J Assist Reprod Genet (2014) 31:485–491
DOI 10.1007/s10815-014-0186-3

ASSISTED REPRODUCTION TECHNOLOGIES

Low testosterone levels in women with diminished ovarian reserve impair embryo implantation rate: a retrospective case-control study

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Received: 19 November 2013 / Accepted: 28 January 2014 / Published online: 14 February 2014

Abstract
Objective To investigate the association of basal testosterone (T) levels with the outcome of in vitro fertilization (IVF) in women with diminished ovarian reserve (DOR).
Methods Complete clinical data on the first 223 IVF cycles in women with DOR were retrospectively analyzed. The associations of basal follicle stimulating hormone, luteinizing hormone, estradiol, and T levels with ovarian response and IVF outcome were studied.
Results Basal T levels were significantly different between pregnant and non-pregnant women. However, basal T levels showed no correlation with controlled ovarian hyperstimulation parameters after adjusting for age. The association of basal T levels with pregnancy rate was significant after adjusting for other impact factors. Using receiver operating characteristic (ROC) analysis, the basal T level of 1.115 nmol/L for predicting pregnancy outcome had a sensitivity of 82.80 % and specificity of 58.09 %. The women were divided into two groups based on this value; although the clinical characteristics and ovarian stimulation parameters were similar, the clinical pregnancy (16.18 % (11/68) vs. 40.15 % (53/132), respectively, p=0.000) and implantation rates (10.07 % (15/149) vs. 22.41 % (65/290), respectively, p=0.002) were significantly different in the low and high T level groups.
Conclusion In women with DOR, the basal T level presented a positive association with pregnancy outcome in IVF. The poor reproductive outcome observed in women with lower basal T levels may be due to the decreased implantation rate.

Keywords Diminished ovarian reserve · Basal testosterone · Ovarian response · Pregnancy · Implantation rate

Capsule Low basal serum testosterone leads to poor in vitro fertilization outcome in women with diminished ovarian reserve by decreasing the implantation rate.

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Introduction

Diminished ovarian reserve (DOR) is often associated with poor ovarian response, a high cancellation rate, and a significant decline in pregnancy rate. Thus, early and accurate assessments of the ovarian response and pregnancy outcome before in vitro fertilization and embryo transfer (IVF-ET) treatment are particularly important. These can not only help physicians to determine treatment protocols, predict the clinical outcome, but also assist individual couples in deciding whether to undergo costly and often demanding and disappointing IVF treatment. The values of commonly used markers of ovarian reserve and IVF outcome, including basal follicle stimulating hormone (FSH), the basal FSH/luteinizing hormone (LH) ratio, estradiol (E_2), antral follicle count (AFC), and anti-Mullerian hormone (AMH), are derived from general infertility populations and have a number of significant

shortcomings [1, 2]. Currently, few data are available regarding IVF outcome predictors in women with DOR, who constitute a large subgroup of assisted reproductive technology (ART) populations.

Under physiological conditions, serum concentrations of dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), and testosterone (T) have been found to steadily decline in women with age, which parallels the age-related decline in reproductive ability [3, 4]. However, the few studies conducted to date addressing the relationship between androgen and IVF outcomes have yielded conflicting results. Serum androgen was first reported to have a negative correlation with IVF stimulation parameters, and low basal T levels predicted poor IVF success rates in women with normal ovarian reserve [5]. Shortly thereafter, however, researchers from the same group rejected this predictive value of pregnancy outcome [6]. A recent study showed that basal T levels could predict the number of large follicles on the HCG day and pregnancy outcome in women with DOR but not in women with normal ovarian reserve [7]. Thus, whether serum basal T levels can influence IVF ovarian response and predict outcomes in women with DOR requires further study.

