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RESEARCH ARTICLE

# Evaluation of a new transdermal testosterone dosage used for 60 days prior to controlled ovarian stimulation in poor responders: preliminary results

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## Abstract

**Objectives:** Studies suggest a beneficial effect of transdermal testosterone (TT) used prior to controlled ovarian stimulation (COS) on reproductive outcomes in poor responders. Our aim was to evaluate the adverse effects and serum concentrations of testosterone, sex hormone-binding globulin (SHBG), and estradiol, following a new TT dosage associated with combined oral contraceptive (COC) before COS in poor responders. **Methods:** Pilot case-control study in which each patient was her own case-control. Women with poor ovarian response fulfilling Bologna Criteria were recruited and submitted to COS (Control Cycle - CC) to perform ICSI. Those who did not become pregnant were subjected to another COS with previous use of TT (25 mg/48 hours, for 60 days), associated with COC (Testosterone Cycle-TC). Blood samples were taken at the beginning of the first COS (D1); day of first oocyte retrieval (D2), first day of transdermal testosterone use (D3); 60 days after Testo-COC usage (D4); and day of second oocyte retrieval (D5). Adverse effects and serum concentrations of total and free testosterone, estradiol, and SHBG were analyzed. **Results:** Six women concluded both cycles. Testosterone was higher at D4 than D1 (p=0.009, p=0.041) and D3 (p=0.010, p=0.031). SHBG was higher at D4 than D1 (p=0.028). Estradiol was higher at D2 than D1 (p<0.001) and at D5 than D3 (p=0.001) and D4 (p=0.002). Mild adverse effects were observed. **Conclusion:** The TT dosage used before COS caused only mild adverse effects and resulted in significantly higher concentrations of total and free testosterone than in basal conditions.

**Keywords:** Assisted Reproductive Techniques; Ovarian Stimulation; Testosterone; Poor Responder

## INTRODUCTION

Over the past few years, the clinical knowledge and technological development of assisted reproduction techniques (ART) have undergone progressive improvement[1]. However, one of the limitations that still exist in the daily routine of ART clinics is related to poor response following Controlled Ovarian Stimulation (COS), which leads to low pregnancy rates per cycle when compared to those of women with normal responses[1,2]. Such limitation affects a group of women known as 'poor responders', which correspond to 9% to 24% of women undergoing ART[3].

In 2011 a study group from the European Society for Human Reproduction and Embryology (ESHRE) suggested a consensual definition of poor responders with the elaboration of the Bologna Criteria[4]. Hence, according to these criteria, in order to define the poor response to COS *in vitro* fertilization (IVF), at least two of the following three features must be present: (i) advanced maternal age or any other risk factor for poor ovarian response (POR); (ii) a previous POR; and (iii) an abnormal ovarian reserve test (ORT). Two episodes of POR following maximal stimulation are also sufficient to define a patient as a poor responder in the absence of advanced maternal age or an abnormal ovarian reserve test[4].

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One of the most prominent adjunctive therapies to increase the gestational success of poor responders is androgen supplementation. *In vitro* tests have shown that the response to the follicle-stimulating hormone (FSH) by granulosa cells constitutes an androgen-modulated process[5]. A recent study in non-human primates using three-dimensional follicular cultivation of secondary follicles showed that the addition of androgens alters follicular survival and growth, as well as steroid and anti-mullerian hormone (AMH) production, in a dosage-dependent manner[6], suggesting a significant role of androgens throughout folliculogenesis. In non-human primates, androgen treatment increased the expression of FSH receptors in granulosa cells[7,8], promoted the initiation of primordial follicle growth[9], and increased the number of growing preantral and small antral follicles[10].

The effect of transdermal testosterone on reproductive outcomes in women with poor ovarian response to COS has been evaluated in some randomized controlled trials (RCT)[11-17], with encouraging results. Data from the latest meta-analyses suggested a potential beneficial effect of transdermal testosterone, used prior to COS, on live birth rates[18,19].

All in all, some *in vitro* assays have shown that the FSH response by granulosa cells comprises an androgen-dependent process[5], by which androgens can synergistically collaborate with FSH in primordial follicle growth[8], playing an essential role throughout folliculogenesis in a dose and time-dependent manner[6]. However, the efficacy and safety of the intervention prior to COS in poor responders, as well as the most appropriate dose and duration of use, have not yet been well-established. Therefore, starting in mid-2017, we began offering to potentially poor responders, who met the Bologna Criteria, the possibility of using the only transdermal testosterone marketed in Brazil (Androgel®), at a dose of 25 mg every 48 hours, coupled with combined oral contraceptive pills (Miranova® or Level®), for 60 days prior to COS. Thus, the main objective of this observational study was to assess the adverse effects of a new, never before tested dose of transdermal testosterone, for a longer exposure period than that used in previous studies, and evaluate serum testosterone concentrations (total and free), sex hormone-binding globulin (SHBG), free androgen index (FAI), and estradiol. As secondary objectives, reproductive outcomes among the control cycle and testosterone cycle were also compared.

