Evidence for different aetiologies of low estradiol response to FSH: age-related accelerated luteinization of follicles or presence of ovarian autoantibodies

J.L.Luborsky¹,⁴, P.Thiruppathi¹, B.Rivnay², R.Roussev³, C.Coulam³ and E.Radwanska¹

¹Department of Obstetrics and Gynecology, Rush Medical College, Chicago, IL, ²Repromedix Corporation, Woburn, MA and ³Sher Institute and Millinova Laboratories, Chicago, IL, USA

⁴To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, 1653 W Congress Parkway, Rush Medical College, Chicago, IL 60612, USA. E-mail: jluborsk@rush.edu

BACKGROUND: We found granulosa cells of low responders (LR) expressed more LH receptors, suggesting that follicles were more luteinized than normal responders (NR). The objectives were to test the hypothesis that follicles of LR were more luteinized than follicles of NR, and to determine if LR with (LR⁺/c⁹⁰⁵⁹) and without (LR⁻) ovarian antibodies differed. METHODS: Hormone levels and ovarian autoantibodies (OV AB) were measured in follicular fluid from mature follicles (>17 mm), and in serum obtained on the day of oocyte retrieval during controlled ovarian stimulation. The gonadotrophin response was defined as a ratio of peak estradiol/number of FSH ampoules.

RESULTS: NR (32.5 ± 4.6 years; n = 11) were similar in age to LR⁺ (33.4 ± 4.2 years; n = 9) and were younger than LR⁻ (37.1 ± 3.8 years; n = 12) (P = 0.03). Likewise, dehydroepiandrosterone sulphate was lower in LR⁻ compared with LR⁺ or NR (P < 0.01). FSH, progesterone, inhibin-A and vascular endothelial growth factor levels were higher in follicular fluid of LR than NR. LR⁻ and LR⁺ differed. For example, the follicular fluid progesterone/estradiol ratio was similar in NR (11.1 ± 8.9) and LR⁺ (9.8 ± 6.6) but was lower than LR⁻ (22.9 ± 19.6) (P = 0.05). Serum hormone levels did not reflect follicular fluid hormone profiles. CONCLUSIONS: In the absence of ovarian antibodies, low responses are associated with higher age and accelerated luteinization of mature follicles, rather than diminished responsiveness. Ovarian antibody may be an additional tool to predict and individualize treatment regimens in poor responders.

Key words: age-related ovarian response/low responders/luteinization/ovarian antibodies/ovarian stimulation

Introduction

Low estradiol responses to FSH occur sporadically or consistently in 20–30% of women undergoing controlled ovarian stimulation (Ben-Rafael et al., 1994; Scott 1996; Ferraretti et al., 2000). Since low responses are associated with poorer treatment outcomes, they present a significant impediment to successful infertility treatment. The basis for low responses to FSH is not well defined (Ferraretti et al., 2000; Surrey and Schoolcraft, 2000).

Low gonadotrophin responses are usually differentiated from normal responses by the level of serum estradiol attained in response to 7–14 days of FSH stimulation during controlled ovarian stimulation. It has become customary to designate low responses as those <1000 mIU/ml estradiol (Scott, 1996; Ferraretti et al., 2000). The number of ampoules of FSH required to attain an adequate response is also an indicator of low responses, although a specific cut-off value has not been defined. A reduced number of oocytes retrieved after hCG stimulation may also be associated with low responses (Dor et al., 1992). However, poor responses associated with a low number of follicles recruited during controlled ovarian stimulation may represent a different clinical entity than low responses to gonadotrophin (Ferraretti et al., 2000; Surrey and Schoolcraft, 2000).

Different aetiologies have been described for low responses. For example, low responses are associated with ageing and low ovarian reserve (Kligman and Rosenwaks, 2001), reduced blood flow (Pellicer et al., 1994), reduced aromatase activity (Hurst et al., 1992), hormone resistance associated with specific FSH receptor polymorphism (Perez Mayorga et al., 2000) and ovarian autoimmunity (Meyer and Lavy et al., 1990; Hovav et al., 1994). Although ageing appears to explain a proportion of low responses, many low responders are young (Jenkins et al., 1991; Kim 1995; Hanoch et al., 1998). Thus, it is likely that low responses represent multiple aetiologies.