DOR appears to represent an androgen-deficient state [8]. Androgen supplementation for DOR, such as with DHEA and transdermal testosterone, not only increased retrieved oocytes and improved pregnancy outcome but also decreased the time to conception with infertility treatments [8–11]. Considering these findings, an improved IVF outcome in DOR may involve an increase in the number and quality of oocytes retrieved by enhancing the T level, as the majority of DHEA in women is converted to T [8, 10]. Therefore, T levels may be related to the ovarian response and IVF pregnancy outcome. However, if the predictive value of T levels for IVF outcome depends on oocyte quality and quantity, the basal T level may not influence the implantation rate. To the best of our knowledge, no published study to date has focused on the impact of basal T levels on the implantation rate for women with DOR. The present study was designed to evaluate the predictive value of basal T levels for IVF outcome in women with DOR and to assess whether basal T levels affect IVF outcome via ovarian response or embryo implantation.

Materials and methods

Study population

After obtaining institutional review board approval from the ethics committee of People's Hospital, Peking University, we performed the following retrospective study. The medical records of 1598 Chinese women undergoing their first IVF-ET cycle and reaching the oocyte pick-up stage at Reproductive Medical Center, People's Hospital, Peking University, between March 2009 and December 2010 were reviewed. DOR was defined as a basal FSH value >10 IU/L or an FSH : LH ratio >3 [1, 7]. Women with DOR were recruited. The exclusion criteria were diagnosis of polycystic ovary syndrome, the presence of endometriosis with laparoscopic or ultrasonographic evidence, and the use of oral contraceptives, DHEA and other hormone therapy in the three preceding months.

Stimulation protocol

The stimulation protocols for IVF-ET were determined by each patient's doctor based on the age and ovarian reserve status of the patient, which included an evaluation of basal endocrine levels, AFC and etc. AFC (3–10 mm) were counted via using ultrasound scan on cycle day 2 or 3. Patients suspected of having a normal response, typically based on the long luteal down-regulation protocol, were injected with 1.25 mg of gonadotropin-releasing hormone agonist (GnRHa, Diphereline, Beaufour-Ipsen Pharmaceuticals Ltd., France) on menstrual day 21. The starting dose of gonadotropins was based on the physician's discretion but always contained a fixed amount of recombinant FSH (rFSH, 75 U/ampoule, Gonal F, Serono Ltd., Switzerland) supplemented with at least one ampoule (75 IU) of human menopausal gonadotropin (HMG). This treatment was initiated on menstrual day 3 or 4 and continued for 4 days of stimulation; thereafter, the gonadotropin dose was adjusted according to the follicular growth, which was monitored by transvaginal ultrasonography and serum E_2 concentrations. Women with a potentially low or poor response to gonadotropins underwent a flare-up agonist or antagonist protocol. For the flare-up agonist stimulation, a fixed dose of rFSH (225 or 300 IU/day) and HMG (75 or 150 IU/day), along with a fixed dose of GnRHa (0.1 mg/day, Triptorelin, Ferring, Germany), was administered beginning on menstrual day 2. The flexible and multi-dose GnRH antagonist protocol was also initiated with a fixed dose of rFSH (225 or 300 IU/day) and HMG (75 or 150 IU/day) on cycle day 2 and co-treatment with a GnRH antagonist (Cetrotide, 0.25 mg/day, Merck-Serono, Turkey) when the leading follicle was 13–14 mm in diameter. Human chorionic gonadotropin (HCG, 10,000 IU, Lizhu Ltd., Guangdong, China) was administered when at least two follicles were 18 mm in diameter. Oocytes were retrieved by transvaginal ultrasound-guided follicular aspiration 36 h later. Depending on the semen quality of the partner, oocytes were fertilized using either standard IVF insemination or intracytoplasmic sperm injection.

Outcome measurement

The fertilization results were assessed 16–20 h after insemination. Two or three embryos per patient were transferred on

the third day after oocyte retrieval, and the luteal phase was supported with 60 mg of progesterone (20 mg/ampoule, Xianju Ltd., Zhejiang, China). Specifically, 2 embryos were transferred to women less than 35 years old and in their first treatment cycle, and 3 embryos were transferred to the others women. A positive pregnancy was defined as a β-hCG level >25 IU/L on the fourteenth day after the embryo transfer. Clinical pregnancy was confirmed by positive fetal cardiac activity and by the number of sacs counted using vaginal ultrasound at a gestation of 6 weeks. High-quality embryos were defined as embryos developed from normal fertilized eggs with no fragmentation or no more than 1/3 fragmentation, no presence of multinucleation, and 7–8 blastomeres 72 h after egg retrieval [7].

