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The content of five sex steroids in human testis

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Summary: In order to assess whether intratesticular hormone content may be helpful for prediction of successful conception in men with fertility problems, five sex steroids, testosterone, dihydrotestosterone, androstenedione, estradiol and, for the first time epitestosterone, were measured in testicular tissue obtained by surgical retrieval from total 84 men. The group consisted of non-obstructive azoospermic men, aged 21-67 years who attended the centre for in vitro fertilization. Steroids after ether extraction and solvent partition were separated by high performance liquid chromatography and then measured by specific radioimmunoassays. The values varied considerably with means±S.D. 2.43 ± 2.47 , 0.27 ± 0.24 , 0.080 ± 0.13 , 0.071 ± 0.089 and 0.31 ± 0.27 for testosterone, dihydrotestosterone, androstenedione, estradiol and epitestosterone, respectively.

Key words: androgens, estradiol, epitestosterone, human testis, biopsy

Introduction

From the point of view of regulation, the testis represents to some extent an autonomous system characterized by its own transport mechanisms and feed-back loops. It is a site of formation of endocrine/paracrine/autocrine acting molecules as growth factors, cytokines and last but not least steroids. Among their endocrine effects, steroids mediate inter-cell communications via their intratesticular receptors. Estradiol, for instance, besides its direct inhibitory action on testicular steroidogenesis, conducts paracrine action between Leydig and Sertoli cells (Levalle *et al.* 1994). Though also glucocorticoids may regulate testicular steroidogenesis by inhibition of LH receptors expression through their own receptors in Leydig cells (Bambino and Hsueh 1981), the principal role in the testis play sex steroids. The importance of intratesticular between-cell communication is demonstrated among others by androgen-binding protein (ABP) mediating androgen transport from the site of their biosynthesis to target cells (Selva and Hammond 2006)

In spite of undoubted importance of steroids in the testis, there are not many reports on their intratesticular content. The first attempts of sex steroids determination in the human testicular tissue are dated from the seventies, for references see e.g. (Takahashi *et al.* 1982). They concerned young men with varicocele (McCoven *et al.* 1979), patients with prostate cancer (Leinonen 1980; Suescun *et al.* 1981; Kuber *et al.* 1991;), various groups of infertile men with emphasis on the gonadotropin regulation of intratesticular sex steroid content (Takahashi *et al.* 1982; Levalle *et al.* 1994; Marie *et al.* 2001; Carreau *et al.* 2004), healthy male volunteers (Jarow and Zirkin 2005; Roth *et al.* 2010) and also post mortem tissues (Vermeulen and Deslypere 1986; Marie *et al.* 2001). The methods used for steroid determination differed in sample processing and not all included sufficient purification of the testicular extract. Meanwhile the methodology advanced enabling determination of minute

amounts of steroids in various matrices including tissues. Here we present the data of four major sex steroids content in the whole human testicular tissue obtained by surgical retrieval, namely testosterone, its precursor androstenedione, dihydrotestosterone and estradiol, after their separation by high performance liquid chromatography (HPLC), in view to assess whether intratesticular hormone content may be helpful for prediction of successful conception in men with fertility problems. In addition, for the first time, epitestosterone concentration was determined, believed to act as an endogenous antiandrogen (Stárka 2003).

Materials and Methods

The whole testicular tissue was obtained from 84 non-obstructive azoospermic men aged 21-67 years (mean±SEM: 35.4±9.9) by surgical retrieval. Patients were evaluated by comprehensive history, physical examination, measurement of testicular size, at least two semen analysis and hormone parameters. The latter included serum follicle stimulating hormone (mean±SEM: 11.61±17.55 U/L), luteinizing hormone (mean±SEM: 6.01±5.06 U/L), prolactin (mean±SEM: 12.05±7.08 µg/L) and total testosterone (mean±SEM: 12.63±5.61 nmol/L) levels. All patients underwent screening for cystic fibrosis, Y chromosome microdeletion and karyotype analysis. Patients underwent microsurgical testicular sperm extraction (M-TESE) using optical x20-25 magnification (microscope OPMI Pico/S100, Carl Zeiss). In each case the informed written consent was obtained from the patients for working up the rests of tissues for scientific purposes. The material was immediately frozen in dry carbon dioxide and stored frozen at -70° until processed. The amounts of tissue varied from 2 to 65 mg. The tissues after thawing were homogenized in 1 ml of physiological solution, at first 1 min under increasing rate from 8000 up to 24 000 rpm, and the additional 1 min at 24 000 rpm using Ultraturrax T25 homogenizer. The homogenate was then extracted twice with

