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Immunomodulatory 7-hydroxylated metabolites of dehydroepiandrosterone are present in human semen

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Abstract

Seminal fluid represents a milieu enabling spermatozoa to break the ovum membrane and suppress its immune response and, at the same time, to protect male germ cells against infects. Among constituents of the seminal fluid various steroids, including dehydroepiandrosterone (DHEA) and its sulphate, were detected. With respect to immunomodulatory and antioxidative properties of the latter steroids and its 7-hydroxylated metabolites, believed to be at least in some instances the locally active species, their presence in seminal fluid is of particular interest. Here for the first time unconjugated 3β,7α-dihydroxy-5-androsten-17-one (7α-OH-DHEA) and its 7β-hydroxyisomer have been detected and quantified in semen. Eight semen samples were extracted with diethyl ether and following evaporation and solvent partition both isomers were detected by gas chromatography–mass fragmentometry using the ions m/z 358 and 343 for quantification. Another portion was separated by HPLC and in the fractions corresponding to 7-OH-DHEA isomers the steroids were measured by recently developed specific radioimmunoassays (RIA). Mean concentrations of 7-OH-DHEA as measured by RIA amounted 5.75 ± 1.29 and 5.39 ± 0.75 nmol/l (mean $\pm$ SEM) for 7α- and 7β-OH-DHEA, respectively.

Keywords: Seminal fluid; Dehydroepiandrosterone; 7-hydroxy-dehydroepiandrosterone

1. Introduction

Seminal fluid represents a milieu enabling spermatozoa to break the ovum membrane and suppress its immune response and, at the same time, to protect male germ cells against infects [1]. Among other constituents seminal plasma contains various steroids that influence sperm production and maturation. Most studies addressing this topic dealt with androgens, of which the most abundant is dihydrotestosterone [2–6]. Besides androgens and their precursors in biosynthetic pathway, dehydroepiandrosterone (DHEA), its sulphate, androstenedione and also the main estrogens were detected in semen [5,7–11]. A number of investigations have been undertaken to find out whether androgen content does correlate with sperm abnormalities, but no unequivocal conclusion was made [5,7,9,10,12–15].

Recently, it was suggested that cytological anomalies of spermatozoa observed in oligo- or azoospermic subjects may be linked at least in part to an oxidative stress through chronic exposure of the cells to reactive oxygen species (ROS) [16,17]. At the same time a considerable body of evidence was assembled on the importance of cytokines, chemokines and other active species present in semen and testis for modulating immune response and consequently the conditions for physiological or pathophysiological development of spermatozoa [17–19].

Dehydroepiandrosterone belongs to steroids, which may influence ROS formation by its effect on peroxisomal enzymes and lipid peroxidation [20–22]. In addition, it possesses immunomodulatory and immunoprotective properties, some of which but not all, are ascribed to its non-genomic antiglucocorticoid activities [23].

Later on it has been demonstrated that not only DHEA itself, but some of its 7-hydroxylated metabolites, until recently believed to lack any biological activ-

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ity, may act as locally active agents [24,25]. The question arose whether they are also present in semen. Here, for the first time evidence is brought that both 7-hydroxyisomers of DHEA are present in human seminal fluid.

2. Materials and methods

2.1. Semen samples

Semen was obtained by masturbation from eight healthy men (22–46 yr) attending the Out-Patient Fertility Centre of the Clinic of Obstetric and Gynecology, 1st Faculty of Medicine, Charles University, Prague. The samples after liquefaction were left frozen in solid carbon dioxide and stored frozen at −20°C until analysed.

2.2. Reagents

[1,2,6,7-^{3}H]testosterone, specific radioactivity 2.9 TBq/mmol, purified by thin layer chromatography (DC-Alufolien, Kieselgel 60 F_{254} from Merck, Darmstadt, Germany) in system dichloromethane–methanol 97:3, and carrier free Na^{125}I for radioiodination were purchased from Radiochemical Centre, Amersham, UK. All chemicals were from Merck (Germany), analytical grade, solvents of HPLC grade.

