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URINARY STEROID PROFILES OF PATIENTS THREE WEEKS AFTER *IN VITRO* FERTILIZATION

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ABSTRACT. The urinary steroid metabolite profiles were studied for patients, who failed to achieve pregnancy and for patients with ongoing pregnancy after *in vitro* fertilization (*IVF*). Ten age-matched women who failed to achieve pregnancy and twelve women with ongoing pregnancy after *IVF* were chosen. The standard "*long*" protocol was used for ovarian stimulation in *in vitro* fertilization, while intracytoplasmic sperm injection was employed for the assisted fertilization. The methoxim-silyl derivatives of twenty two steroids were determined by gas chromatography/mass spectrometry and the results were analysed by statistical evaluation. We found, that the concentrations of pregnanediol and pregnanetriol were significantly higher, and the concentration of tetrahydrocortisol was significantly lower in the pregnant patients than in women who failed to achieve pregnancy. The study concludes that the production of pregnanediol, pregnanetriol and tetrahydrocortisol is altered in early pregnancy.

Keywords: *In vitro fertilization, early pregnancy, urinary steroids, gas chromatography-mass spectrometry*

INTRODUCTION

Several studies have examined the early hormonal values and their relationship to pregnancy outcome after *in vitro* fertilization (*IVF*). Most of these studies have focused on the clinical pregnancy (>6 weeks after the last menstrual period). In most cases, however, miscarriages happen earlier, within the first 3 weeks after conception (nearly 5 weeks after the last menstrual period) (1), which is the time for the development of the structural and functional units of the placenta (2). Studies focused on clinical pregnancy miss the most critical period for pregnancy continuance.

Early pregnancy loss represents an important event in the natural and in the *IVF* cycles. Using different ultrasensitive β-hCG assays during the entire luteal phase in women who want to conceive, it has been found that

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the possibility of a positive pregnancy test is 22 - 57 % (3-7). At the same time, 31 - 62 % of these cycles will end in miscarriage. Interestingly, the rate of clinical abortion is between the 5 - 20 % range. However, the early pregnancy loss can be as high as 92 % of the total number of miscarriages (6). This affirms that the early events occurring in the endometrial cavity at the time of implantation are more important, than what we can detect with routine pregnancy tests or vaginal ultrasound (US) scans. Therefore any attempt to examine implantation efficiency in *IVF* should also note the chance of early pregnancy loss.

Progesterone represents a key hormone involved in endometrial receptivity. Endometrial receptivity, a term first introduced by Psychoyos (8), remains one of the major limiting factors for a successful pregnancy.

The aim of the present study was to get more information about the steroid hormone metabolite changes of early pregnancy after *IVF*.

RESULTS

The urinary steroid metabolite profiles of female patients were determined by gas-chromatography mass spectrometry analyses. The urine samples were collected 3 weeks after the embryo transfer in the *in vitro* fertilization procedure. The separation of the urinary steroid metabolites of a 34 year old woman, who failed to achieve pregnancy (member of Group 1) is shown in Figure 1.

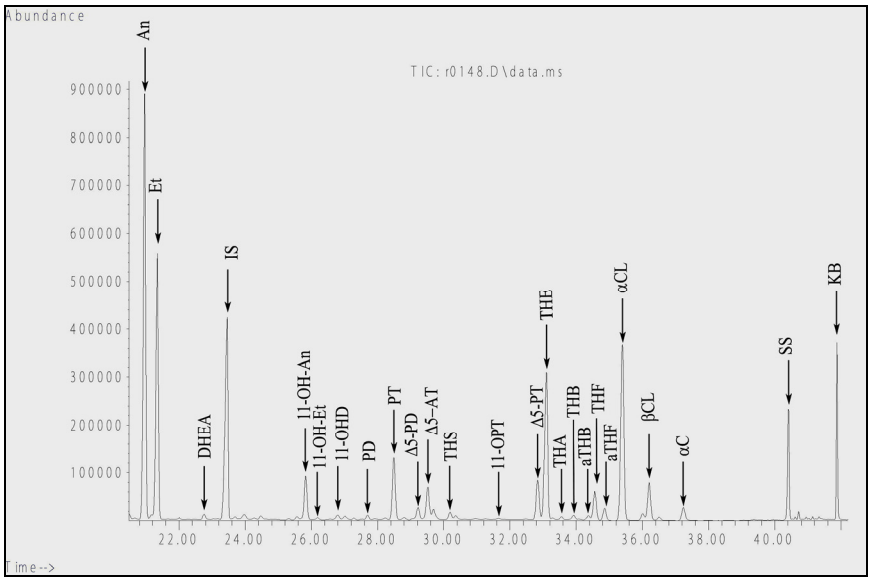

Figure 1. Separation of urinary steroid metabolites of a woman, who failed to achive pregnancy. The urine sample was collected three weeks after the embryo transfer in the *in vitro* fertilization procedure

The steroid profile of a 32 year old woman with ongoing *IVF* pregnancy (member of Group 2) is shown in Figure 2.