2 ml of diethyl ether, the ether extracts were combined and evaporated to dryness. The dry residues were partitioned between n-hexane (1 ml), methanol (2 ml) and water (0.5 ml). The upper hexane-methanolic phase containing the excessive fat was sucked off and the lower phase containing the steroids was evaporated again in the vacuum rotation evaporator. The dry residues were dissolved in 15 % acetonitrile (50 μ l, v/v) and mixed properly to rinse the tube walls. The samples were centrifuged (2000 g, 3 min, 22 °C) and decanted solutions were transferred into chromatographic vials.

All the chemicals (analytical grade) and solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany).

HPLC was used for separation of androstenedione (AD), testosterone (T), dihydrotestosterone (DHT), epitestosterone (ET) and estradiol (E2). The system (Dionex Softron, Germering, Germany) consisted of a HPLC pump P680 equipped with automatic flow rate control, an automated sample injector ASI-100, a thermostatic column compartment TCC-100, a photodiode array detector PDA-100 with wavelength range 190-600 nm and a fraction collector Foxy Jr. (Teledyne ISCO, Lincoln, NE). The separation was carried out on reverse phase EC 250/4 NUCLEOSIL® 100-5 C18 column (250 x 4 mm) with particle size of 5 μ m (MACHEREY-NAGEL, Düren, Germany). To avoid possible column contamination the Phenomenex SecurityGuard system with cartridge C18 (4.0 x 3.0 mm) (Phenomenex, Torrance, CA) was used. For hormone separation the following protocol was used: The temperature in the column was maintained at 42 °C and the flow rate of the mobile phase was kept constant at 0.7 ml/min. The following gradient profile was used: 0.0-1.5 min constant mobile phase acetonitrile-water (10:90), 1.5-17.0 min linear gradient from methanol-acetonitrile-water (57: 4.8: 38.2) to methanol-acetonitrile-water (68: 4.8: 27.2), 17.0-21.0 min constant mobile phase acetonitrile-water (10:90). The column was equilibrated prior to sample injection. The standard solutions of AD, T, DHT, ET and E2 were used to set up the

collecting windows. AD, T and ET were detected at 244 nm, DHT and E2 at 206 nm. The retention times of authentic AD, DHT, T, EpiT and E2 were 14.98, 15.45, 16.47, 17.77 and 15.38 min, respectively. The collected fractions were evaporated at 55 °C as above and the dry residues were analyzed by radioimmunoassay using the methods developed previously in the author's laboratory, or by using commercial kits as follows: AD according to Putz *et al.* (1982), DHT by Hampl *et al.* (1990) and ET by Bilek *et al.* (1987). The corresponding intra-assay and inter-assay coefficients of variation (CV) in per cents were 5.8 and 11.6 (AD), 7.4 and 9.6 (ET), and 8.7 and 12.1 (DHT). Testosterone was measured by commercial RIA kit from Immunotech (Czech Republic division of Beckman Coulter, Marseille, France), estradiol by radioimmunoassay kit Spectria Estradiol RIA (Orion Diagnostica Oy, Espoo, Finland). The respective intra- and inter-assay CVs were 2.9 to 9.7 % (T) and 2.3 to 10.2 % (E2).

The losses during sample processing (extraction, solvent partition, HPLC) were estimated by spiking the pooled rests of testicular tissues (40 mg/sample) with radioactive tracers (50 000 dpm/sample) and by measuring the remaining radioactivity by liquid scintillation spectrometry (Beckman LS 6000 Liquid scintillation spectrometer). The radioactive tracers – [1,2,6,7-³H]Testosterone, [1,2,6,7-³H]Estradiol, [1,2,6,7-³H]Androstenedione and [1,2,4,5,6,7-³H]Dihydrotestosterone were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). The recovery of tracers varied from 36.7 to 45.6 with mean 41.1 %. The results including those for ET were corrected for this value.