Hormone assays

On day 2 or 3 of the menstrual cycle, basal serum levels of FSH, LH, E2, and T were determined for each subject using electrochemiluminescent immunoassay with UniCel DXI 800 Access Immunoassay System (Beckman Coulter, Inc., USA). The intra-assay and inter-assay coefficients of variation were <4 % and <7 % for FSH, <3 % and <8 % for LH, <5 % and <8 % for E_2, <3 % and <7 % for T, and <6 % and <9 % for β-hCG, respectively.

Statistical analysis

The baseline characteristics of women in the pregnancy and non-pregnancy groups were compared using Student's t-test, and the metrics assessed included age; body mass index (BMI); fasting insulin; fasting glucose; AFC; basal levels of FSH, E2, LH, and T; starting dose of gonadotropins; and the number of oocytes retrieved. The associations between the basal T level and the following metrics were determined using Pearson correlation analyses and partial correlation tests: age, AFC, FSH, starting dose of gonadotropins, days of stimulation and total dose of gonadotropins, number of follicles >14 mm on the HCG day, and number of oocytes retrieved. To find the optimum cutoff values for pregnancy, receiver operating characteristic (ROC) analysis was performed on age, AFC, FSH and basal T levels, and the area under the curve was determined. Multiple logistical regression analysis with backward stepwise selection method was used to assess the effect of basal T (as a categorical variable) and several other impact factors, including age, AFC and FSH, on IVF outcome. Adjusted odds ratio (OR) and its 95 % confidence interval (CI) were calculated based on the analysis. The χ^2 test and Fisher's exact test were used to assess the pregnancy and implantation rates. The data were analyzed using the SPSS statistical package (SPSS version 17.0, Chicago, IL). All tests were two-tailed and a p-value of 0.05 was considered significant.

Results

Among the 223 recruited patients with DOR, 53, 127, and 43 women underwent the long luteal down-regulation protocol, the short flare-up agonist protocol, and the antagonist protocol, respectively. Twenty-three women did not transfer embryos, including 20 women due to the lack of an embryo, one woman for fever and two women for thin endometrium. Table 1 compared the demographic characteristics and controlled ovarian hyperstimulation (COH) parameters of pregnant and non-pregnant women; data for patients who did not undergo embryo transfer were excluded. Age and FSH level were significantly lower in the pregnant women than in the non-pregnant women ($p<0.05$), whereas the AFC and basal T level were higher in the pregnant women. The pregnant women also had a higher number of retrieved oocytes resulting in more high-quality embryos. Thus, in addition to the traditional markers of IVF outcome, the basal T level appeared to have predictive value.

Basal T levels were positively associated with E_2 levels on the HCG day ($r=0.138$, $p=0.046$) and the number of oocytes retrieved ($r=0.148$, $p=0.027$), based on Pearson rank correlation analysis, but were negatively associated with age ($r=-0.180$, $p=0.007$) and starting dose of gonadotropins ($r=-0.141$, $p=0.036$). However, none of the associations reached a statistically significant level after adjusting for age.

The discriminative abilities of basal T levels, age, AFC and FSH for the prediction of pregnancy per transfer were low, as indicated by the AUC from the ROC curve analysis (Table 2). The women were then divided into different subgroups according to different cutoff values for basal T level, age, AFC and FSH level. Then, multiple logistical regression analysis with backward stepwise selection method identified the basal T level as an independent determinant of IVF outcome among those parameters (Table 3). For women with a basal T level lower than 1.115 nmol/L had a significantly decreased pregnancy rate (16.18 %, 11/68) compared with those with high basal T levels (40.15 %, 53/132), and this decrease was significant as shown by multivariate logistic regression analysis ($P=0.005$). This result indicates that the basal T level presents a positive association with pregnancy outcome in IVF.