In a previous study, we examined the possibility that differences in hormone response were due to differences in gonadotrophin receptor expression. We showed that FSH receptor expression was similar on granulosa cells of low and normal responders but LH receptor expression was slightly...
higher on cells of low responders (Thiruppathi et al., 2001). In addition, more of the granulosa cells from low responders had the large rounded, granular morphology of luteal cells (unpublished observation). Similarly, Whitman et al. observed that follicles of oocytes that failed to fertilize contained more luteinized cells (Whitman et al., 1988, 1989). These observations suggested that follicles of low responders might be more luteinized.

More advanced luteinization has been reported in follicles of older women during natural ovarian cycles (Klein et al., 1996a,b,c; Klein and Soules, 1998). Based on serum measures, premature luteinization was observed in women with infertility (Ubaldi et al., 1996; Lindheim et al., 1999; Muttukrishna et al., 2000). However, markers of luteinization have not been examined at the follicular level as a basis for altered responsiveness to gonadotrophin.

The objective of this study was to test the hypothesis that follicles of low responders were more luteinized than follicles of normal responders. Endocrine markers of luteinization such as progesterone, inhibin-A and vascular endothelial growth factor (VEGF) were assessed in follicular fluid and serum of low and normal responders at oocyte retrieval. A secondary objective was to determine if hormone profiles in the presence or absence of ovarian antibodies differed.

Materials and methods

Study group

Women undergoing IVF for infertility were entered into the study. Women with polycystic ovarian syndrome (PCOS) or recurrent spontaneous fetal loss were excluded. Women with PCOS were excluded based on a history of oligomenorrhoea (menses interval >42 days) and ultrasound evidence of cystic ovary (Franks, 1989). Women with recurrent fetal loss were excluded based on a history of more than three unexplained spontaneous abortions. The primary infertility diagnosis included male factor (n = 12), pelvic factor (n = 5), uterine factor (n = 2), tubal factor (n = 4) and unexplained (n = 9). The average age of the study group was 34.5 ± 4.6 years (range 27–42). Early follicular phase FSH levels were not routinely measured, particularly in younger women with menstrual cycles since the information has limited predictive value (Schipper et al., 1998; Bancsi et al., 2000). The average years of infertility was 3.3 ± 2.0 (range 1–8). The average number of prior IVF cycles was 1 ± 1 (range 0–4). A blood sample was obtained by routine venipuncture on the day of oocyte retrieval. Follicular fluid was collected during oocyte retrieval (n = 32). All procedures were reviewed and approved by the Institutional Review Board.

Ovarian stimulation was initiated by pituitary desensitization with GnRH during the midluteal phase of the preceding cycle. In the subsequent cycle, hMG, urofollitropin, or urofollitropin (HP) was injected daily starting with an initial dose of 300 IU. Highly purified urinary FSH (Fertinex®; Serono, Norwell, MA, USA) was used for 70% of the patients, with the remaining FSH preparations distributed similarly among the study groups. Doses were adjusted based on follicle growth by ultrasound and serum estradiol monitored every other day. Ovulation was induced with 10 000 IU of hCG (Profasi®; Serono) after follicles were ≥17 mm in diameter and estradiol had increased over 8 days. The average estradiol response to gonadotrophin in the study cycle was 1341 ± 605 (range 564–2714) and the average number of ampoules of FSH administered was 52 ± 28 (range 16–120). Oocytes were obtained 34 h after hCG administration under transvaginal ultrasound guidance.