Results

The results of determination of five sex steroids in samples of testicular tissues from eighty four non-obstructive azoospermic men are shown in Table 1. The means, standard deviations,

medians and upper and lower quartiles are provided. In the Table 2 the data are compared with those reported by other authors.

Discussion

The concentrations of testosterone are lower than those reported by early studies which did not use chromatographic separation of the ether extract but agree well with more recent ones. The values of estradiol and androstenedione are comparable with previous findings (Levalle *et al.* 1994, Takahashi *et al.* 1982). We have found higher intratesticular concentrations of dihydrotestosterone but the groups of subjects in other reports were much smaller. For the first time the values are provided for all steroids of interest in one sample, namely testosterone, its precursor androstenedione and 5alpha-reduced metabolite dihydrotestosterone, together with estradiol and epitestosterone. The number of samples and their heterogeneity do not allow statistical evaluation of the relation to the patient's state, especially the sperm parameters, but the method is satisfactory for use in a larger number of samples.

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Table 1

Intratesticular levels of five sex steroids from 84 azoospermic men, in nmol/g tissue

Steroid	Mean	SD	Median	Lower quartile	Upper quartile
T	2.43	2.47	1.52	0.80	3.20
DHT	0.274	0.244	0.204	0.104	0.337
AD	0.080	0.128	0.043	0.017	0.106
E2	0.071	0.089	0.036	0.015	0.096
ET	0.309	0.274	0.223	0.114	0.390

Table 2

Survey of reported data on intratesticular steroids in humans

Author, year	Sample (n)	Unit	T	DHT	AD	E2	ET	Method
McCowen et al. 1979	9 Young males with varicocele after bilateral testicular biopsy	nmol/g tissue	5.16±0.32	0.14±0.071				RIA after extraction and thin layer chromatography
Pirke et al. 1979	45 Males with azoospermia or oligozoospermia	nmol/g tissue	1.88					RIA after homogenization and extraction
Leinonen et al. 1980	25 Males orchidectomized for prostate cancer	nmol/g tissue				0.0092±0.017		Extraction on Lipidex
Suescun et al. 1981	16 Males with prostate cancer + 1 with carcinoma of penis	nmol/g tissue	1.83±0.22	0.082±0.01				RIA after homogenization and extraction
Takahashi et al. 1982	40 Infertile men after testicular biopsy	nmol/g tissue	4.58±2.95	0.067±0.040				RIA after extraction and separation on Sephadex LH 20
Vermeulen and Deslypere 1986	Cadaverous testes from 34 men	nmol/g tissue	1.55±0.22	0.064±0.013	0.058±0.045	0.019±0.002		RIA after homogenization, extraction and PC

Levalle et al. 1994	11 Infertile men differing in FSH levels	nmol/g tissue	1.14 - 1.99			0.015 - 0.11			RIA after homogenization and extraction
Marie et al. 2001	10 Infertile men after testicular biopsy Cadaverous testes from 20 healthy men	nmol/g tissue	6.14±1.33		0.62±0.27	0.64±0.41			RIA after homogenization and extraction
Carreau et al. 2004	Three groups of aged men (total 37 men)	nmol/g tissue	1.68±0.26			0.047 ± 0.015			Immunoassay after homogenization and extraction
This paper 2011	84 Males with various fertility disorders	nmol/g tissue	2.43±2.47	0.27±0.24	0.080±0.128	0.071±0.089	0.309±0.274		RIA after homogenization, extraction and HPLC
Jarrow and Zirkin 2005	9 Male volunteers after bilatellar testicular aspiration	nmol/ml testicular volume	1.98±0.35	0.046±0.006		0.058±0.008			Tandem liquid chromatography- mass spectrometry
Roth et al. 2010	10 Healthy male volunteers	nmol/ml testicular volume	1.49 - 3.11	0.004 - 0.016		0.0048 - 0.009			Tandem liquid chromatography- mass spectrometry