Further analyses were performed to clarify the difference in pregnancy rates between the low basal T group ($n=68$) and the high basal T group ($n=132$) (Table 4). The implantation rate in the low basal T group (10.07 %, 15/149) was significantly lower than that in the high basal T group (22.41 %, 65/290, $p=0.002$), even though the clinical characteristics and ovarian stimulation parameters, such as AFC, starting dose of gonadotropins, E_2 levels on the HCG day, number of oocytes retrieved, number of high-quality embryos, and number of embryos transferred, were similar.

Table 1 Demographic characteristics and controlled ovarian hyperstimulation parameters in pregnant and non-pregnant women with DOR

Variables	Entire group (n=223)	Non-pregnant cycles (n=136)	Pregnant cycles (n=64)	p-value
Age (years)	34.57±5.11	35.30±5.11	32.23±4.32	0.000
BMI (kg/m^2)	22.22±2.79	22.37±2.63	21.72±2.79	0.112
Fasting glucose (mmol/L)	5.10±0.64	5.12±0.70	5.05±0.51	0.477
Fasting insulin (uIU/mL)	9.00±5.91	8.86±5.65	9.32±6.79	0.620
Antral follicle count (n)	6.59±3.66	6.25±3.23	8.06±4.21	0.001
Basal FSH levels (U/L)	10.55±3.90	10.46±3.27	9.44±2.60	0.029
Basal LH levels (U/L)	3.57±1.88	3.56±1.99	3.42±1.41	0.619
Basal E_2 levels (nmol/L)	0.15±0.10	0.15±0.10	0.15±0.13	0.998
Basal T levels (nmol/L)	1.37±0.62	1.31±0.62	1.54±0.55	0.010
Starting dose of gonadotropins	288.17±55.67	292.00±56.38	276.95±49.06	0.068
Days of stimulation	8.65±1.87	8.79±1.88	8.66±1.76	0.641
Total dose of gonadotropins (U)	2667.47±958.90	2765.13±1027.03	2490.44±725.04	0.056
Number of follicles >13 mm	6.20±2.95	6.05±2.87	7.34±2.76	0.003
LH levels on the HCG day (U/L)	4.21±5.76	3.93±3.68	3.56±2.24	0.471
E_2 levels on HCG day (nmol/L)	6.88±4.10	6.75±4.35	7.96±3.46	0.061
T levels on HCG day (nmol/L)	2.16±0.87	2.08±0.81	2.38±0.92	0.049
P levels on HCG day (nmol/L)	3.60±2.09	3.62±1.96	3.76±2.45	0.676
Number of oocytes retrieved	7.73±4.01	7.51±3.82	9.47±3.50	0.001
Number of MII oocytes (n)	6.30±3.57	5.99±3.24	8.16±3.21	0.000
Number of 2PN (n)	3.75±2.61	3.61±2.37	4.91±2.56	0.001
Number of high-quality embryos	1.33±1.37	1.29±1.32	1.73±1.38	0.032
Number of embryos transferred	2.20±0.61	2.15±0.64	2.28±0.52	0.169
Endometrial thickness on HCG day (cm)	1.01±0.26	1.04±0.29	1.00±0.189	0.237

Data other than the p-values indicate means±SD

BMI body mass index; *DOR* diminished ovarian reserve; *E_2* estradiol; *FSH* follicle-Stimulating hormone; *HCG* human chorionic gonadotropin; *LH* luteinizing hormone; *T* testosterone; *2PN* two pronuclei

Discussion

The available data confirm that androgen plays an important role in female fertility via the androgen receptors (ARs) on the human ovary and endometrium [12, 13]. It is generally accepted that androgen activates primordial follicles through the phosphatidylinositol 3-kinase-Akt-Foxo3α pathway, promotes follicular development beyond the preantral stage, and reduces follicular atresia by stimulating granulosa cells [14, 15]. Thus, androgen supplementation may yield more oocytes, resulting in more embryos and improving pregnancy outcomes in women with DOR [8–11]. However, very few studies have investigated the effects of androgen on endometrial cyclic development and embryo implantation.