Measurement of hormones and antibodies in follicular fluid and serum

For each individual, the follicular fluid from three follicles, 18–20 mm in diameter, was pooled in order to obtain adequate volume for multiple measures. The similarity of results from individual follicles and the pooled follicular fluid of six individuals was verified (Thiruppathi et al., 2001). The follicular fluid was centrifuged to remove cells and debris and frozen at −20°C until assay. Serum was separated from whole blood and stored at −20°C. Hormones were measured by immunoassay with commercial kits. Progesterone and estradiol were measured in serum and follicular fluid (Cayman Chemical, Ann Arbor, MI, USA) with detection limits of 10 and 9 pg/ml respectively. Androstenedione was measured in follicular fluid and dehydroepiandrosterone sulphate (DHEAS) were measured in serum (Diagnostic Systems Laboratories, Houston, TX, USA) with detection limits of 0.03 and 15 ng/ml respectively. VEGF was measured in follicular fluid by enzyme immunoassay (R&D Systems, Minneapolis, MN, USA). FSH was measured in follicular fluid (Genzyme, CA, USA). Inhibin-A and inhibin-B were measured with Serotec kits (Harlan, Raleigh, NC, USA). The sensitivity of the inhibin-A immunoassay was 10 pg/ml and of inhibin-B was 15 pg/ml. Hormone levels in follicular fluid were significantly higher than the range of the immunoassay standard curves. Therefore, samples were diluted with sample dilution buffer supplied by the manufacturer, until the levels were in the mid-range of the standard curve. The inter-assay and intra-assay coefficients of variation of all commercial assays were <10%.

Ovarian and oocyte antibodies in serum were determined as described previously (Luborsky et al., 1990; Luborsky and Pong, 2000). In summary, anti-ovarian antibodies were detected by enzyme-linked immunosorbent assay (ELISA). Ovaries were obtained from rats superovulated with pregnant mare’s serum gonadotrophin and HCG. The 10 000 g pellet of ovarian homogenates was used as antigen (0.1 µg/0.1 ml Tris buffer/well). Results with rat or human ovary were highly correlated (r = 0.95, P < 0.001) (Luborsky and Pong, 2000). Anti-oocyte antibodies were detected in ELISA using homogenates of oocytes from mature follicles of rat ovaries. Oocytes were aspirated from follicles at the ovary surface, incubated in 0.5% collagenase (Sigma, St Louis, MO, USA) and 0.01% DNAase (Sigma) in PBS (30 min, 37°C) and separated from cells by gradient centrifugation in 3 ml of Hypaque (Sigma) (400 g, 30 min). After washing, oocytes were homogenized in batches of 100 in a 1 ml microfuge tube with a hand-held micro-pestle. Oocytes were added to ELISA plates at a concentration of ~1 oocyte/0.1 ml Tris buffer/well. Results with rat or human oocytes were correlated (r = 0.85, P < 0.001).

Sera were tested at a dilution of 1:100 and follicular fluid at a dilution of 1:10. Negative control sera from normally cycling women (n = 10) and a positive control were included in each assay. For follicular fluid, four control samples from women with male factor infertility were used as negative controls. The captured autoantibodies were identified by a goat antihuman IgG–alkaline phosphatase conjugate (FAB specific; Sigma). Bound alkaline phosphatase was reacted with substrate (AP substrate; Sigma) and the product read at 405 nm (Thermomax; Molecular Devices, Sunnyvale, CA, USA).

Data and statistical analysis

Statistical differences between normal and low responders and among the response groups were assessed with the Mann–Whitney U-test. Pearson’s correlation coefficient (r) was calculated to evaluate associations between variables. P < 0.05 was considered significant.
For detection of ovarian and oocyte auto antibodies, samples were assessed in duplicate against four negative control samples and a positive control. Inter-assay and intra-assay coefficients of variation for specific optical density signals were <15% and <10% respectively. In this study, individuals positive for one or both antibodies were considered positive and results are referred to in the text as ‘ovarian antibody’ positive for simplicity. Although ovarian antibodies were correlated in follicular fluid and serum (r = 0.4, P = 0.035), the serum test results were used to differentiate positive and negative low responders. Results were compared by $\chi^2$-analysis.

**Results**

**Characterization of low and normal responders**

In order to integrate the FSH dose and the estradiol response, the ratio of the peak estradiol/number of ampoules of FSH (gonadotrophin response) was used to define low and normal responses. This method of defining low responses was more consistent with resistance to FSH and was used to define response groups as described previously (Thiruppathi et al., 2001). The gonadotrophin response ratio ranged from 7 to 89; low responders were those <42. Ovarian antibodies were detected in low responders but not normal responders. There were 9/32 (28.1%) low responders with ovarian antibodies, 12/32 (37.5%) low responders without ovarian antibodies and 11/32 (34.4%) normal responders. The mean gonadotrophin response ratio was higher for normal responders than low responders, but did not differ for low responders with and without ovarian antibodies (Table I).