## METHODS

### Study design, settings, and participants

The present prospective pilot case-control study was approved by the National Commission for Ethics in Research, under Certificate of Presentation for Ethical Consideration # 62062216.1.0000.5440. All participants signed an informed consent form for inclusion in the study. Women were recruited at our IVF Unit, located at the Clinical Hospital of the Ribeirão Preto Medical School – University of São Paulo, from July 2017 to September 2019.

The study population comprised women with poor ovarian response, fulfilling Bologna Criteria[4]. The study's exclusion criteria were: perimenopause in amenorrhea or an irregular cycle; basal FSH > 20 IU/L; uterine abnormalities; any current untreated endocrine abnormality; unilateral or bilateral hydrosalpinx; contraindications to gonadotropin use; serious illness requiring regular treatment; androgen use during the past three months, and azoospermia.

### Experimental design

Initially, poor responders were submitted to COS for intracytoplasmic sperm injection (ICSI) using one of the two clinical protocols currently used at the clinic, briefly described below, initiating on the second or third day of a natural cycle (Control Cycle - CC). Patients who did not become pregnant when participating in the CC were offered the possibility of using transdermal testosterone (Androgel® - 25 mg, one sachet every 48 hours) associated with COC (Level®) for 60 days, prior to the next COS cycle for ICSI. Those who agreed to use this intervention and underwent the new COS cycle constituted the Testosterone Cycle (TC) group. Thus, each patient was their own case-control. In the TC, COS started four and two days after discontinuation of COC and transdermal testosterone application, respectively, using the same COS protocol used in the CC (Figure 1). These patients were instructed to apply transdermal testosterone on their thighs and lower abdomen[20].

### Protocols of controlled ovarian stimulation

The patients underwent COS using one of the two clinical protocols currently used at the clinic:

- *High-dose protocol*: Daily use of high dose (300 IU) – human menopausal gonadotropin (HMG - Menopur®, Ferring, SWZ) started on the second or third day of a natural cycle (CC) or four days after discontinuation of COC use and two days after discontinuance of transdermal testosterone application (TC), and maintained until a follicle reached at least 17 mm;