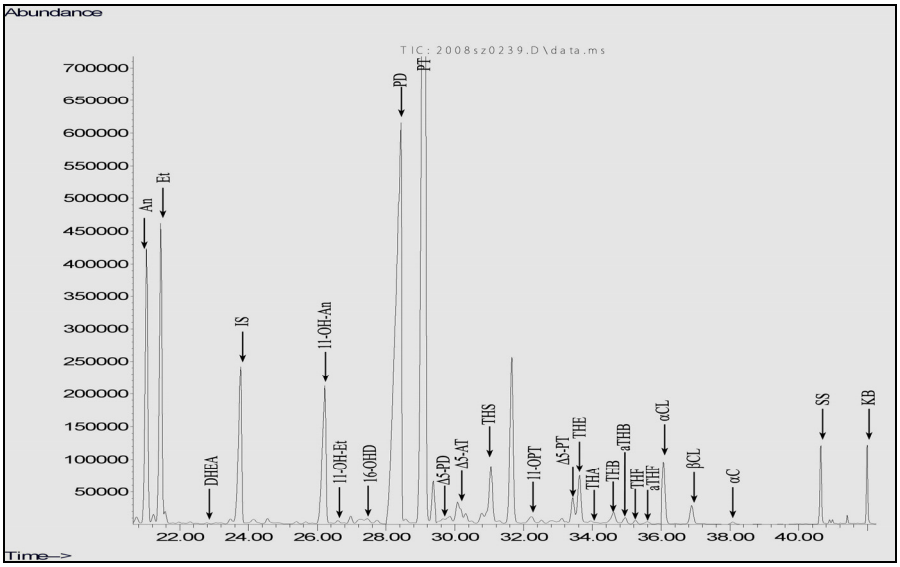

Figure 2. Separation of urinary steroid metabolites of a woman with successful pregnancy. The urine sample was collected three weeks after after the embryo transfer in the *in vitro* fertilization procedure

Altogether twenty two steroid metabolites were determined in a single run for each sample. The steroids were determined after the usual derivatization procedure, described in the Experimental.The two kinds of the profiles obtained for the two groups showed significant differences. Statistical data of the quantitative determination (nmol/24h) of the metabolites for the two groups (patients, who failed to achieve pregnancy, and patients with ungoing pregnancy) including the mean values, median (Me) and quartiles (Q1, Q2) of the amounts of the androgens, progesterone and corticoids in the “non-pregnant” group (Group 1) and in the “pregnant” group (Group 2) is summarized in Table 1.

The Mann-Whitney nonparametric test was applied to compare the concentrations of the derivatized metabolites of androgens, corticoids and progesterone in the urine of the non pregnant and pregnant patients. We found that the concentrations of pregnanediol (PD) and pregnanetriol (PT) were significantly higher (*p*-values are lower than 0.05), and the concentration of tetrahydrocortisol (THF) was significantly lower (*p*-value is lower than 0.05) in the pregnant than in the non pregnant patients.

Table 1. Statistical data of steroid metabolites of patients with successful and unavailing *in vitro* fertilization three weeks after the embryo transfer

	Group 1. (Non pregnant)					Group 2. (Pregnant)					
Derivatized steroids	Data number	Mean	Q1	Me	Q3	Data number	Mean	Q1	Me	Q3	P-values of M-W test
Androsterone (An)	10	1321	533	1017	1408	12	2961	654	1753	3250	0,262
Etiocholanolone (Et)	10	1638	669	1056	2257	12	3229	651	1832	4484	0,391
Dehydroepiandrosterone (DHEA)*	6	112	55	110	165	8	228	83	143	369	0,439
11-hydroxy-androsterone (11-OH-An)*	9	270	150	311	364	11	236	86	195	358	0,518
11-hydroxy-etiocholanolone (11-OH-Et)*	8	215	68	176	382	6	181	76	178	288	0,699
16-hydroxy-DHEA (16-OHD)*	7	432	119	542	728	12	269	137	303	337	0,398
Pregnanediol (PD)	10	7061	278	2091	5810	12	34305	17427	25399	48449	***0,001***
Pregnanetriol (PT)	10	1941	162	429	1001	12	6452	1090	5340	9424	***0,025***
Pregnenediol (Δ5-PD)*	5	261	27	145	553	8	864	334	707	1545	0,079
Androstenetriol (Δ5-AT)*	8	276	127	167	446	11	190	63	182	247	0,457
Tetrahydro-11-deoxycortisol (THS)*	5	130	55	87	227	7	277	135	304	359	0,062
11-keto-pregnanetriol (11-OPT)*	1	40	40	40	40	5	63	23	67	100	0,770
Pregnenetriol (Δ5-PT)*	7	209	114	175	312	10	306	115	213	377	0,495
Tetrahydrocortisone (THE)*	9	1150	425	1381	1531	11	685	273	408	1000	0,160
Tetrahydro-11-dehydrocorticosterone (THA)*	7	181	50	119	303	11	487	146	417	580	0,077
Tetrahydro-corticosterone (THB)*	9	259	165	182	432	10	522	245	495	736	0,072
Allo-tetrahydro-corticosterone (aTHB)*	7	249	123	196	372	10	423	144	344	596	0,283
Tetrahydrocortisol (THF)*	9	471	154	403	637	8	164	72	156	223	***0,034***
Allo-tetrahydrocortisol (aTHF)*	8	246	99	142	420	4	117	49	103	199	0,234
α-cortolone (α-CL)*	8	389	154	336	682	9	282	82	209	409	0,441
β-cortolone (β-CL)*	7	221	89	226	378	5	167	91	137	259	0,570
α-cortol (α-C)*	5	84	48	65	130	3	52	25	34	96	0,180

