The follicular hormonal profile in low-responder patients undergoing unstimulated cycles: is it hypoandrogenic?

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STUDY QUESTION: What is the final hormonal milieu of pre-ovulatory follicles of low-responder (LR) patients undergoing unstimulated cycles?

SUMMARY ANSWER: Neither androgen secretion nor LH was impaired in pre-ovulatory follicles of LR women.

WHAT IS KNOWN ALREADY: Therapies currently used to improve ovarian response in LR women have an impact on the final hormonal follicular milieu, and these changes are believed to be partially responsible for determining the success rate in these women. Surprisingly, as far as we know, there is no report of the final hormonal profile of LR women undergoing unstimulated cycles or evidence that follicular androgen secretion in LR women is impaired.

STUDY DESIGN, SIZE AND DURATION: A prospective case–control study including 94 women, 36 normal controls and 58 LR patients (19 Young ≤35 years LR and 39 Aged >35 years LR) from 2009 to 2011.

PARTICIPANTS/MATERIALS, SETTING AND METHODS: Fifty-eight LR women were divided into two groups: Young LR (age ≤35; n = 19) and Aged LR (ALR; age >35; n = 39). The control group (group C) comprised 36 egg donors undergoing an unstimulated cycle in our IVF unit. Serum and follicular fluid hormonal concentrations for estradiol (E2), progesterone, testosterone and androstendione were measured. The spindle parameters of metaphase II oocytes generated from these groups were also analysed.

MAIN RESULTS AND THE ROLE OF CHANCE: Pre-ovulatory follicles from LR patients had similar androgenic and LH concentrations to those observed in the control group. However, higher intrafollicular concentrations of FSH and progesterone were observed in ALR. Moreover, no differences were found for the spindle evaluation of oocytes between groups by the Oosight technology.

LIMITATIONS, REASONS FOR CAUTION: The controls were younger and had a lower BMI than the LR women. The sample size available restricted statistical power.

WIDER IMPLICATIONS OF THE FINDINGS: This study suggests that the problem with LR women is not the final pre-ovulatory follicular androgen concentration since this is similar to normal responders, but in the ability to respond to controlled ovarian stimulation protocols. Therefore, efforts should be focused on long-interval androgen priming to potentially increase the recruitment of small antral follicles rather than increasing the intravarian androgen levels within the current cycle.

STUDY FUNDING/COMPETING INTEREST: The present project has been supported by the R+D programme from the Generalitat Valenciana (Regional Valencian Government) IMPIVA MIDTF/2010/95. The authors have no conflict of interest to declare.

Key words: androgen / low responder / unstimulated cycle / pre-ovulatory follicle / hormonal profile
Introduction

Low-responder (LR) patients to controlled ovarian stimulation (COS) represent 9–25% of the women included in assisted reproduction technology programmes (Keay et al., 1997). The efforts made to increase ovarian response using different approaches have been generally unsuccessful, but some strategies have been developed with the ultimate goal of increasing serum androgens in order to deliver high androgens levels to growing follicles (Kyrou et al., 2009).

These strategies are based on the important role that androgens play during ovarian physiology. They have a trophic function during follicular growth at the pre-antral and antral stages (Hillier and Tetsuka, 1997), and are the natural precursors for estradiol (E2) synthesis after FSH binding to its receptor to promote granulose cells proliferation (Hillier et al., 1994; Vendola et al., 1999a,b).

Treatment of primates with both testosterone and dehydrotestosterone in the luteal phase of a menstrual cycle was able to stimulate androgens levels to growing follicles (Kyrou et al., 2009).