The predictors of IVF pregnancy outcome in women with DOR are still under investigation. Herein, we found that in addition to the traditional predictive markers of IVF pregnancy outcome and ovarian response, such as age, AFC, and basal FSH, basal T levels were significantly different between the pregnancy and non-pregnancy groups. Although the sensitivity was low, ROC analysis indicated that low basal serum T levels were associated with poor IVF treatment performance. The logistic regression model showed that pregnancy was significantly related with basal serum T levels after controlling

Table 2 Receiver operating characteristic curve analysis of study variables for the prediction of IVF outcome

Variable	AUC_{roc}	Optimum cutoff	Sensitivity %	Specificity %	95 % CI	P value
Age (years)	0.668	33.500	63.43	35.94	0.591–0.744	0.000
Antral follicle count (n)	0.637	6.500	62.50	38.23	0.557–0.717	0.002
Basal FSH level (U/L)	0.587	11.720	30.88	12.50	0.505–0.669	0.047
Basal T level (nmol/L)	0.621	1.115	82.80	58.09	0.541–0.701	0.006

AUC_{roc} area under the receiver operating characteristic curve, *CI* confidence interval

Table 3 Multiple logistical regression analyses of possible determinants for pregnancy

Factor		Pregnancy rate (%)	OR (95 % CI)	*P* value
Age (years)	<33.50	45.05 % (41/91)	2.109 (1.081–4.117)	0.029
	>33.50	21.10 % (23/109) [a]	1	
Antral follicle count (n)	<6.50	22.22 % (24/108)	1	0.024
	>6.50	43.48 % (40/92) [a]	2.139 (1.103–4.149)	
Basal T level (nmol/L)	<1.115	16.18 % (11/68)	1	0.005
	>1.115	40.15 % (53/132) [a]	2.966 (1.386–6.348)	

The OR was calculated from the multiple logistic regression analyses after controlling for other variables

[a] Significantly ($P<0.01$) different compared with the relevant groups

for the possible confounding effect of variables. These findings suggest that basal serum T levels may be a new prognostic factor for IVF treatment outcome in women with DOR.

To gain further insight into the role of androgens in pregnancy outcome, statistical analysis was performed on the demographic, COH variables and the implantation rate between the high and low basal T groups using the ROC threshold (Table 4). The results showed much higher rates of implantation in women with high basal T levels compared with those with low basal T levels, despite similar values for metrics of ovarian response between the two groups. This result suggests that the basal T level plays an important role in embryo implantation and levels below 1.115 nmol/L are indicative of lower implantation rates in women with DOR.

Although the precise mechanism by which the basal T level influences embryo implantation has not yet been elucidated, AR is known to be abundantly expressed in both human stromal and glandular endometrial cells and even detectable

Table 4 Demographic characteristics, controlled ovarian hyperstimulation parameters and pregnancy rates in the low and high basal T groups of women with DOR

Variables	Basal T<1.115 nmol/L (n=68)	Basal T≥1.115 nmol/L (n=132)	p-value
Age (years)	35.76±5.56	33.95±4.76	0.012
BMI (kg/m^2)	22.15±2.79	22.27±2.79	0.774
Fasting glucose	5.09±0.67	5.10±0.62	0.979
Fasting insulin	8.17±4.89	9.45±6.35	0.127
Antral follicle count (n)	6.35±3.49	6.72±3.75	0.473
Basal FSH levels (U/L)	11.31±4.83	10.15±3.25	0.034
Basal LH levels (U/L)	3.67±1.88	3.52±1.90	0.586
Basal E_2 levels (nmol/L)	0.16±0.15	0.14±0.06	0.062
Basal T levels (nmol/L)	0.71±0.29	1.72±0.42	0.000
Starting dose of gonadotropins	295.29±58.69	284.42±53.83	0.166
Days of stimulation	8.81±2.03	8.57±1.79	0.372
Total dose of gonadotropins (U)	2822.90±1041.67	2585.50±905.23	0.079
Number of follicles >13 mm	5.88±3.08	6.36±2.86	0.248
LH levels on HCG day (U/L)	4.53±8.54	4.03±3.58	0.637
E_2 levels on HCG day (nmol/L)	6.42±4.24	7.11±4.02	0.258
T levels on HCG day (nmol/L)	1.81±0.74	2.37±0.88	0.000
P levels on HCG day (nmol/L)	3.48±2.34	3.74±1.71	0.173
Number of oocytes retrieved	7.29±4.03	7.96±3.98	0.233
Number of MII oocytes (n)	5.88±3.62	6.53±3.53	0.203
Number of 2PN (n)	3.42±2.65	3.80±2.57	0.292
Number of high-quality embryos	1.39±1.58	1.30±1.26	0.658
Number of embryos transferred	2.19±0.72	2.20±0.55	0.949
Endometrial thickness on HCG day (cm)	1.01±0.29	1.02±0.24	0.885
Pregnancy rate	16.18 % (11/68)	40.15 % (53/132)	0.000
Implantation rate	10.07 % (15/149)	22.41 % (65/290)	0.002