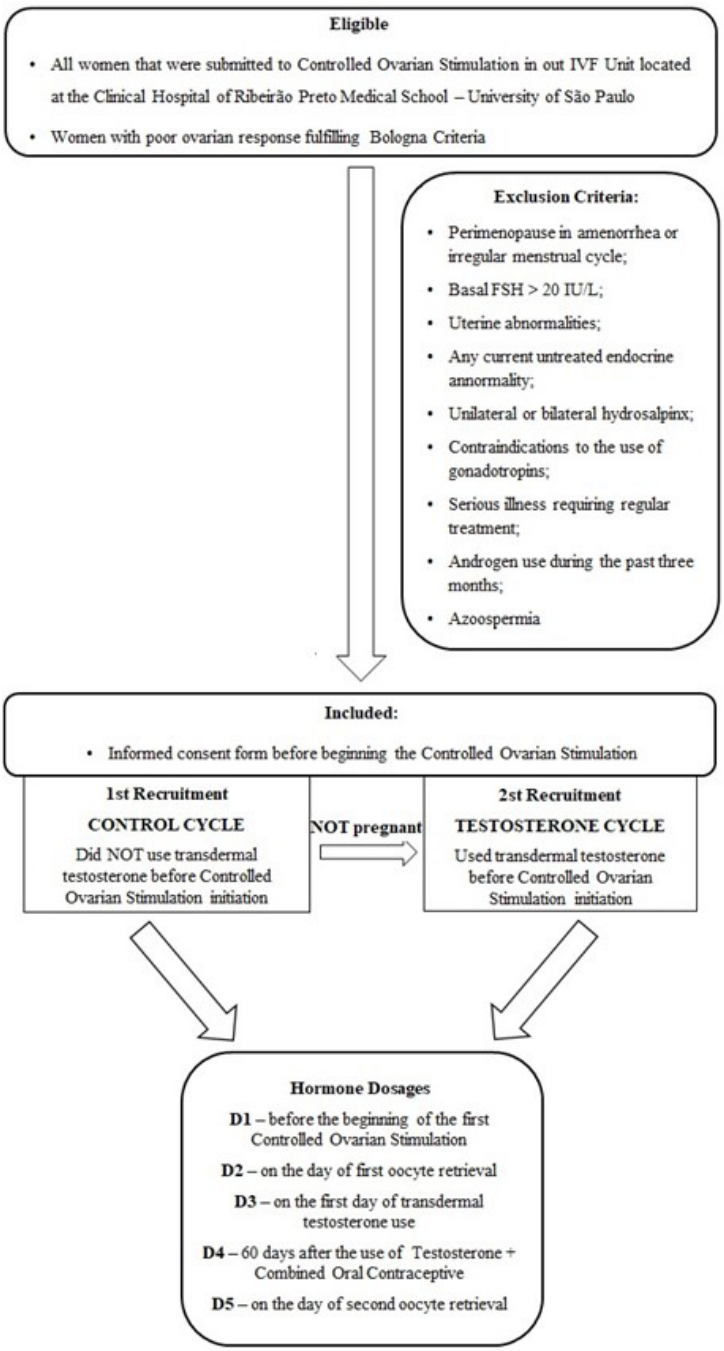


**Figure 1.** Experimental design.
**Note:** IVF: *in vitro* fertilization; FSH: follicle-stimulating hormone

- *Minimal stimulation protocol*: Daily use of clomiphene citrate (100 mg) for five days, started on the second or third day of a natural cycle (CC) or four days after discontinuation of COC use and two days after discontinuation of transdermal testosterone application (TC). Use of 150 IU of human menopausal gonadotropin (HMG - Menopur®, Ferring, SWZ) on alternate days (on the second and fourth day of COS) and daily from the sixth day of COS until a follicle reached at least 17 mm.

The choice of the COS protocol depended on the financial conditions of the couples (medication costs were covered by each couple), as well as the number of antral follicles on the day that treatment started, previous responses to COS (for patients undergoing previous treatments), and the clinical experience of the physician who assisted each patient.

All women were monitored by transvaginal ultrasonography (UStv) every two days, beginning on the 6th day of COS, to assess follicular growth. For pituitary suppression, an antagonist of the gonadotropin-releasing hormone (GnRH) – Orgalutran® (Schering-Plow, USA) or Cetrotide® (Merck, USA) – was used, at a dose of 0.25 mg/day, starting when at least one follicle reached a mean diameter of 14 mm, up to the day of administration of recombinant chorionic gonadotropin (r-hCG) (Ovidrel®, Serono, ITA).

Cycle cancellation was carried out when no follicles with a mean diameter of ≥ 10 mm were seen after 10 days of COS, or when no follicles reached 17 mm in diameter. Oocyte retrieval was performed 35 to 37 hours after the administration of human chorionic gonadotropin (hCG) for final follicular maturation, and the follicles were aspirated transvaginally into a pool. The ICSI procedure was performed in mature oocytes, characterized by the presence of the first polar body. Fertilization was evaluated approximately 16-18 hours after ICSI and it was verified by the presence of two pronuclei and two polar bodies[21].

Embryo transfer was performed two to three days after oocyte retrieval[22], with a maximum of two embryos per woman being transferred[23]. If there were more than two embryos on the day of transfer, those with 4 cells on the second day or 8 cells on the third day after fertilization, and with less fragmentation, would be selected for transfer[21]. The surplus embryos were cryopreserved for possible future transfer.

## Outcomes, variables, and data sources

- *Primary outcomes*: adverse effects related to the use of testosterone plus COC and serum concentrations of testosterone (total and free), SHBG, and estradiol;
- *Secondary outcomes*: duration of COS, total gonadotropins used, number of oocytes retrieved, mature oocytes, fertilized oocytes, formed embryos, and transferred embryos in the Control and Testosterone Cycles;
- *Variables of clinical characterization of study population*: age, body mass index (BMI), cause of infertility, duration of infertility, and the number of previous COS cycles.

All data were obtained from medical records.

## Blood sample collection and processing

Blood collection and hormonal measurements were performed prior to the beginning of the first COS (Dosage 1 - D1), on the day of first oocyte retrieval (Dosage 2 - D2), on the day of the initiation of transdermal testosterone + COC use (Dosage 3 - D3), 60 days after use of transdermal testosterone + COC (Dosage 4 - D4), and on the day of second oocyte retrieval (Dosage 5 - D5).

Approximately 10 mL of venous blood was collected in a vacuum tube containing clot activator. Blood samples were centrifuged at 3000 g for 10 minutes and serum was obtained and stored in a cryotube at -80ºC until dosing.

## Serum hormone measurements

All serum hormonal measurements were performed at Fleury Laboratory of São Paulo. Estradiol was assessed by competitive electrochemiluminescence using Cobas® 6000 E601 (Roche Diagnostics) equipment, with a coefficient of variation (CV) of 5.0% and sensitivity of 0.5 ng/mL. The SHBG dosage was carried out by an electrochemiluminometric immunoassay method using the same estradiol-dosing equipment, with a CV of 5.0% and sensitivity of 0.8 nmol/L. Total testosterone was assayed by high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using Thermo Scientific TSQ Altis LC/MS-MS equipment, which is considered the gold standard for testosterone measurement in women and children. The sensitivity of the testosterone assay was 9.0 ng/dL, with an intra-assay error of 2.2 to 3.7% and an inter-assay error of 2.0 to 4.2%, both depending on the dosed concentration. In order to determine the total testosterone concentration in nmol/L, the value obtained in ng/dL was multiplied by the conversion factor 0.0347. To obtain the estradiol concentration in pg/mL, the value obtained in ng/dL was multiplied by 10. The Free Androgen Index (FAI) was calculated by the following formula: FAI = TT (nmol/L)/SHBG (nmol/L) x 100 (where TT refers to total testosterone in serum, and SHBG, to sex-hormone binding globulin)[24]. In order to calculate the free testosterone, the total testosterone values were multiplied by the respective FAI, divided by 100.