Several strategies whose common goal was to increase androgen levels such as the use of testosterone (Balasch et al., 2005; Gleicher et al., 2005) and IGF-1 and the IGF-1 receptor (Weil et al., 1999). A similar scenario may occur in humans since an intimate association has been reported in small antral follicles between the follicular fluid levels of androgen and the mRNA expressions of both androgen and FSH receptors in immature granulosa cells (Nielsen et al., 2011). Several strategies whose common goal was to increase androgen levels such as the use of testosterone (Balasch et al., 2006; Massin et al., 2006; Fabregues et al., 2009), dehydroepiandrosterone (Casson and Carson, 1996; Casson et al., 2000; Barad and Gleicher, 2005; Gleicher et al., 2011), LH to stimulate androgen production within the follicle or aromatase inhibitors to increase androgen substrate (Rubio et al., 2000; Garcia-Velasco et al., 2005; Sonmez et al., 2010) have reported different outcomes in terms of embryo quality and pregnancy success. Surprisingly, as far as we know there is no report of the final hormonal profile of LR women undergoing unstimulated cycles or evidence to report that follicular androgen secretion in LR women is impaired.

Since the final hormonal microenvironment may eventually affect oocyte quality, we also tested the oocyte spindle parameters. Indeed, spindle assembly checkpoint malfunctions are among the possible causes affecting oocyte quality, especially in advanced maternal age (Hasold et al., 2007) which, in many cases, is associated with low ovarian response.

To this end, we designed a prospective study to analyse the final intrafollicular hormonal milieu, focusing on androgen concentration and oocyte quality by means of a non-invasive polarized microscopy technique in normal and LR women to clarify this issue.

Materials and Methods

Subjects and protocol

The study was approved by the CEIC IVI Valencia Ethical Committee (IBS), and informed written consent was obtained from each participant. The study group comprised a total of 58 LR patients who were divided into two groups: Young LR (YLR), comprising women aged 35 years or less, (n = 19) and Aged LR (ALR), comprising women aged over 35 years (n = 39). All of the patients met the ESHRE consensus criteria for LR (Ferrareti et al., 2011) retrospectively. LR women with severe or grade IV endometriosis were excluded from the present study. The average number of oocytes and previous cycles for both the YLR and ALR groups were as follows: 1.7 ± 1.6 oocytes and 7.1 ± 2.5 cycles and 1.3 ± 1.9 oocytes and 6.5 ± 2.0 cycles, respectively.

All participants underwent unstimulated cycles instead of COS as a strategy to obtain oocytes to be vitrified and stored for further accumulation before ICSI (Cobo et al., 2012).

Additionally, 36 egg donors undergoing unstimulated cycles who participated in clinical trial registration number NCT00707525 were used as the control group (C group).

Cycle monitoring started on cycle days 6–11. When follicular size ranged between 16 and 18 mm, all patients received a single dose of 250 µg of Ovitrelle® (Merck-Serono) that was equivalent to ~6500 IU of hCG. To avoid a spontaneous LH surge, GnRH antagonist (Orgalutran, Merck Sharpe Dome) was administered the day before and on the day of hCG.

Sample protocol

Oocyte retrieval was scheduled 36 h after hCG administration. Usually, only one pre-ovulatory follicle was present and was aspirated. Occasionally, two pre-ovulatory follicles were present, in which case only the aspirate from the dominant one was included in this study.

The volume of the follicular fluids was measured and they were immediately centrifuged (1500g, 10 min) to separate out cellular contents and debris; supernatants were aliquoted and stored at ~80 °C for subsequent analyses. Only those follicles containing metaphase II (MII) oocytes were included in the study. All the oocytes associated with the aspirated follicles were kept in culture individually. Mature MII oocytes were vitrified following the Cryotop method, as previously described by Cobo et al. (2008), in order to accumulate several oocytes before an ICSI cycle.