* The data number (number of patients) in the determination of the data for DHEA, 11-OH-An, 11-OH-Et, 16-OHD, Δ5-PD, Δ5-AT, THS, 11-OPT, Δ5-PT, THE, THA, THB, aTHB, THF, aTHF, α-CL, β-CL, α-C differ in the two groups (non pregnant and pregnant patients) since the concentration of these steroids were below the lower limit of quantification (LLOQ) in those subjects

Although higher amounts were observed in the levels of tetrahydro-11-deoxycortisol (THS), tetrahydro-11-dehydrocorticosterone (THA) and tetrahydro-11-corticosterone (THB) in Group 2 compared to Group 1, this difference could not be confirmed statistically.

DISCUSSION

The hormonal events in early pregnancy are very complex processes, and still poorly understood. We found, that the concentrations of PD and PT (progesterone metabolites) are significantly higher, and the concentration of THF (a cortisol metabolite) is significantly lower in pregnancy.

Progesterone is produced mainly by the corpus luteum up to weeks 5-6 of gestation, but after the twelfth week the placenta will be the dominant site of biosynthesis. Csapó et al. found, that the corpus luteum (CL) is needed to be maintained until the placenta takes over the production of progesterone and that luteectomy before this event induces abortion (13). However, they demonstrated that pregnancy could be supported even after removal of the CL by external administration of progesterone (14). Johnson and colleagues (15, 16) measured high concentrations of progesterone during the first trimester. Progesterone also promotes uterine musculature quiescence and local vasodilatation, by inducing nitric oxide synthesis in the decidua (endometrium of pregnancy) (17). Inadequate uterine contractility may lead to ectopic pregnancies, miscarriages, retrograde bleeding with dysmenorrhea and endometriosis (17).

The endocrine system and the immune system interact closely during maintenance of pregnancy. At the decidua, under the influence of sex steroids, there is a dramatic increase of a unique population of lymphocytes, the uterine natural killer (uNK) cells in early pregnancy. The role of these cells in human pregnancy is still not definitively established. However, they are believed to promote placental and trophoblast growth and provide immunomodulation at the maternal-fetal interface. Uterine natural killer cells are hormonally regulated by progesterone, estrogen and prolaktin (18).

In the pregnant group the lower level of THF (compared to the group failing to achieve pregnancy) is probably a maternal adaptation to the pregnancy. In the non-pregnant group the higher THF level suggests the chance of possible miscarriages (19). The increased cortisol level may decrease the production of progesterone around the time of implantation (20). The discovery of corticotrophin-releasing factor receptors on the ovary (21) is also consistent with the possible existence of a down-regulatory effect of stress on steroidogenesis (22). Furthermore, immune challenges in mice appear to promote a shift in the Th1/Th2 cytokine ratio, which has been associated with low progesterone levels and early spontaneous abortion (23, 24). It was found in rabbit cell-culture studies that glucocorticoids can cause degeneration and premature aging of the trophoblast (25, 26).

CONCLUSIONS

The aim of this study was to get information about the steroid hormone metabolite levels of early pregnancy. The significantly higher concentrations of pregnanediol (PD) and pregnanetriol (PT) – progesterone metabolites, – help to maintain pregnancy, supporting uterine musculature quiescence and local vasodilatation. The low concentration of tetrahydrocortisol (THF) in the pregnant patients compared to the non pregnant patients shows a maternal adaptation to the pregnancy.