Normal responders and low responders with ovarian antibodies were significantly younger than low responders and also significantly younger than low responders without antibodies (Table I and Figure 1). The average number of FSH ampoules administered and the peak estradiol were significantly different between normal and low responders but did not differ between low responders with and without ovarian antibodies (Table I). The mean number of oocytes retrieved and the mean percentage of fertilized oocytes were not different between the groups (Table II). However, the lowest number of oocytes retrieved and the lowest percentage of oocytes fertilized occurred in low responders with ovarian antibodies, as reflected in the standard deviations.

**Hormones in follicular fluid**

Hormone levels in follicular fluid of mature follicles >17 mm were measured in order to determine if the local environment of maturing follicles differed for normal and low responders.

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 11)</th>
<th>Low (n = 21)</th>
<th>Low negative (n = 12)</th>
<th>Low positive (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>32.5 ± 4.6</td>
<td>35.5 ± 4.3</td>
<td>37.1 ± 3.8</td>
<td>33.4 ± 4.2</td>
</tr>
<tr>
<td><strong>Ampoules of FSH</strong></td>
<td>32.7 ± 13.9</td>
<td>61.5 ± 29.1</td>
<td>55.8 ± 22.4</td>
<td>69.2 ± 36.2</td>
</tr>
<tr>
<td><strong>Peak estradiol (pg/ml serum)</strong></td>
<td>1883 ± 538</td>
<td>1057 ± 419</td>
<td>1118 ± 469</td>
<td>978 ± 353</td>
</tr>
<tr>
<td><strong>Ratio peak estradiol/ampoules FSH</strong></td>
<td>62.0 ± 18.6</td>
<td>19.7 ± 8.3</td>
<td>21.1 ± 5.9</td>
<td>17.7 ± 10.7</td>
</tr>
</tbody>
</table>

$\text{LR} = \text{low responders}; \text{NS} = \text{not significant.}$

**Figure 1.** Age of normal and low responders shown as box plots. Responses to FSH were defined as a ratio of the peak estradiol/number of FSH ampoules. In addition, the presence (POS) or absence (NEG) of ovarian antibodies (OVAB) was used to define groups. The median, range (whiskers), 25th–75th percentile (box) and outliers (circles) are shown. The mean values and significance of differences are summarized in Table I.

Estradiol and androstenedione levels were similar between normal and low responders (Table III). Progesterone levels were significantly higher in follicles of low responders compared with normal responders (Table III). The mean progesterone level did not differ significantly between low responders with or without ovarian antibodies, although it tended to be lower and more variable in low responders with ovarian antibodies (Figure 2). Progesterone was significantly higher in low responders without ovarian antibodies compared with normal responders (Table III). The progesterone/estradiol ratio was significantly higher for low responders without ovarian antibodies compared with low responders with ovarian antibodies or normal responders (Table III).

Inhibin-A, inhibin-B and VEGF levels were measured as additional indicators of luteinization. Inhibin-B levels in follicular fluid of normal and low responders were similar (Table III). Inhibin-A and the inhibin-A/inhibin-B ratio were higher in follicles of low responders although the difference was not statistically significant compared with normal responders (Table III). Only low responders without ovarian antibodies had significantly higher inhibin-A levels compared with normal responders (Figure 2). Similarly, only low responders without ovarian antibodies had significantly higher VEGF levels in follicular fluid compared with normal responders (Table III).
Table II. Oocyte parameters: descriptive statistics (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 11)</th>
<th>Low (n = 21)</th>
<th>Low negative (n = 12)</th>
<th>Low positive (n = 9)</th>
<th>Normal versus Low P-value</th>
<th>Normal versus LR– P-value</th>
<th>Normal versus LR+ P-value</th>
<th>LR– versus LR+ P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of oocytes</td>
<td>22.1 ± 7.7</td>
<td>18.1 ± 8.5</td>
<td>19.8 ± 7.9</td>
<td>15.8 ± 9.2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% mature oocytes</td>
<td>44.0 ± 6</td>
<td>42.5 ± 10</td>
<td>43.0 ± 4.0</td>
<td>41.0 ± 16.0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% fertilized oocytes</td>
<td>52.1 ± 17.3</td>
<td>53.4 ± 22.3</td>
<td>56.6 ± 12.8</td>
<td>49.0 ± 31.3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

LR = low responders; NS = not significant.