Serum and follicular fluid hormonal determination

Serum and follicular fluid E2 and progesterone were analysed using a commercially available microparticle enzyme immunoassay kit (Abbott Laboratories, Abbott Park, IL, USA). The inter- and intra-assay coefficients of variation for E2 at a concentration of 40 pg/ml were 8.8 and 4.6%, respectively. The serum progesterone kit had a sensitivity of 0.2 ng/ml, with inter- and intra-assay coefficients of variation of 7.9 and 4.5%, respectively. Serum and follicular fluid testosterone and androstenedione were evaluated by radioimmunoassay (Biosource, Nivelles, Belgium). The inter- and intra-assay coefficients of variation for testosterone were 8.4 and 4.4%, respectively, and were 6 and 3.3% for androstenedione. Intrafollicular FSH and LH concentration were also measured by an enzyme immune analysis. The inter- and intra-assay coefficients of variation of both FSH and LH were 6.9 and 4.1% and 5.9 and 3.8% for FSH and LH, respectively.

Visualization of spindle using Oosight

Two hours after egg retrieval the cumulus cells were removed from the oocytes, which were then placed in 5 µl drops of Quinn’s Washing Media (SAGE, Biocare, Rome, Italy) to be overlaid with 4 ml of mineral oil in a glass-bottomed culture dish (Willico Wells, The Netherlands).

Before taking images, oocytes were immobilized with a holding pipette to optimize spindle visualization. The liquid crystal (LC) Oosight controller (CRI USA) and CCD camera were adapted to an Olympus IX71 inverted
microscopic. The location, length, width and mean retardance for the meiotic spindles were analysed by means of an LC computerized image analysis system. Spindles were considered to be of a normal structure if they were complete, barrel-shaped and showed strong retardance. Only true MII oocytes were included in the evaluation of spindle structure. All the measurements were taken at 37°C, which was maintained using a Tokai Hit warming stage placed on the microscope (Tokyo, Japan).

**Statistical analysis**

Data were expressed as mean ± SEM. A Student’s t-test was used to compare the means of the quantitative variables. A Chi-square test was employed to compare the categorical data. The statistical analysis was performed by means of the Statistical Package for Social Sciences 17.0 (SPSS Inc., Chicago, IL, USA), and data were considered statistically different when \( P < 0.05 \).

**Results**

**Subject**

The mean average ages of the three study groups were LR 32.3 ± 2.8, 40.2 ± 2.5 and 25.4 ± 4.2 for the YLR, ALR and C groups, respectively. Differences were found for BMI between the YLR and ALR groups (25.3 ± 6.9, 23.5 ± 3.8, respectively), and both only reached statistical significance when compared with group C (21.5 ± 2.0), however, except for the YLR group where the mean BMI was in the lower part of overweight range, both the ALR patients and controls were within the range of normality; see Table I. Serum hormone concentration.

The basal serum concentrations of FSH and E2 on Day 3 of the menstrual cycle for the YLR, ALR and C groups are shown in Table I. Higher levels of FSH and lower levels of E2 were observed in both the LR groups compared with controls. Furthermore, no differences were seen at the peripheral level in terms of E2 or progesterone concentration on the day of hCG administration.

**Follicular fluid hormone concentration**

Similar-sized pre-ovulatory follicles were obtained from all three groups. Despite no differences being observed at the peripheral level in terms of E2 or progesterone concentration, the E2 concentration for the intrafollicular milieu was significantly higher in the YLR group if compared with both the ALR and the C group (2071, 1298 and 1927 nM), respectively. For progesterone concentration, ALR showed a significantly higher concentration when compared with group C (2071, 1298 and 1927 nM), respectively. Differences were found for BMI between the YLR and ALR groups (25.3 ± 6.9, 23.5 ± 3.8, respectively), and both only reached statistical significance when compared with group C (21.5 ± 2.0), however, except for the YLR group where the mean BMI was in the lower part of overweight range, both the ALR patients and controls were within the range of normality; see Table I. Serum hormone concentration.