2.3. Sample preparation

The ejaculate (2 ml) was spiked with 50 μl (100 000 dpm) of ethanolic solution of purified [^{3}H]testosterone and extracted twice with diethyl ether (8 ml). The water phase was separated by freezing in solid carbon dioxide and the solvent was evaporated to dryness. The dry residue was dissolved in methanol (5 ml), water (4 ml) and light petroleum ether (1 ml) and mixed. Following separation of the phases the upper organic phase was sucked off carefully with a Pasteur pipette and the water-methanolic phase was evaporated in speed-vac centrifuge. The dry residue was re-dissolved in 2 ml of ethanol and the solution was divided into three portions: 100 μl in duplicate were taken for determination of recovery by measuring [^{3}H]testosterone radioactivity by liquid scintillation counting (Beckman LSC Model 1801). The mean recovery ± S.D. was 68±4%. Another two 600 μl portions were evaporated under nitrogen and used for HPLC separation of 7-OH-DHEA isomers from DHEA and from each other, followed by specific radioimmunoassay (RIA) in each fraction. The third portion (200 μl in duplicate) was used for gas chromatography–mass spectrometry (GC-MS).

2.4. HPLC

Dry residues equivalent to 600 μl of seminal fluid were re-dissolved in the mixture 50% methanol–water and 50 μl portions (equivalent 300 μl of semen) were chromatographed on HPLC using the reverse C_{18} column ET 250/4 Nucleosil 100-5 C18 (Macheray-Nagel, Germany), as described previously [26]. The fractions (400 μl each) corresponding to authentic 7α-OH-DHEA, its 7β-isomer and DHEA were collected, evaporated and used for RIA. No more losses were recorded after HPLC checked also by the simultaneous chromatography of [^{3}H]testosterone.

2.5. Radioimmunoassay

The dry residues from HPLC fractions containing the above-mentioned steroids, were dissolved in 300 μl of 20 mmol/l sodium phosphate-saline buffer containing sodium azide and bovine serum albumin (100 mg/100 ml each), and 100 μl aliquots in duplicates were used for specific RIAs. 7β-OH-DHEA and its 7α-isomer were determined by methods given in Refs. [26,27], DHEA was measured by RIA kit from Immunotech (France). The results were corrected to losses during extraction and solvent partition.

2.6. Gas chromatography–mass spectrometry

The GC-MS system from Shimadzu (Japan) consisted of the gas chromatograph GC-17 A, the simple quadrupole analyzer QP 5050A equipped with electron impact ionization on 70 eV, with the mass range from 10 to 900 amu.

Ethanolic solutions corresponding to 200 μl of deproteinized polar fraction of seminal fluid were transferred quantitatively to reaction vials, evaporated and derivatized in the mixture of 30 μl of pyridine, 10 μl bis(trimethysilyl)-trifluoracetamide and trimethychlorsilane (99:1) at 65°C for 30 min. After derivatization, 50 μl of acetonitrile and 200 μl of isooctane were added and after 1 min of vigorous mixing, the non-polar layer was removed, dried and adjusted to final volume 10 μl in isooctane, from which 2 μl, corresponding to 40 μl of original fluid, were injected to GC. The calibration curve was processed in the same way as the samples. The GC column Zebron ZB-50 15 M × 0.25 mm, film thickness 0.15 μm, cat. No. 7EG-G004-05 was used. Samples were analyzed using the following program: temperature, 140°C, 40°C/min gradient to 220°C, then 8°C/min gradient to 300°C. Pressure, 30 kPa, 11 kPa/min gradient to 52 kPa, then 2.3 kPa/min gradient to 75 kPa. The total analysis time was 12 min per sample. The injection was splitless in high-pressure injection mode 200 kPa for 1 min. The detection was performed in SIM mode at $m/z = 358.3$ amu with checking on

$m/z = 343.3$ amu. The retention times for 7α-OH-DHEA and 7β-OH-DHEA were 4.915 and 5.603 min, respectively.