### Adverse effect evaluation

Adverse effects associated with transdermal testosterone were evaluated in all patients included in the study by the same researcher. They were evaluated at D4, which means 60 days after the use of transdermal testosterone associated with COC, and were self-reported by each of the patients.

### Bias

In an attempt to avoid potential sources of bias, all patients deemed eligible during the study period were invited to participate in the survey. All samples collected were processed and assayed by a technician without access to the patients' clinical information. The statistician who analyzed the data also did not have access to clinical information and to which group the samples belonged.

### Sample size

The sample size was represented by the total number of eligible patients who agreed to participate in the study, performed both cycles (CC and TC), and had five blood samples collected from July 2017 to September 2019.

### Statistical analysis

The variables (age, BMI, infertility duration, and the number of previous cycles) were represented by the median and interquartile range and presented descriptively. Exploratory data analysis was conducted by measuring the central position (mean and median) and dispersion (standard deviation, interquartile range, minimum and maximum values). A paired t-test was performed to compare the following variables: duration of COS, total gonadotropins used, numbers of oocytes retrieved, mature oocytes, fertilized oocytes, formed embryos, and transferred embryos between the Control and Testosterone Cycles.

The comparison between collection periods and cycles for each variable was carried out considering orthogonal contrasts using the mixed linear regression model, *i.e.*, with two sources of variability, between individuals within the same dosing period (Control Cycle and Testosterone Cycle), and within the same individuals at different dosing periods (D1, D2, D3, D4, and D5). Statistical comparisons were made between D1 and D2, D1 and D3, D1 and D4, D2 and D5, D3 and D4, D3 and D5, and D4 and D5. All statistical analyses were performed in the SAS program, version 9.4 (SAS Institute Inc., USA), with a significance level of 5%.

## RESULTS

### Study participants

Medical records of 416 patients were analyzed, of whom 363 were not eligible since they did not fulfill the Bologna Criteria for poor ovarian response (Figure 1). Of the 53 poor responders or expected poor responders, only 21 were eligible because 32 of them had some exclusion factor: 11 were excluded for having hydrosalpinx, 4 for not having the financial means to afford new treatment, 3 for not being interested in participating in the study, 3 due to the use of androgens in the past three months, 3 due to azoospermia, 2 due to untreated endocrine abnormalities, 2 due to irregular menstrual cycle, 1 due to uterine abnormality, 1 due to serious illness requiring regular treatment, 1 due to COC use prior to COS, and 1 due to pregnancy before study recruitment. Therefore, 21 eligible women were interviewed and after signing an informed consent form, were part of the Control Cycle. Of the 21 patients who participated in the Control Cycle, 12 did not complete the Testosterone Cycle: 8 for not having financial conditions for new treatment, 2 who gave up the ART, 1 who became pregnant while participating in the Control Cycle, and 1 who became pregnant after the ICSI was converted into Intrauterine Insemination (IUI). Among the 9 patients who participated in the Control Cycle, as well as in the Testosterone Cycle, 3 were not submitted to oocyte retrieval in any of the studied cycles (1 for having no follicular response in both the Control and Testosterone Cycles, 1 for having no follicular response in the Testosterone Cycle, and 1 for having given up the Testosterone Cycle after using transdermal testosterone, however before the beginning of the COS). Thus, only six women concluded both cycles and had blood samples collected at the five times provided for this study (Figure 2).

### Characterization of the study population

The six patients who completed the Control and Testosterone Cycles had the clinical variables age, BMI, infertility time, and the number of previous cycles descriptively analyzed using the median and interquartile range (Table 1). Two patients used the high dose COS protocol, while four used the minimal stimulation COS protocol.

