

Ovarian Features after 2 Weeks, 3 Weeks and 4 Weeks Transdermal Testosterone Gel Treatment and Their Associated Effect on IVF Outcomes in Poor Responders

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ABSTRACT : This study was performed to investigate the effect of of transdermal testosterone gel (TTG) on controlled ovarian stimulation (COS) and IVF outcomes and ovarian morphology according to pretreatment duration in poor responders. A total of 120 women were recruited for this pilot study. They were randomized into control, 2 weeks, 3 weeks or 4 weeks TTG treatment groups. For three TTG treatment groups, 12.5 mg TTG was applied daily for 2 weeks, 3 weeks or 4 weeks in preceding period of study stimulation cycle. After 3 weeks of TTG pretreatment, significant increase of antral follicle count (AFC) and significant decreases of mean follicular diameter (MFD) and resistance index (RI) value of ovarian stromal artery were observed ($p=0.026$, $p<0.001$, $p<0.01$, respectively). The total dose of rhFSH administered for COS significantly decreased after 3 and 4 weeks TTG treatment both compared with control group ($p<0.001$, $p<0.001$). The numbers of oocytes retrieved and mature oocytes were significantly higher in 3 and 4 weeks TTG treatment groups than control group ($p<0.001$, $p<0.001$ in the number of oocytes retrieved; $p<0.001$, $p<0.001$ in the number of mature oocytes). The clinical pregnancy rate and live birth rate were increased only in 4 weeks TTG treatment group compared with control group ($p=0.030$ and $p=0.042$, respectively). These data demonstrated that TTG pretreatment for 3 to 4 weeks increases AFC and ovarian stromal blood flow, thereby potentially improving the ovarian response to COS and IVF outcome in poor responders undergoing IVF/ICSI.

Key words : Transdermal testosterone gel, COS, IVF, Poor responders

INTRODUCTION

Androgen leads follicular atresia in murine experimental models (Fabregues et al., 2009). However, in primates, androgens had positive effects about follicular development and some synergistic effects with follicle stimulating hormone

(FSH). Androgen supplementation such as testosterone or dihydrotestosterone recruited more growing follicles, and increased granulosa and theca cell proliferation (Vendola et al., 1998). Furthermore, androgen makes follicular FSH receptor expression in granulosa cells (Weil et al., 1999). These androgen actions showed some helpful outcomes

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during in vitro fertilization (IVF) treatment especially for poor ovarian responders (Fabregues et al., 2009; Barad & Gleicher, 2006).

Poor ovarian response to ovarian stimulation usually indicates a reduced follicular response during assisted reproduction. According to a consensus developed by The European Society for Human Reproduction and Embryology, less than 3 oocytes could be retrieved with a conventional stimulation protocol in poor responders by definition (Ferraretti et al., 2011). So a variety of stimulation protocols have been used, however none of them have been so effective for poor ovarian response. Androgen supplementation protocol is one of them (Gonzalez-Comadran et al., 2012), which have been investigated upon data from experimental animals described above and speculation from women with poly-cystic ovarian syndrome (Massin et al., 2006).

In human data, there was controversy about androgen priming during assisted reproduction. The oral dehydroepiandrosterone (DHEA) supplementation in women with diminished ovarian reserve showed increased ovarian responsiveness and demonstrated significant improved IVF outcomes (Barad & Gleicher, 2006; Casson et al., 2000). Transdermal testosterone pretreatment also showed increased ovarian sensitivity to gonadotropin (Fabregues et al., 2009).

However, Massin et al. could not demonstrate any beneficial effect on ovarian response to gonadotropin depending on testosterone application. They proposed several reasons about the absence of any beneficial effects of testosterone application, and timing and duration of androgen supplementation were thought to be critical in adequate follicle stimulation and total amount of androgen supplementation was also considered to be an important factor (Massin et al., 2006).

Therefore we conducted a pilot study to investigate the effect of transdermal testosterone gel (TTG) among subgroups classified by use duration in patients who had experienced

a poor ovarian response to prior IVF cycles. We observed and compared ovarian morphologies of each groups including antral follicle count, follicular diameter and intra-ovarian blood flow after TTG treatment.