3. Results

The concentrations of 7α-OH-DHEA and its 7β-isomer in individual semen samples, measured by both RIA and by GC-MS, along with DHEA measured by RIA only, are shown in Table 1.

The levels of 7-OH-DHEA measured by both methods and of DHEA were mutually correlated using Spearman's and Pearson's correlations. The respective correlation matrixes are shown in Table 2. Due to low number of samples and several outliers (Table 1), the only significant correlation found by both statistical methods were between 7α- and 7β-OH-DHEA when measured by GC-MS and between 7α-OH-DHEA and DHEA measured by RIA.

4. Discussion

For the first time 7-OH-DHEA isomers have been detected and quantified in human semen by two independent methods. The levels of both isomers are even slightly higher than those found in blood, which in healthy men did not exceed 6.6 nmol/l (mean 2.33 and 2.26 nmol/l for 7α- and 7β-OH-DHEA, respectively) [26,27]. This raises the question of their origin in the semen. Our group demonstrated 7-hydroxylation of DHEA in human testis and epididymis as early as in 1972 [28], but recent report showed that seminal fluid itself contain various steroid transforming enzymes [29]. It would not be thus surprising if 7-hydroxylation takes place also in the ejaculate and we plan the next experiment to answer this question.

The molecular mechanism of immunomodulatory action of 7-OH-DHEA is unknown so far. On the other hand, recent study demonstrated that locally formed 7-hydroxylated DHEA metabolites can elicit a specific

Table 1
Content of 7α- and 7β-hydroxy-DHEA and unconjugated DHEA in eight semen samples as measured by RIA after HPLC separation and by GC-MS (DHEA was measured by RIA only)

Sample	7α-OH-DHEA (nM)		7β-OH-DHEA (nM)		DHEA (nM)
	RIA	GC-MS	RIA	GC-MS	
1	6.67	4.85	6.47	5.87	36.4
2	6.31	5.45	6.08	8.67	13.6
3	7.88	7.69	5.75	10.9	40.3
4	4.2	2.84	4.37	3.83	17.1
5	5.88	6.74	5.75	10.5	28.4
6	5.09	6.48	5.29	10.2	11.4
7	5.98	4.01	4.53	5.11	16.3
8	4.01	4.15	4.89	8.13	8.49
Mean	5.75	5.28	5.39	7.90	21.50
Median	5.93	5.15	5.52	8.40	16.70
SEM	1.29	1.62	0.75	2.68	11.97

Table 2
Correlation matrixes showing mutual correlations of the levels of 7-OH-DHEA isomers as measured by RIA and GC-MS and of DHEA, using Spearman's and Pearson's methods[a]

Spearman's method				
7α-OH-EA RIA	0.6190	0.7785	0.4762	0.7619
0.1015	7α-OH-DHEA GC-MS	0.6108	0.9762	0.3571
0.0394	0.1061	7β-OH-DHEA RIA	0.4910	0.3832
0.2077	**0.0098**	0.1939	7β-OH-DHEA GC-MS	0.2143
0.0436	0.3477	0.3106	0.5707	DHEA
Pearson's method				
7α-OH-DHEA RIA	0.6786	0.6909	0.39590	0.7988
0.0643	7α-OH-DHEA GC-MS	0.5950	0.9208	0.4967
0.0578	0.1197	7β-OH-DHEA RIA	0.4666	0.5784
0.3315	**0.0012**	0.2439	7β-OH-DHEA GC-MS	0.1970
0.0174	0.2105	0.1331	0.6400	DHEA

[a] The values above and below the diagonal represent the correlation coefficients and the levels of statistical significance, respectively. Significant values of at least 95% probability level are in bold letters.

signal for the T-lymphocyte differentiation of B cells into specific IgG producing plasma cells [25]. This might be of particular importance for maintaining a unique milieu, which a semen fluid represents from the immunological point of view, rich in interleukines responsible for lymphocyte differentiation: after having now identified 7-OH-DHEA isomers in semen, a study is in preparation aiming to put in relation the concentrations of 7-OH-DHEA to interleukine content in semen from subjects with various immune disorders.

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