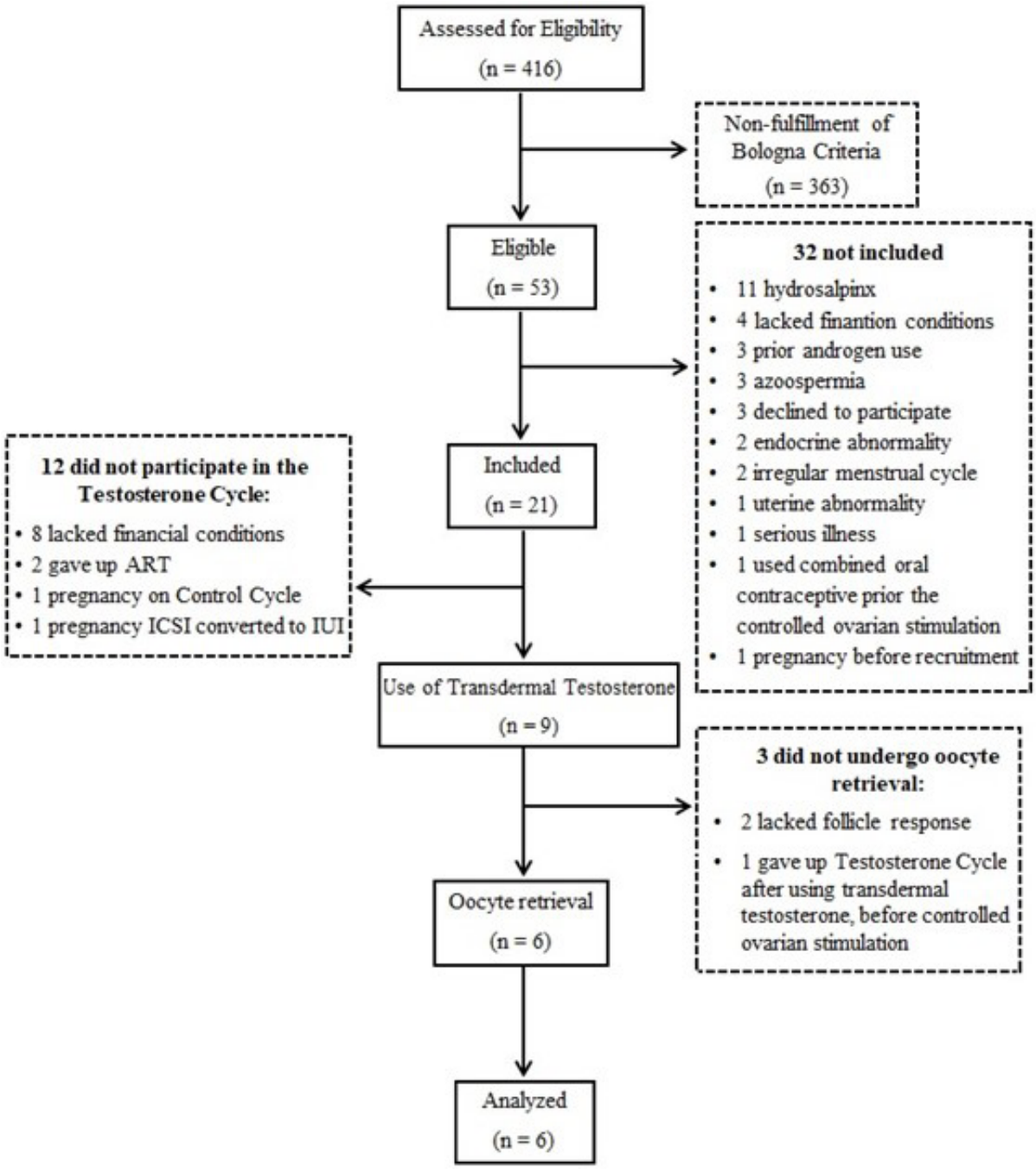


**Figure 2.** Flowchart of the study.
**Note:** ICSI: Intracytoplasmic Sperm Injection, IUI: Intrauterine Insemination, ART: Assisted Reproduction Techniques.

**Table 1.** Descriptive data of patients in the Control and Testosterone Cycles

| Variable | Control Cycle Median (IQR) | Testosterone Cycle Median (IQR) |
|---|---|---|
| Age (years) | 37.5 (36 - 39) | 37.5 (37 - 39) |
| BMI (kg/m$^2$) | 21.96(20.93 – 25.39) | 21.96(20.93 – 25.39) |
| Duration of infertility (months) | 90(60 – 104) | 95(64 – 108) |
| # of previous cycles | 2 (2 – 2) | 3 (3 – 3) |

**Note:** IQR (interquartile range 25 - 75); BMI (body mass index)

## Primary results

### Adverse effects reported during use of transdermal testosterone gel

The following adverse effects were reported when using transdermal testosterone gel: irregular vaginal bleeding (6/6 patients; 100%), breast tenderness (2/6 patients; 33.3%), and oily skin and acne (1/6 patients; 16.6%). However, the adverse effects were mild and did not interfere with regular medication use. None of these effects were reported after the CC.

### Analysis of hormonal measurements conducted at different moments in the study

The serum concentrations of SHBG, total testosterone, free testosterone, and estradiol, performed before the start of the first COS (D1), on the day of oocyte retrieval of the first COS (D2), on the start day of transdermal testosterone use (D3), 60 days after transdermal testosterone + COC use (D4), and on the day of second oocyte retrieval (D5), were analyzed (Table 2).