MATERIAL AND METHODS

1. Patients

The study population consisted of total 120 women, and they had been showed poor ovarian responses during prior failed IVF cycles. In detail, women were enrolled who failed to produce over 3 follicles with a mean diameter of ≥ 16 mm, and then less than 3 follicle were retrieved even a high total dose of recombinant human follicle stimulating hormone(rhFSH, $> 2,500$ IU). All the patients were in good health within normal limits of thyroid, kidney and hepatic laboratory results, and they had regular menstruation period with duration 21-35 days. None of them had ever taken any infertility medication over past 3 months. Our pilot study was performed at a university-based infertility clinic at the Asan Medical Center, Seoul, South Korea. The study population consisted of 120 poor responders who had undergone 120 IVF/ICSI cycles between Jan 2006 and Jan 2010. Patients were randomly allocated into three TTG treatment groups (TTG use for 2 weeks, 3 weeks or 4 weeks) (n=30 in each groups) and one control group (n=30) by the use of sealed envelopes and a computer-generated list. The sequence of allocation to the four groups was provided to the investigating physicians and randomization was performed as planned according to the randomization list order.

2. Ovarian stimulation protocol

Before starting COS cycle, all of the patients had taken estrogen and progesterone pretreatment for 25 days using E2 valerate (Progynova; Bayer Schering Pharma, Berlin, Germany) 1 mg/d and norethindrone (Primolut; Bayer Schering Pharma) 5 mg/d. In all subgroups, GnRH antagonist multiple-

dose protocol (MDP) was used for ovarian stimulation. Briefly, 5 days after stopping hormonal pretreatment, rhFSH (Gonal-F; Merck Serono, Geneva, Switzerland) 300 IU/d was started when establishing ovarian and uterine quiescence by using transvaginal ultrasonography. The dose adjustment was based on an individual ovarian response assessed by serial transvaginal ultrasonography every 3 or 4 days. GnRH antagonist cetrorelix (Cetrotide; Merck Serono) 0.25 mg/d was started when the leading follicle is 13 to 14 mm in diameter and maintained daily till the day of recombinant hCG (rhCG; Ovidrel; Merck Serono) 250 mg administration. The criteria to induce follicular maturation was the presence of one or more follicle ≥ 18 mm.

Oocyte aspiration was performed under transvaginal ultrasound guidance 35-36 hours after hCG injection, and up to four embryos per patients, after IVF or ICSI, were replaced on the third day after oocyte aspiration. Luteal phase was supported by vaginal progesterone gel (90 mg/day, Crinone gel 8%; Merck Serono) once daily from the day of oocyte aspiration. Pregnancy diagnosis was made by rising serum b-hCG concentrations and ultrasonographic evidence of a gestational sac. Measurement of b-hCG was performed by radioimmunoassay using an hCG MAIAclone kit (Serono Diagnostics, Woking, U.K.); interassay and intraassay variances were $<10\%$ and $<5\%$, respectively.

3. TTG pretreatment

For three TTG pretreatment subgroups, 12.5 mg TTG (Testo gel 1%; Laboratories Besins International, Paris, France) with a 1.25 mg/d nominal delivery rate of testosterone was applied once daily in the morning, which was administered by the patient onto clean dry healthy skin over both upper arms. This testosterone gel formulation is a hydroalcoholic gel containing 1% testosterone, and approximately 9-14% of the steroid in the gel is available in the body (Wang et al., 2004). The dose of TTG, 12.5 mg/d was determined on the basis of experimental data in

primates (Vendola et al., 1998; Vendola et al., 1999). Once gel applied, patients were instructed to avoid washing the application sites, for 5 hours, but washed their hands thoroughly with soap and water. The duration of TTG pretreatment was varied among the study subgroups, which were 2, 3 and 4 weeks respectively in preceding period of study stimulation cycle. Ovarian stimulation was started next day of last testosterone gel application.

4. Outcome measures

The primary end point was the total number of mature retrieved oocytes. The secondary endpoints were the number of growing follicles, numbers of fertilized oocytes and good-quality embryos. We also evaluated and analyzed total dose and days of rhFSH administered, implantation rate, clinical pregnancy rate per cycle, and live birth rate per cycle. Clinical pregnancy was indicated in case of the presence of a gestational sac by ultrasonography, and live birth was indicated in case of the delivery of a fetus with signs of life after 20 completed weeks of gestational age. Miscarriage rate per clinical pregnancy was defined as the proportion of patients who failed to continue development before 20 weeks of gestation in all clinical pregnancies.