Table III. Follicular fluid hormone levels: descriptive statistics (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 11)</th>
<th>Low (n = 21)</th>
<th>Low negative (n = 12)</th>
<th>Low positive (n = 9)</th>
<th>Normal versus Low P-value</th>
<th>Normal versus LR– P-value</th>
<th>Normal versus LR+ P-value</th>
<th>LR– versus LR+ P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (mIU/ml)</td>
<td>11.1 ± 9.1</td>
<td>30.9 ± 23.8</td>
<td>31.3 ± 23.0</td>
<td>30.3 ± 26.2</td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Estradiol (ug/ml)</td>
<td>1.3 ± 1.3</td>
<td>1.1 ± 0.67</td>
<td>0.98 ± 0.5</td>
<td>1.3 ± 0.8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Progesterone (µg/ml)</td>
<td>7.2 ± 3.5</td>
<td>13.3 ± 8.7</td>
<td>15.7 ± 7.5</td>
<td>10.2 ± 9.6</td>
<td>0.02</td>
<td>0.004</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Progesterone/estradiol ratio</td>
<td>11.1 ± 8.9</td>
<td>17.3 ± 16.6</td>
<td>22.9 ± 19.6</td>
<td>9.8 ± 6.6</td>
<td>NS</td>
<td>0.05</td>
<td>NS</td>
<td>0.05</td>
</tr>
<tr>
<td>Androstenedione (ng/ml)</td>
<td>39.1 ± 27.3</td>
<td>32.8 ± 9.7</td>
<td>35.4 ± 10.0</td>
<td>29.5 ± 8.6</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Estradiol/androstenedione ratio</td>
<td>61.3 ± 106.8</td>
<td>33.1 ± 18.9</td>
<td>27.9 ± 13.1</td>
<td>40.0 ± 23.7</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Inhibin-A (ng/ml)</td>
<td>109.9 ± 44.7</td>
<td>142.2 ± 44.2</td>
<td>149.0 ± 37.0</td>
<td>133.2 ± 53.3</td>
<td>NS</td>
<td>0.04</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Inhibin-B (ng/ml)</td>
<td>87.0 ± 55.3</td>
<td>88.5 ± 71.5</td>
<td>76.4 ± 47.2</td>
<td>105.2 ± 96.9</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Inhibin-A/Inhibin-B ratio</td>
<td>1.7 ± 1.1</td>
<td>2.9 ± 2.4</td>
<td>3.2 ± 2.5</td>
<td>2.6 ± 2.2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>2052 ± 1256</td>
<td>3148 ± 1550</td>
<td>3288 ± 1631</td>
<td>2961 ± 1510</td>
<td>NS</td>
<td>0.04</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

LR = low responders; NS = not significant; VEGF = vascular endothelial growth factor.

The correlation of gonadotrophin response and indicators of luteinization were also assessed without separating the study group into low and normal responses. Response to gonadotrophin was negatively correlated with age (r = –0.4, P = 0.04), follicular fluid FSH levels (r = –0.5, P = 0.003) and the inhibin-A/inhibin-B ratio (r = –0.5, P = 0.013), but was not correlated with follicular fluid estradiol, progesterone or the progesterone/estradiol ratio. The lack of correlation of the progesterone/estradiol ratio with gonadotrophin response reflects a difference in the distribution of progesterone levels for normal and low responders. As seen in Figure 3, progesterone was correlated with gonadotrophin response in normal responders (r = 0.72, P = 0.006) but was not correlated among low responders (r = 0.05, P = 0.4, not significant). When ovarian antibody positive individuals were excluded, the correlation of response with progesterone (r = 0.4, P = 0.055) and inhibin-B (r = 0.4, P = 0.067) approached significance.

The number of ampoules of FSH and levels of FSH in follicular fluid were correlated regardless of the inclusion (correlation = 0.71, P = 0.001) or exclusion (correlation = 0.76, P = 0.001) of individuals with ovarian antibodies. FSH in follicular fluid was positively correlated with age (r = 0.5, P = 0.003), progesterone (r = 0.6, P = 0.001), VEGF (r = 0.6, P < 0.001) and the ratio of inhibin-A/inhibin-B (r = 0.6, P = 0.001) in follicular fluid, and negatively correlated with inhibin-B (r = –0.5, P = 0.013). The correlation of FSH with the progesterone/estradiol ratio (r = 0.4, P = 0.067) approached significance in the absence of individuals with ovarian antibodies while other correlations remained similar.