**Table 2.** Serum concentrations of SHBG, total testosterone, free testosterone, free androgen index (FAI), and estradiol at different periods of treatment

| Hormonal Dosage | D1 Median (IQR) | D2 Median (IQR) | D3 Median (IQR) | D4 Median (IQR) | D5 Median (IQR) |
|---|---|---|---|---|---|
| SHBG (nmol/L) | 116 (81 – 127) [a] | 148 (96 – 168) | 114 (100 – 162) | 140,5 (121 – 179) [a] | 120.5 (105 – 131) |
| Total Testosterone (ng/dL) | 19 (13 – 24) [a] | 34 (32 – 52) | 20 (15 – 23) [b] | 36 (28 – 99) [a,b] | 31.5 (21 – 44) |
| Free Testosterone (ng/dL) | 0.11 (0.06 – 0.18) [a] | 0.36 (0.25 – 0.66) | 0.12 (0.06 – 0.18) [b] | 0.38 (0.19 – 1.65) [a,b] | 0.35 (0.15 – 0.55) |
| Free Androgen Index (%) | 0,62 (0.51 – 0.77) [a] | 1,07 (0.8 – 1.28) | 0.62 (0.42 – 0.8) [b] | 1.07 (0.68 – 1.67) [a,b] | 1.13 (0.73 – 1.26) |
| Estradiol (pg/mL) | 49 (35 – 59) [a] | 345 (202 – 351) [a] | 52 (50 – 73) [b] | 12.5 (8 – 23) [c] | 209 (91 – 254) [b,c] |

**Note:** % (percentage); IQR (interquartile range); D1 (before the start of the first COS); D2 (on the day of oocyte retrieval of the first COS); D3 (on the start day of transdermal testosterone; D4 (60 days after use of transdermal testosterone + COC); D5 (on the day of second oocyte retrieval). Statistical analysis was performed between D1 and D2, D1 and D3, D1 and D4, D2 and D5, D3 and D4, D3 and D5, and D4 and D5. Equal letters in the same line indicate statistically significant difference ($p<0.05$).

When comparing the serum concentrations of SHBG, we observed significantly higher levels at D4 than D1 (p=0.028). Regarding total and free testosterone serum concentrations, we noted significantly higher concentrations at D4 than D1 (p=0.009 and p=0.041, respectively) and D3 (p=0.010 and p=0.031, respectively). The free androgen index (FAI) was also significantly higher at D4 than D1 (p=0.041) and D3 (p=0.031). Additionally, when analyzing estradiol serum concentrations, we found significantly higher serum concentrations at D2 than D1 ($p<0.001$) and at D5 than D3 (p=0.001) and D4 (p=0.002).

### Other analyses – Response to COS and ICSI results of the Control and Testosterone cycles

The results of the COS and ICSI responses were compared between the Control and Testosterone Cycles. No significant differences were observed regarding the following parameters: total gonadotropins used (p=0.1063), the duration of COS (p=0.099), the number of retrieved oocytes (p=0.4186), the number of mature oocytes (p=0.4186), the number of fertilized oocytes (p=0.1852), and the number of embryos formed (p=0.1747) (Table 3).

**Table 3.** COS response and ICSI results of poor responding patients in the Control and Testosterone Cycles

| Variable | Control Cycle Median (IQR) | Testosterone Cycle Median (IQR) | p-value |
|---|---|---|---|
| Total gonadotropins (IU) | 750 (450 –1800) | 1275 (975 - 2250) | 0.1063 |
| Duration of COS (days) | 6.5 (6 – 7) | 10 (8 – 12) | 0.099 |
| # retrieved oocytes | 0 (0 – 1) | 1.5 (0 – 3) | 0.4186 |
| # mature oocytes | 0 (0 – 1) | 1.5 (0 – 3) | 0.4186 |
| # fertilized oocytes | 0 (0 - 0) | 0.5 (0 – 2) | 0.1852 |
| # formed embryos | 0 (0 - 0) | 0.5 (0 – 2) | 0.1747 |
| # transferred embryos | 0 (0 - 0) | 0 (0 – 2) | - |

**Note:** IQR (Interquartile range 25 - 75); COS (controlled ovarian stimulation); p-value ($p<0.05$).

## DISCUSSION

Data from the latest meta-analyses suggested a potential beneficial effect of transdermal testosterone, used prior to COS, on live birth rates following ART in poor responders[18,19]. However, the efficacy and safety of transdermal testosterone prior to COS in poor responders, as well as the most appropriate dose and duration of treatment, have not yet been established. In the present study, the choice for Androgel® was due to the fact that it is the only transdermal testosterone presentation marketed in Brazil that is approved by the National Health Surveillance Agency (ANVISA), but for use by hypogonadotropic men. Androgel® was available in two dosages (25 and 50 mg). We chose to use the lowest dosage since very high serum testosterone concentrations could promote harmful effects on folliculogenesis, according to studies in rodents[25-27]. Also, in order to not promote the excessive elevation of serum testosterone concentrations, we chose to use 25 mg every 48 hours (and not daily), associated with COC containing progestin levonorgestrel (Level®), since COCs cause the elevation of SHBG, with a consequent reduction in free concentrations of sex steroids[28]. The choice for the period of use (60 days prior to COS), which is longer than that described in surveys that investigated the effectiveness of transdermal testosterone in poor responders[11-17,29-31], was due to the fact that studies in non-human primates show a potential beneficial role of testosterone since the beginning of folliculogenesis[6].