Assessment of meiotic spindle parameters using the polyscope

A similar proportion of immature oocytes were observed in the three groups of women, as follows: YLR 5.3%, ALR 5.1% and control 4.8%. Moreover, a similar average of oocytes was punctured: 1.6 ± 1.0, 1.7 ± 1.0 and 1.7 ± 1.1 in the YLR, ALR and C groups, respectively. A meiotic spindle analysis was performed in 53 oocytes: 17 in the YLR group, 21 in the ALR group and 15 in the C group. The retardance, length, width and perimeter of the spindles were analysed and recorded for each group. Additionally, the meiosis status and structure of the spindles were also considered. As shown in Table III, no differences were seen for any of the spindle variables.

**Discussion**

This study found no differences in terms of the final pre-ovulatory follicle androgen concentration from young and ALR women undergoing unstimulated cycles compared with the C group. Only a higher progesterone and a lower E2 concentration were observed in the ALR group despite an elevated FSH level and a similar LH concentration being found inside the follicle.

Currently, a variety of different clinical approaches have been used in LR women to achieve a higher intra-ovarian androgen concentration, and sometimes better embryo quality was achieved by such strategies. Among them, administration of LH or systemic administration of either DHEA or testosterone have been considered; in fact, this approach is utilized by approximately one-third of all IVF centres (for a review, see Fanchin et al., 2011; Gleicher et al., 2011).

The rationale for adopting these strategies is based on animal experimental results demonstrating that androgens are able to increase the proliferation of theca and granulosa cells in the rhesus monkey. It is noteworthy that the expression of androgen receptors negatively correlated with apoptosis in those studies (Weil et al., 1999). Recent studies in humans have also confirmed the model observed in primates, showing how the androgen concentration in small antral follicles positively correlated with FSH receptors (Nielsen et al., 2011), which is fundamental to make small follicles more sensitive to FSH stimulation.

However, as reviewed by Fanchin et al. (2011), current clinical approaches that aim to increase androgen availability in the ovary provide conflicting results; some publications show enhanced fertilization ability and a number of embryos available for transfer, or pregnancy and implantation rates, whereas others failed to repeat the results.

In the present study, we aimed to define the final hormonal profile in pre-ovulatory follicles from LR women undergoing a more natural approach in order to better understand the subject of follicular health in this type of patient. Our results indicate that individual pre-ovulatory dominant follicles from ALR women present more differences than the younger ones when compared with the C group. In other words, YLR women have more in common with the C group than their Aged counterparts. These differences were mainly due to the final greater progesterone and FSH and lower E2 concentrations. Concerning the final intrafollicular hormonal concentration, it is important to take into account that in the present study, ovulation triggering was performed when the size of the follicles ranged between...
Intrafollicular hormones in low-responder patients

Table I Demographic data and comparison of the serum E2 and progesterone concentrations in unstimulated cycles in the Young (≤35 years) and Aged (>35 years) LR groups undergoing IVF for oocyte vitrification and unstimulated cycles in oocyte donors (controls).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Young LR (n = 19)</th>
<th>Aged LR (n = 39)</th>
<th>Control (n = 36)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>32.2 ± 2.8a</td>
<td>40.2 ± 2b</td>
<td>25.4 ± 4.2a</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI</td>
<td>25.3 ± 6.9b</td>
<td>23.5 ± 3.8b</td>
<td>21.5 ± 2.0b</td>
<td>0.01</td>
</tr>
<tr>
<td>Basal FSH (mIU/ml)</td>
<td>11.7 ± 4.5a</td>
<td>12.9 ± 10.7a</td>
<td>6.6 ± 2.5b</td>
<td>0.01</td>
</tr>
<tr>
<td>Basal E2 (nM)</td>
<td>0.12 ± 0.1a</td>
<td>0.13 ± 0.07b</td>
<td>0.29 ± 0.07b</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum E2 day of hCG (nM)</td>
<td>1.1 ± 0.8a</td>
<td>1.0 ± 0.5b</td>
<td>1.2 ± 2.7a</td>
<td>NS</td>
</tr>
<tr>
<td>Serum progesterone day of hCG (nM)</td>
<td>1.2 ± 0.7a</td>
<td>1.2 ± 0.6a</td>
<td>1.3 ± 0.9a</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD.
Different superscripts denote statistical differences among the groups (P < 0.01). Same superscripts denote no significant differences between the groups.