5. Statistical analysis

Results were presented as the mean \pm standard deviation (SD) with 95% confidence interval. All statistical analysis was performed using SPSS statistical package for Windows, version 11.0 (SPSS Inc, Chicago, IL). Analysis of variance (ANOVA) was performed to compare the mean values among four groups. Chi-square test, and Fisher's exact test were performed for the comparisons of fraction, where applicable. $P < 0.05$ was considered to be obtained statistical significance.

RESULTS

As presented in Table 1, patients' characteristics including

Table 1. Patient characteristics

	Control	2 wks treatment	3 wks treatment	4 wks treatment	<i>P</i>
No. of patients	30	30	30	30	
Age of patients (yrs)	37.6 ± 3.4	37.9 ± 2.9	38.3 ± 3.1	38.1 ± 3.0	NS
Age of husbands (yrs)	43.3 ± 4.3	42.9 ± 3.3	43.5 ± 4.2	43.0 ± 3.6	NS
Infertility duration (mon)	51.8 ± 25.8	49.7 ± 23.9	55.2 ± 33.4	53.3 ± 28.5	NS
BMI (kg/m ²)	21.6 ± 2.4	21.4 ± 2.1	21.8 ± 2.3	21.6 ± 2.2	NS
No. of nullipara (%)	60.0 (18/30)	63.3 (19/30)	56.7 (17/30)	66.6 (20/30)	NS
No. of patients with AFC ≤ 5	90.0 (27/30)	90.0 (27/30)	96.7 (29/30)	86.7 (26/30)	NS
AFC	4.7 ± 0.8	4.6 ± 0.7	4.4 ± 0.6	4.8 ± 1.0	NS
Basal FSH (IU/L)	8.7 ± 1.2	8.5 ± 0.8	8.9 ± 1.1	8.8 ± 1.1	NS

Values are means ± SDs. BMI = body mass index; AFC = antral follicle count; FSH = follicle stimulating hormone; NS = not significant

age, duration of infertility, body mass index (BMI), basal FSH, and antral follicle count (AFC) were similar in four groups. Each three TTG pretreatment and control groups consisted of 30 cycles initiated, corresponding to 30 patients. Three cycles (10.0%) of control group and 2 cycles (6.7%) of 2 weeks TTG group were cancelled, because of no follicular development before oocyte aspiration or no oocytes obtained despite a follicular aspiration for oocyte retrieval. Statistical difference was not found in overall cancellation rate among all TTG pretreatment and control groups (Table 2).

Both serum T and free T levels were significantly higher in 2 weeks, 3 weeks and 4 weeks TTG treatment groups compared with control group ($p < 0.05$, $p < 0.01$, $p < 0.01$, respectively in serum T level; $p < 0.05$, $p < 0.01$, $p < 0.01$ respectively in serum free T level). After 2 weeks of TTG treatment, AFC, mean follicular diameter (MFD) and resistance index (RI) value of ovarian stromal artery (OSA) were similar to those in control group. COS results and IVF outcome did not show any significant differences between 2 weeks TTG treatment and control groups (Table 2). However, TTG pretreatment for 3 weeks resulted in

significant increase of AFC and significant decreases of MFD and RI value of OSA ($p = 0.026$, $p < 0.001$, $p < 0.01$, respectively) (Table 2). Total dose and days of rhFSH used were significantly fewer in 3 weeks TTG group than those in control group ($p < 0.001$, $p < 0.01$, respectively). The numbers of oocytes retrieved, mature oocytes and fertilized oocytes were significantly higher than in control group ($p < 0.001$, $p < 0.001$, $p < 0.001$). There were no significant differences in clinical pregnancy rate (CPR) per cycle initiated and live birth rate between 3 weeks TTG treatment and control groups. After 4 weeks of TTG pretreatment, AFC increased, and MFD and RI value of OSA decreased, compared with control group ($p = 0.002$, $p < 0.001$, $p < 0.001$, respectively). Total dose and days of rhFSH administered were significantly fewer than those in control group ($p < 0.001$, $p < 0.001$). The numbers of oocytes retrieved, mature oocytes and fertilized oocytes, CPR per cycle initiated and live birth rate were significantly higher in 4 weeks TTG treatment group than those in control group ($p < 0.001$, $p < 0.001$, $p = 0.030$, $p = 0.042$, respectively) (Table 2). No local or systemic adverse effect according to TTG application was reported.