We found that the use of transdermal testosterone at a dose of 25 mg, given as one sachet every 48 hours, caused mild adverse effects, mainly represented by irregular vaginal bleeding, breast tenderness, and oily skin and acne. This dose of transdermal testosterone given in the present study was higher than those used in other studies in potentially poor responders[11-16,29,30] and was administered for a longer period of time as compared to previously described in the literature (maximum time of 4 weeks, in the study by Kim et al.)[15].

Similar results have been described in other studies, one of which used transdermal testosterone adhesives at a dose of 150 µg/day and 300 µg/day in a 52-week study of postmenopausal women with low sexual desire[32]. The authors reported irregular vaginal bleeding, oily skin and acne as some of the adverse effects described by the patients. Another similar study used transdermal testosterone adhesives at a dose of 300 µg/day for six months in naturally menopausal women with low libido and reported adverse effects including irregular vaginal bleeding, oily skin and acne, and breast tenderness[33].

Our findings were different from those evidenced by Kim et al.[15], who used 12.5 mg of transdermal testosterone gel daily in potentially poor responders for 2, 3, or 4 weeks prior to COS, with no adverse effects being reported by the users. Therefore, it is possible that the use of a higher dose of transdermal testosterone in the present study, for a longer time, may be responsible for the adverse effects observed. In any event, none of the patients herein discontinued treatment, indicating acceptable tolerability regarding the dose and time of administration of the testosterone gel.

In baseline conditions of both cycles (Control Cycle - D1 and Testosterone Cycle - D3), we did not observe any significant differences of serum concentrations of SHBG, total testosterone, free testosterone, estradiol, and free androgen index, indicating a lack of effect of the first COS on these variables at D3. Also, it is noteworthy that the serum concentrations of SHBG, as well as the other analyzed hormones, are within the normal range for women aged 20 to 49 years[34].

When analyzing the Control Cycle of potentially poor responders, we observed significantly higher serum concentrations of estradiol after COS (D2) as compared to before the procedure (D1). Controlled ovarian stimulation using gonadotropins aims at multiple follicular growths[35], stimulating aromatase expression in granulosa cells, with consequent increase in estradiol levels[36]. Since poor responders have few follicles that can be recruited through COS, a mild increase in estradiol concentration between the start day of COS and the day of oocyte retrieval, observed in the present study, is consistent with the low ovarian reserve and small number of oocytes retrieved from these patients[37]. Serum concentrations of total and free testosterone and SHBG increased, although not significantly, after COS, a fact that may be due to the small sample size evaluated herein, which is underpowered for these comparisons. Some studies showed that serum testosterone concentration has a positive correlation with the estradiol peak after COS and also after hCG administration[38,39]. On the other hand, the concentration of SHBG also tends to increase as a result of the increase in estradiol secondary to COS, which binds to steroid hormones, such as estradiol and testosterone, leaving only a fraction of them bioavailable[40]. In the present study, we observed significantly higher total and free testosterone concentrations following the use of transdermal testosterone associated with COC (D4) when compared to their baseline levels before the first COS (D1) and prior to their administration in the Testosterone Cycle (D3). A higher dose of transdermal testosterone for a longer time was used in order to promote a slightly significant increase in serum concentrations of biologically active hormones (not bound to SHBG), which could cause deleterious effects on follicular recruitment, as reported in rodents[25-27]. Therefore, we chose to associate testosterone with the COC, which, by promoting the elevation of SHBG[41], would control the increase in free androgen levels. As expected, we observed higher SHBG serum concentrations following the use of transdermal testosterone associated with COC than their baseline concentrations (D1).

An interesting finding was a non-significant reduction in estradiol serum concentrations following the administration of transdermal testosterone associated with COC, which may be due to increased SHBG serum concentrations. The strategy

was effective in promoting elevated serum concentrations of free testosterone, which were significantly higher in D4 than D1 and D3. Our findings corroborate those of Massin et al.[11], Sipe et al.[13], and Bosdou et al.[30], who used transdermal testosterone in potentially poor responders, at the following doses and durations of treatment: 10 mg/day for 15 days, 2.5 mg/day for 12 days, and 10 mg/day for 21 days.