in the decidua of early pregnancy [13, 16]. AR controls genes essential for cytoskeletal organization and cell cycle regulation and cooperates with the progesterone receptor (PR) to promote endometrial stromal fibroblast differentiation into secretory epithelioid-like decidual cells [16]. Androgen also enhances the process of decidual transformation and inhibits apoptosis of decidual endometrial fibroblasts by promoting resistance to oxidative stress [17]. Moreover, androgen increases AR expression in normal endometria and then amplifies the effects of AR expression [16]. All these activities imply that androgen plays an important role via AR in modulating the decidualization process and decidual-trophoblast interactions, which are the critical processes that control embryo implantation.

As DOR appears to represent an androgen-deficient state, lower basal T levels (as defined in the present study) in women with DOR may indicate an extreme shortage of androgen for endometrial development and embryonic implantation, resulting in a low probability of embryo implantation. This hypothesis is supported by evidence that transient androgen supplementation significantly increased the embryo implantation rate and clinical pregnancy rate in poor responders [9]. However, embryo implantation defects coinciding with aberrant expression of avb3 integrin, glycodelin, estrogen receptor a, and HOXA10 have also been observed in polycystic ovarian syndrome patients with chronic hyperandrogenism [17, 18]. These paradoxical phenomena reveal that endometrial development and embryonic implantation may require appropriate androgen levels, but the relationship will require further investigation.

Recently published data have shown that androgen supplementation may improve ovarian reserve and have beneficial effects on the ovarian response in women with DOR [8–11]. Considering the results of the present study in this context, we can infer that T has a dual function of modulating folliculogenesis and regulating embryo implantation in female reproduction. This hypothesis is supported by the observations of reduced fertility in homozygous female AR knockout mice, which is not only associated with fewer oocytes after super-ovulation and follicular atresia but also with impaired uterine hypertrophy and endometrial growth, which are required for implantation [19, 20]. However, in the present study, basal T levels showed no correlation with ovarian stimulus metrics, such as the E_2 levels on the HCG day, number of oocytes retrieved, and the total dose of gonadotropins. The differences in the implantation and pregnancy rates only became significant when the study population was divided based on the ROC threshold of the basal T level. Thus, very low T levels, such as those below 1.115 nmol/L in women with DOR may have a great negative impact on implantation but not on ovarian response.

In this study, 20 women were excluded from the analysis of IVF outcome because no embryo transferred. When the demographic characteristics and COH parameters of these women were compared with the others, only age (35.5±5.62 years vs. 33.67±4.63 years, respectively) and basal T levels (0.73± 0.28 nmol/L vs. 1.72±0.43 nmol/L, respectively) had significant differences. Their ages and basal T levels were similar to those of the low basal T group. Therefore, the data for these women may not have a great influence on the results of this study.

In summary, the present study demonstrated for the first time that low basal T levels are associated with reduced rates of implantation and pregnancy in IVF. Increasing basal T levels through androgen supplementation may represent a new therapeutic target for improving the implantation rate and IVF treatment outcome, but larger prospective studies will be required to assess this approach.

Acknowledgments The authors thank Dr. Jianfeng Li for providing scientific advice.

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