Table 2. Comparison of controlled ovarian stimulation results and IVF-ET outcome

	Control	2 wks treatment	3 wks treatment	4 wks treatment
No. of cycles initiated	30	30	30	30
No. of cycles retrieved	28	29	30	30
No. of ET cycles	27	28	30	30
No. of cycles cancelled	3 (10.0%)	2 (6.7%)	0	0
On stimulation day 1				
AFC	4.0 ± 1.3	4.1 ± 1.2	4.9 ± 1.1 ^a	5.2 ± 1.0 ^b
MFD	5.9 ± 0.6	5.7 ± 0.5	5.2 ± 0.4 ^c	4.7 ± 0.4 ^c
RI of OSA	0.95 ± 0.03	0.94 ± 0.03	0.92 ± 0.03 ^d	0.89 ± 0.03 ^c
Total dose of rhFSH (IU)	3,025.0±425.9	2,765.7±567.8	2,596.7±335.3 ^c	2,643.5±389.0 ^c
Days of rhFSH administered	10.6±1.5	10.2±1.7	9.6±1.1 ^c	9.8±1.0 ^c
No. of oocytes retrieved	3.9±1.3	4.3±1.6	5.3±2.0 ^c	5.8±1.9 ^c
No. of mature oocytes	3.1±1.1	3.6±1.3	4.5±1.8 ^c	4.9±1.6 ^c
No. of fertilized oocytes	3.1±1.1	3.5±1.3	4.2±1.7 ^c	4.6±1.6 ^c
No. of grade I, II embryos	1.5±0.6	1.6±0.6	2.2±0.6 ^a	2.1±0.7 ^a
No. of embryos transferred	2.9±0.9	2.7±0.7	2.9±0.9	2.9±0.8
CPR per cycle initiated (%)	10.0 (3/30)	16.7 (5/30)	30.0 (9/30)	36.7 (11/30) ^e
Miscarriage rate per clinical pregnancy (%)	33.3 (1/3)	20.0 (1/5)	22.2 (2/9)	18.2 (2/11)
Live birth rate per cycle initiated (%)	6.7 (2/30)	13.4 (4/30)	20.0 (6/30)	30.0 (9/30) ^f
Multiple pregnancy rate (%)	0	0	22.2 (2/9)	18.2 (2/11)

Values are means ± standard deviations. ET = embryo transfer; AFC = antral follicle count; MFD = mean follicular diameter; RI = resistance index; OSA = ovarian stromal artery; rhFSH = recombinant human follicle stimulating hormone; CPR = clinical pregnancy rate

^a $p < 0.050$ vs control group, ^b $p = 0.002$ vs control group, ^c $p < 0.001$ vs control group, ^d $p < 0.010$ vs control group, ^e $p = 0.030$ vs control group, ^f $p = 0.042$ vs control group

DISCUSSION

This pilot study demonstrated the beneficial effect of transdermal testosterone gel treatment on COS and IVF in women with poor ovarian responses during prior failed IVF cycles. Indeed the testosterone effect was occurred at least 3 weeks after application of the gel. The TTG treated patients for 3 to 4 weeks responded to FSH more than control or 2 weeks treated patients with result of more numbers of growing follicles during stimulation and oocytes retrieved. Total dose of consumed gonadotropin

decreased, and there were no local or systemic adverse side effects.

The 25% of circulating testosterone comes from ovary in regularly cycling women, and these levels appeared to decrease with age (Barbieri et al., 2005). Similarly, women with lower ovarian reserve were predicted to have low testosterone levels like aging women, even though it's subclinical. According to testosterone threshold analysis during IVF cycles, higher baseline testosterone level appeared to be associated with successful pregnancy (Frattarelli & Peterson, 2004). Barbieri et al. reported significant positive

correlation between serum testosterone and numbers of oocytes retrieved (Barbieri et al., 2005). In this perspective, basal hormonal secretion should be more carefully assessed for poor ovarian responders and they could be affected by testosterone level more than who are not a poor responders during gonadotropin stimulation.