Table II Comparison of the follicular E2, progesterone, testosterone, androstenedione, FSH and LH concentrations in unstimulated cycles in the Young (≤35 years) and Aged (>35 years) LR women undergoing IVF for oocyte vitrification and in unstimulated cycles in oocyte donors (Controls).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Young LR (n = 19)</th>
<th>Aged LR (n = 39)</th>
<th>Control (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular fluid (ml)</td>
<td>4.2 ± 1.0a</td>
<td>3.4 ± 0.8a</td>
<td>4.2 ± 1.3a</td>
</tr>
<tr>
<td>Follicular E2 (µM)</td>
<td>2.1 ± 0.13a</td>
<td>1.3 ± 0.96</td>
<td>1.9 ± 0.96a</td>
</tr>
<tr>
<td>Follicular progesterone (µM)</td>
<td>27.5 ± 1.07a</td>
<td>31.4 ± 1.52a</td>
<td>23.2 ± 1.30a</td>
</tr>
<tr>
<td>Follicular testosterone (nM)</td>
<td>20 ± 6.4a</td>
<td>20 ± 9.5a</td>
<td>22 ± 10.2a</td>
</tr>
<tr>
<td>Follicular androstenedione (nM)</td>
<td>200 ± 173a</td>
<td>160 ± 219a</td>
<td>230 ± 315a</td>
</tr>
<tr>
<td>E2/testosterone ratio</td>
<td>104 ± 52a</td>
<td>71 ± 51a</td>
<td>93 ± 59a</td>
</tr>
<tr>
<td>Follicular FSH (mIU/ml)</td>
<td>4.6 ± 2.6a</td>
<td>6.2 ± 4.0a</td>
<td>3.6 ± 1.7a</td>
</tr>
<tr>
<td>Follicular LH (mIU/ml)</td>
<td>12.6 ± 7.8a</td>
<td>9.9 ± 6.8a</td>
<td>13.8 ± 8.4a</td>
</tr>
</tbody>
</table>

Values are means ± SD.
Different superscripts denote statistical differences among the groups (P < 0.03). Same superscripts denote no significant differences between the groups.

16 and 18 mm, which were smaller than final mature pre-ovulatory follicles generated in spontaneous ovulation, and therefore some differences might be expected from those. Nevertheless, since all the patients were treated in the same way and no differences in terms of follicular volumes were found among study groups, we consider that the results from these comparisons may be still useful for the purpose of the study.

Granulosa cells are very active producers of progesterone under the action of FSH, however, thecal cells also produce it (Fleming and Jenkins, 2010), thus, perhaps the higher intrafollicular levels of FSH found in the Aged LR group could be responsible for intrafollicular synthesis of progesterone and could, therefore, explain the higher progesterone concentrations observed in this group. According to the 2-cell two-gonadotrophin theory, progesterone is metabolized to androgens by theca cells under the influence of LH. However, based on the hormonal profile from the follicle of LR patients, where no differences in androgens and a similar LH concentration were observed, it seems that rather than presenting a deficit in intra-follicular androgen production, both the Young and ALR patients seemed to have problems in the follicle reservoir and growth.

Therefore, we think that what may happen is that the lower peripheral androgen concentration in LR women, which is assumed to decrease with age (Jacob et al., 2010), might not be able to maintain the pool of small follicles for recruitment. However, this defect might be compensated by the rise of peripheral FSH that can rescue the follicle and facilitate follicular growth. Hence, the low peripheral androgen concentration might not be related to the final follicle androgen concentration once the follicle has been recruited and becomes a pre-ovulatory follicle. In fact, for the dominant follicles from healthy older reproductive women, it was seen that androstenedione and testosterone presented similar levels to young women undergoing natural cycles (Klein et al., 2005).