In the Testosterone Cycle, similarly to the Control Cycle, higher estradiol serum concentrations were found after COS (D5) than before and after the use of testosterone associated with COC (D3 and D4, respectively). This finding was expected since the use of exogenous gonadotropins prevents negative feedback by increasing endogenous estradiol concentrations[42] produced by growing follicles. These results corroborate those of Guo et al.[43] when comparing estradiol dosages before and after the COS of normoresponding patients and poor responders, according to the Bologna Criteria. The authors found that both groups, after gonadotropin use, had significantly higher estradiol concentrations when compared to the baseline concentrations before COS.

The interpretation of secondary outcomes in the control and testosterone cycles was quite limited, mainly due to the small sample size and the fact that the patients used two different COS protocols throughout the study (four used the minimal stimulation protocol with clomiphene citrate and a low dose of menotropin, and two used the high-dose protocol with menotropin), which hampered the separate analysis of the users of either protocol. No statistical difference regarding any of the secondary outcomes analyzed (total dose of gonadotropins, duration of COS, number of retrieved oocytes, mature oocytes, and fertilized oocytes, and the number of formed and transferred embryos) was observed in the Control and Testosterone cycles, corroborating the results found by Massin et al.[11], Sipe et al.[13], and Bosdou et al.[30]. Nevertheless, our findings suggest the need for a higher total dose of gonadotropins and longer duration of COS after transdermal testosterone administration at a dose of 25 mg every 48 hours for 60 days than in the Control Cycle. Although our findings suggest the possible need for a higher total dose of gonadotropins and longer duration of COS by patients in the Testosterone Cycle, they did not present a statistical difference. These findings differ from some studies, which have shown that transdermal testosterone use requires a lower total dose of gonadotropins and a shorter duration of COS[12,14,15,29].

We also observed a non-statistical increase in the number of retrieved oocytes, mature oocytes, and fertilized oocytes, as well as the number of embryos formed after transdermal testosterone administration. These data corroborate those of Kim et al.[14,15], Doan et al.[16], Saharkhiz et al.[17], and Bercaire et al.[31], who reported a significant increase in these variables when compared to poor responders who did not use transdermal testosterone. Even if such an increase was not statistically significant, an increment in retrieved oocyte number from potentially poor responders is already associated with an improvement in the patient's chance of pregnancy[44].

In the present study, the Cohen effect size measurement[45] for the number of oocytes retrieved was 0.6. Considering this measure of effect size and assuming a test power of 80%, we would need to analyze 24 patients in order to evidence a statistical difference in the use of testosterone regarding the number of oocytes retrieved. Therefore, although in the present study no significant beneficial effect of transdermal testosterone gel, used at the new dosage within 60 days prior to COS, in potentially poor responders, was observed on ART results, underpowered (0.56) considering the small sample analyzed, this does not allow us to state that there is no difference. Thus, studies with larger casuistry are necessary to better evaluate these outcomes.

As previously mentioned, an important limitation of this study was the small sample size analyzed, justified by the restrictive eligibility criteria and the financial limitations presented by patients for purchasing gonadotropins. Although we included only patients referred by the Unified Health System (SUS), in our clinic, patients pay for the medications used for COS. Therefore, the COS protocols also depend on the patient's available resources, a fact that justified the use of two different COS protocols in the present study. Another significant limitation of our study was the observational design (case-control study), with randomized controlled trials (RCT) being the most appropriate design for testing new therapeutic interventions. One way to try to minimize the limitation regarding the observational design was to consider each patient as their own control. Nonetheless, sometimes the patient performed the Control Cycle but was unable to afford the Testosterone Cycle, thus further reducing the sample.

## CONCLUSION

In conclusion, our findings suggest that the use of transdermal testosterone gel at a dose of 25 mg every 48 hours for 60 days, associated with COC before COS, in potentially poor responders, was well-tolerated, promoting mild adverse effects, mainly represented by irregular vaginal bleeding, reported by all patients included in the study. Although the administration of testosterone gel for 60 days was associated with the use of COC, promoting increased SHBG, we observed significantly higher serum concentrations of total and free testosterone after its administration (D4) than those noted before the first (D1) and second (D3) COS. The free androgen index was also significantly higher in D4 than D1 and D3. As expected, serum concentrations of estradiol were significantly higher after COS, both without and after

transdermal testosterone use. In our study, it was not possible to evidence the benefit from the administration of 25 mg of testosterone gel on alternate days for 60 days, associated with the use of COC, prior to COS, in potentially poor responders to ART. However, the sample and the power of testing were insufficient to substantiate these findings. Further well-designed randomized controlled trials are required to support our findings.

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**Authors contribution**

Conception and design of study: VSIG, PAN; enrolled the subjects: PLMC; analysis of data and writing of the manuscript: PLMC; critical revision of the article for intellectual content: PAN, PLMC, RAF. All authors read and approved the final manuscript.