Poor ovarian response was reported as 9-25% of assisted reproduction (de los Santos et al., 2013). Several strategies have not fulfilled to increase reproductive outcomes for these poor responders, and commonly used approach is to make a high androgen intra-ovarian environment. So, dehydroepiandrosterone (DHEA), transdermal testosterone, aromatase inhibitor or recombinant luteinizing hormone (LH) was commonly administered in clinical practice (de los Santos et al., 2013; Fanchin et al., 2011). The roles of androgens in ovarian folliculogenesis have been proposed, and basically androgens are essential substrates for ovarian steroidogenesis according to 2-cell two- gonadotropin theory (Ryan et al., 1968). Mediated by androgen receptor, testosterone and dihydrotestosterone (DHT) have made preantral and small antral follicular growth significantly in primates' ovaries. Granulosa and theca cell proliferation were significantly increased also in these follicles (Vendola et al., 1998). Weil et al. documented androgen receptor gene expression mostly in granulosa cells was up-regulated and granulosa cell FSH receptor mRNA was increased also by testosterone (Weil et al., 1998). Insulin-like growth factor I (IGF-I) and IGF-I receptor mRNA were markedly increased in primordial follicle oocytes in the testosterone or DHT-treated monkeys (Vendola et al., 1999). They suggested androgens stimulate early, gonadotropin independent stages of follicular growth and enhance responsiveness to gonadotropin (Vendola et al., 1998; Weil et al., 1998).

In the study about human granulosa cells and follicular fluids of small antral follicles, significant correlative mRNA expression was observed between androgen receptor and FSH receptor. Furthermore, follicular fluid testosterone

levels were highly significantly correlated with FSH receptor mRNA expression (Nielsen et al., 2011). There were a few studies about testosterone priming for COS, but the results were controversial and limited. Fabregues et al reported increased ovarian follicular responses to gonadotropin stimulation in previous low responder IVF patients. They used transdermal testosterone patch with 2.5 mg/day nominal delivery rate during 5 days preceding gonadotropin treatment for testosterone priming. However, there was no statistical difference about the numbers of retrieved oocytes and embryos, and the rate of clinical pregnancies (Fabregues et al., 2009). Another prospective, randomized trial performed by Massin et al. failed to demonstrate any significant beneficial effect of testosterone on ovarian response to FSH. In this trial, testosterone transdermal gel was applied for 2 weeks before ovarian stimulation, allowing a daily dosing of 10 mg of testosterone with 10% of absorption approximately. They restricted the duration of application to prevent any side effects (Massin et al., 2006). The timing and duration of testosterone supplementation, and moreover the total amount of supplementation were presented as critical factors for absence of testosterone priming effect.

In this point, our trial has some strengths and favorable results. Most of all, the study was designed to reveal testosterone effect according to the duration of application. The positive effects to ovarian stimulation were observed and moreover IVF outcomes were improved after 3 to 4 weeks of testosterone gel application. CPR were significantly increased up to 36.7% especially after 4 weeks TTG treatment. These results could be highlighted because the study population was poor responder. However, data on the dose-response effects of androgens are still limited. In primates study, the threshold effect of androgens on follicular function has been postulated. When high dose of androgens were used for 5 days before and continuing through out FSH and LH treatment, antagonistic actions to

gonadotropin stimulation were observed (Zeleznik et al., 2004). Furthermore intra-ovarian bioavailability of androgens administered systemically at nonvirilizing dose needs further investigation (Fanchin et al., 2011). The proper duration of androgen pretreatment should be taken into consideration also. Considering androgens' effect to early stage follicles, pretreatment duration could be taken for several weeks before ovarian stimulation (Weil et al., 1998), but TTG should be applied for the shortest duration with positive effect.

Ovarian morphology in circumstances of androgen excess is well known from patients of polycystic ovarian syndrome (PCOS). It is characterized by excessive AFCs, which have been positively correlated with circulating androgen concentrations (Jonard et al., 2003; Pigny et al., 2003). In case of congenital adrenal hyperplasia or some virilizing tumors, which exposed women to androgen for long duration, comparable ovarian morphologies in PCOS were observed (Fanchin et al., 2011). After high doses testosterone taken by noncastrated female to male transsexuals, multifollicular ovarian pattern like PCOS was observed by some investigators (Futterweit & Deligdisch, 1986; Pache et al., 1991). In our study, after 3 weeks of TTG treatment, AFCs were significantly increased and MFD was significantly decreased. Because androgen promotes early stages of follicular growth, MFD had been thought to be decreased. The improvement of intra-ovarian blood flow was demonstrated also, and further investigations are required.

In conclusion, TTG pretreatment for 3 to 4 weeks before ovarian stimulation increases AFC and ovarian stromal blood flow and therefore, potentially can improve the ovarian response to COS and IVF outcome in poor responders undergoing IVF/ICSI.

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