As we said, the E2 levels were significantly lower in the ALR group; one possible explanation could be an alteration in aromatase activity, although the E2/testosterone ratio in the ALR group was not significantly different from the other groups.

Another explanation could lie in the differences in antagonist doses since it has been published that GnRH antagonist has a direct effect by inducing lower granulosa cells aromatase activity (Garcia-Velasco et al., 2001). However, no differences were seen in terms of the total doses administered among the groups.
Traditionally, higher levels of intrafollicular E$_2$ and progesterone, lower androstenedione and testosterone, as well as high E$_2$/androgen ratios, have been considered indicative of healthy ovarian follicles and of a greater chance of achieving pregnancy (Pellicer et al., 1987; Lenton et al., 1988). Elevated E$_2$ and E$_2$/androgen ratios in follicular fluid indicate a more advanced stage of oocyte maturation. In the ALR group, this ratio appeared lower than in the YLR group, and intrafollicular progesterone was also higher despite no differences being seen at the peripheral level on the day of hCG administration. Such a hormonal profile may be compatible with a drop in oocyte quality, which is known to happen with age. However, we could not find any differences in terms of the meiotic status or spindle structure parameters found among the groups.

The meiotic spindle analysis and meiotic status of the oocytes generated from the Young (<35 years) and Aged (>35 years) LR and from oocyte donors (Controls) undergoing unstimulated cycles.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Young LR (n = 19)</th>
<th>Aged LR (n = 39)</th>
<th>Control (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes analysed</td>
<td>17</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>Anaphase I n (%)</td>
<td>2 (11.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Telophase I n (%)</td>
<td>3 (17.6)</td>
<td>3(14.3)</td>
<td>4 (26.7)</td>
</tr>
<tr>
<td>Metaphase II n (%)</td>
<td>12 (70.6)</td>
<td>18 (85.7)</td>
<td>11 (73.3)</td>
</tr>
<tr>
<td>Retardance</td>
<td>1.6 ± 0.8</td>
<td>1.4 ± 0.5</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>Width (nm)</td>
<td>6.6 ± 3.8</td>
<td>7.9 ± 2.6</td>
<td>8.6 ± 3.0</td>
</tr>
<tr>
<td>Length (nm)</td>
<td>11.71 ± 7.4</td>
<td>14.1 ± 4.2</td>
<td>12.8 ± 5.2</td>
</tr>
<tr>
<td>Perimeter (nm)</td>
<td>30.0 ± 21.3</td>
<td>39.3 ± 10.8</td>
<td>32.3 ± 11.2</td>
</tr>
<tr>
<td>Area (nm$^2$)</td>
<td>51.7 ± 37.3</td>
<td>79.1 ± 22.7</td>
<td>75.5 ± 25.8</td>
</tr>
<tr>
<td>% spindle with good structure</td>
<td>50</td>
<td>55</td>
<td>64</td>
</tr>
</tbody>
</table>

Values are means ± SD unless stated otherwise. No significant differences were found among the groups.

Table III

In conclusion, our results support the idea that neither the follicular androgen secretion nor the LH effect on LR women is impaired and that, perhaps other mechanisms like lower FSH receptor expression or aromatase activity insufficiency might be actually taking place. In line with this, it might be interesting to analyse the LH receptor expression or 17 alpha-hydroxylase activity in theca cells, or the expression of the FSH receptors in granulosa cells.

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Authors’ roles

M.J.d.S: conception and design, analysis and interpretation of data, drafting the article and final approval of the version to be published. V.G.L. and D.B.: acquisition and sample processing, analysis and interpretation of data, drafting and revising the article.

J.L.Z.: hormonal assays. E.L.: analysis and interpretation of data, revising the article. P.A.: acquisition of data from control group, analysis and interpretation of data, revising the article. J.C.: acquisition of data from low-responder patients, analysis and interpretation of data, revising the article. E.B.: analysis and interpretation of data, drafting and revising the article, final approval of the version to be published.

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Conflict of interest

None declared.

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