Hormones in serum

Serum levels of hormones were measured as an indicator of the overall activity of follicles in the ovary. Serum inhibin-A and inhibin-B (Table IV) and peak estradiol (day of hCG) (Table I) were significantly lower in low responders than normal responders. Estradiol (day of oocyte retrieval), the inhibin-A/inhibin-B ratio, progesterone and the progesterone/estradiol ratio were not significantly different between low and normal responders. DHEAS was measured as an indicator of somatic ageing and of adrenal steroid biosynthesis and differed significantly between normal and low responders (Table IV). However, DHEAS in low responders without ovarian antibodies was significantly higher than DHEAS in low responders with ovarian antibodies and in normal responders (Figure 4).

Responses to gonadotrophin were positively correlated with serum estradiol (day of oocyte retrieval) (r = 0.4, P = 0.03), inhibin-A (r = 0.6, P < 0.001) and inhibin-B (r = 0.6, P = 0.001) in serum, but were not correlated with progesterone, the progesterone/estradiol ratio, or the inhibin-A/inhibin-B ratio. Unlike the divergent distribution of progesterone levels in follicular fluid in relation to gonadotrophin response,
Low responders, luteinization and ovarian antibodies

Figure 2. Comparison of steroid hormone and inhibin levels in follicular fluid (FF) of mature follicles. Progesterone was elevated in low responders. Estradiol levels did not differ among normal and low responders. Compared with normal responders, the progesterone/estradiol ratio and inhibin-A was significantly higher only for low responders without ovarian antibodies. Specific mean values and the significance of differences are summarized in Table III. OVAB = ovarian antibodies; POS = present; NEG = absent.

Figure 3. Relationship of gonadotrophin response to progesterone in follicular fluid (FF) (left panel) and serum (right panel). Response was defined by the ratio of peak estradiol/number of FSH ampoules. Progesterone levels were correlated with gonadotrophin response in normal responders. In contrast, levels of progesterone varied widely in follicles of low responders, and were not correlated with gonadotrophin response. Correlations are summarized in the text. OVAB = ovarian antibodies; POS = present; NEG = absent.

progesterone levels in serum were more similarly distributed with a single trend from low to high in relation to gonadotrophin response (Figure 3). DHEAS (as an indicator of somatic age) was correlated with gonadotrophin response \( (r = 0.7, P = 0.001) \) only when ovarian antibody positive individuals were excluded from analysis.

The number of FSH ampoules was negatively correlated with serum inhibin-B \( (r = -0.5, P = 0.01) \) regardless of the
Relationship between serum and follicular fluid hormone levels

With a few exceptions, serum hormone levels did not reflect patterns of follicular fluid hormones regardless of the inclusion or exclusion of ovarian antibody positive individuals in the analysis. For example, progesterone in serum and follicular fluid were not correlated ($P = 0.08$, not significant). When ovarian antibody positive individuals were excluded, serum estradiol (day of oocyte retrieval) ($r = -0.53$, $P = 0.02$) and serum inhibin-A ($r = -0.46$, $P = 0.04$) were negatively correlated with the follicular fluid inhibin-A/inhibin-B ratio. In other words, the absence of ovarian antibodies, the lower the serum estradiol and the higher the inhibin-A/inhibin-B ratio the more likely that individual maturing follicles would exhibit accelerated luteinization.

Discussion

In this study, progesterone was the predominant sex steroid in mature follicles. This was expected since hCG initiates luteinization and progesterone production. However, progesterone was strikingly higher in follicles of low responders compared with normal responders. Likewise VEGF, inhibin-A and the inhibin-A/inhibin-B ratio were higher in mature follicles of low responders, consistent with more advanced luteinization. In contrast, hormone levels in serum did not reflect the accelerated luteinization of maturing follicles in low responders. Instead, serum hormone levels tended to be lower in low responders, consistent with reduced ovarian reserve. This is the first direct demonstration that low responses to gonadotrophin and accelerated luteinization of maturing follicles are associated.