The initiation and maintenance of pregnancy depends on complex interactions between the maternal environment and the embryo. Changes in the metabolism can be one of the lots of agents that can cause *IVF* success or failing.

This study indicates a possible correlation between *IVF* pregnancy and the level of certain steroid metabolites, showing differences between patients grouped according to the success or failure of ongoing *IVF* pregnancy, but these data does not demonstrate differences between non pregnant women in general and pregnant women in general, since non-*IVF* data are not included.

EXPERIMENTAL SECTION

Patients and sample collection

Individuals were selected from women with tubal factors, male factors and unexplained infertility. Patients with endocrine disease were not included. Exclusion criteria were an ovarian functional cyst, polycystic ovarian syndrome, ovarian endometrioma, an unilateral ovarian resection or ovariectomy. Other exclusion criterion was the body mass index above 30 kg/m^2. A written informed consent was obtained from the patients.

All patients underwent the previously described standard "*long*" protocol (9), consisting of pre-treatment with GnRH analog (GnRH-a) (0.05 mg/day), followed by stimulation with recombinant follicle-stimulating hormone (rFSH) and highly purified human menopausal gonadotropin (HMG) for controlled ovarian stimulation. 250 μg recombinant hCG was administered before 36 hours the oocyte retrieval. The luteal phase was supported by progesterone (Utrogestan, 3x600mg/day).

Age-matched individuals undergoing their first *IVF* were grouped according to the clinical outcome of *IVF*. Group 1. (n=10) were defined as patients who failed to achieve pregnancy (the mean ± S.D. ages were 34,9 ± 6,11 years). Group 2. (n=12) included patients with ongoing *IVF* pregnancy (the mean ± S.D. ages were 33,3 ± 2,74 years).

Ongoing *IVF* pregnancy was judged by hCG measurement and ultrasound.

This clinical research programme was permitted by the Regional Research Ethics Committee of the Medical Center, University of Pécs, Hungary (permission number: 2997. 28. September 2007.)

Sample preparation

The urine samples were collected over 24 h, 3 weeks after the embryo transfer and stored at -20°C until analysis. The method of the extraction and derivatization has been described in ref. 10, with some modifications described in ref. 11. Shortly, solid phase extraction, enzymatic hydrolysis and methoxime-trymethyl-silyl derivatization and extraction on Lipidex 5000 columns were carried out to obtain the sufficient derivatives for the GC-MS analyses.

Gas-chromatography-mass spectrometry

The GC/MS analysis was carried out on an Agilent 6890N gas chromatograph (Agilent, Santa Clara, USA) coupled to an Agilent 5975 mass spectrometer. The separation was performed in a HP-1-MS column with a length of 25 m, internal diameter 0.2 mm and a film thickness of 0.33 μm. The GC system was operated in constant flow mode at a flow rate of 1.5 ml/min with helium carrier gas. Splitless injection mode was employed. 2 μl of derivatized sample extract was taken into the heated injector (300°C). The GC temperature program was as follows: the initial temperature was 50°C, which was raised at 30°C/min to 190°C, and it was held for 5 min. Then the temperature was increased at 2.1°C/min to 300°C and maintained for 10 min. The transferline temperature was 300°C, and the temperature of the ion source was 200°C. The MS data were acquired at 70 eV, in selected ion-monitoring (SIM) mode.

The androgen metabolites, androsterone (An), etiocholanolone (Et), dehydroepiandrosterone (DHEA), 11-hydroxy-androsterone (11-OH-An), 11-hydroxyetiocholanolone (11-OH-Et), 16-hydroxy-DHEA (16-OHD), androstenetriol (Δ5-AT); the progesterone and intermediate metabolites, pregnanediol (PD), pregnanetriol (PT), pregnenediol (Δ5-PD), 11-keto-pregnanetriol (11-OPT), pregnenetriol (Δ5-PT); and the corticoid metabolites tetrahydro-11-deoxycortisol (THS), tetrahydrocortisone (THE), tetrahydro-11-dehydrocorticosterone (THA), tetrahydro-corticosterone (THB), allotetrahydro-corticosterone (aTHB), tetrahydro-cortisol (THF), allo-tetrahydrocortisol (aTHF), α-cortolone (α-CL), β-cortolone (β-CL), α-cortol (α-C) were quantified and identified with their characteristic mass ions, *i.e.*, the Target Ions (Tg Ion) and the Qualifier Ions (Q Ions) specific for each steroid (12).

Calculation and statistical analysis

Statistical evaluation was made by the Mann-Whitney nonparametric test, using SPSS 16.0 software (SPSS Institute Inc., Chicago, IL, USA). A *p* value of lower than 0.05 was considered to be statistically significant. The data were described by sample size (n), median (Me), and the quartiles (Q1, Q3).

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