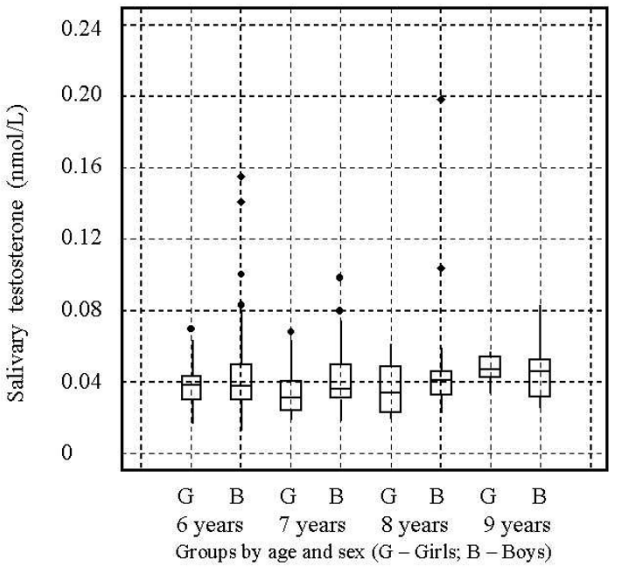

Figure 3
Box and whiskers plot for salivary testosterone levels (nmol/L) in girls and boys by age groups. The central horizontal lines within the box represent the median of the sample group. Lower and upper lines represent two quartiles of the measured value. The vertical line indicates the range of values falling within 1.5.times the interquartile. Asterisks denote values that are greater than 1.5 times the interquartile range.

In our study of 126 boys and 77 girls the younger age categories were investigated. The youngest age categories numerically overrepresented the older age categories of prepubertal children. With respect to circadian and also seasonal fluctuation known to occur in adults [17] much attention was paid to standardization of sample collection. The sampling of all our subjects was performed in November, since salivary testosterone in adult subjects was found to be higher in autumn compared with spring levels [15]. Sampling was carried out at a standardized time (between 9–11 a.m.), and the mean of two successive samples was taken into consideration.

The changes during the followed period have shown moderate insignificant decrease in salivary testosterone concentrations in 7-years old girls, which is in line with the results of Sizonenko et al. [19] who found the fall in plasma testosterone concentrations in girls of the bone age 7 in their study. The insignificant rise of salivary testosterone levels was seen in 9 years old girls. This is supposed to occur due to well-known rise of adrenal androgens, which occurs years before puberty resulting in adrenarche. Rise of adrenal androgen levels are not sufficient to replace testicular androgens in men, but are the major source of androgens in women.

Our results confirmed significant differences of salivary testosterone levels between both sexes. Prepubertal boys had higher testosterone levels in saliva than prepubertal girls.

Since we tried to avoid as much as possible the problems with sample collection, our data can serve as reference values of salivary testosterone in preadolescent age. Several reports indicate that there exists association between psychosocial development including learning abilities and salivary testosterone [10–12]. This work is the part of a more complex research program on the relationship between testosterone and intellectual ability of children.

Conclusions

Generally it can be concluded, that salivary testosterone levels in our prepubertal subjects remained stable. There was no significant increase of salivary testosterone levels from the age of 6 until the age of 9 in both sexes.

Our results confirmed significant differences of salivary testosterone levels between both sexes. Prepubertal boys had higher testosterone levels in saliva than prepubertal girls.

Competing Interests
None declared.

Authors' Contributions
D.O. participated in the design and coordination of the study and drafted the manuscript.

K.P. participated in the sequence alignment and performed the statistical analysis.

Z.P. participated on the development of the method and carried out the immunoassays.

M.D. participated in the sequence alignment.

A.M. participated in the sequence alignment.

R.H. participated in the design of the study and substantially contributed to the final version of the article.

All authors read and approved the final manuscript

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