Only low responders without ovarian antibodies showed a clear trend towards accelerated luteinization and higher age. Low responders with ovarian antibodies tended to be similar to normal responders with respect to age and hormone profiles, rather than to low responders without ovarian antibodies. Also, hormone levels and oocyte parameters varied more in low responders with ovarian antibodies.

The higher VEGF observed in this study is consistent with previous reports that higher levels of VEGF were associated with lower peak estradiol levels, lower number of oocytes retrieved, higher age and higher number of FSH ampoules required (Friedman et al., 1997, 1998). In addition, Neulen et al. showed that the ratio of VEGF/soluble VEGF receptor (sVEGFR-1) was inversely related to the number of oocytes retrieved, suggesting that decreased bioavailability of VEGF is associated with poor responses defined by oocyte yields (Neulen et al., 2001). VEGF is required for corpus luteum formation and increases with progressive luteinization and vascular growth (Ferrara and Davis-Smyth, 1997; Ferrara et al., 1998). VEGF production in luteinizing granulosa cells is stimulated by FSH and LH (Christenson and Stouffer 1997; Laitinen et al., 1997; Geva and Jaffe, 2000; Stouffer et al., 2001). Likewise, VEGF was correlated with FSH in this study. However, since doses of FSH are not equivalent, it is not clear if the higher levels of VEGF are a consequence of higher doses of FSH, if luteinization is enhanced by a slightly higher

![Graph of DHEAS levels](image)

**Figure 4.** Comparison of dehydroepiandrosterone sulphate (DHEAS) in serum (day of oocyte retrieval). DHEAS was significantly lower in low responders without ovarian antibodies compared with normal and low responders with ovarian antibodies. As an indicator of somatic age, DHEAS levels are consistent with mean chronological ages of each group shown in Figure 1. Specific mean values and the significance of differences are summarized in Table IV. OVAB = ovarian antibodies; POS = present; NEG = absent.

Table IV. Serum hormone levels: descriptive statistics (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 11)</th>
<th>Low (n = 21)</th>
<th>Low negative (n = 12)</th>
<th>Low positive (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (ng/ml)</td>
<td>0.96 ± 0.45</td>
<td>0.69 ± 0.36</td>
<td>0.72 ± 0.37</td>
<td>0.67 ± 0.38</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>56.1 ± 21.0</td>
<td>44.3 ± 28.6</td>
<td>43.6 ± 19.4</td>
<td>45.1 ± 38.7</td>
</tr>
<tr>
<td>Ratio progesterone/estradiol</td>
<td>0.97 ± 0.43</td>
<td>113.6 ± 119</td>
<td>91.3 ± 98.2</td>
<td>141.6 ± 274</td>
</tr>
<tr>
<td>Inhibin-A (pg/ml)</td>
<td>333.9 ± 166.6</td>
<td>152.9 ± 89.8</td>
<td>157.4 ± 98.2</td>
<td>1475.5 ± 84.4</td>
</tr>
<tr>
<td>Inhibin-B (pg/ml)</td>
<td>630.8 ± 231.3</td>
<td>354.9 ± 250.5</td>
<td>363.7 ± 286.9</td>
<td>344.0 ± 215.1</td>
</tr>
<tr>
<td>Ratio inhibin-A/inhibin-B</td>
<td>0.57 ± 0.3</td>
<td>0.51 ± 0.32</td>
<td>0.49 ± 0.36</td>
<td>0.53 ± 0.29</td>
</tr>
<tr>
<td>DHEAS (ng/ml)</td>
<td>1175 ± 573</td>
<td>1064 ± 866</td>
<td>602 ± 230</td>
<td>1640 ± 1034</td>
</tr>
</tbody>
</table>

LR = low responders; NS = not significant; DHEAS = dehydroepiandrosterone sulphate.
content of LH in the FSH preparation (Filicori et al., 2001) or if low responders have reduced control of the rate of follicle growth and differentiation (Eppig, 2001).

Serum hormone levels did not reflect the hormone profiles of mature follicles. Instead, serum hormone levels tended to be lower in low responders. Both low responders with and without ovarian antibodies had lower levels of ovarian hormones consistent with reduced ovarian reserve that is thought to precede ovarian failure. In serum, the hormone levels reflect the additive activity of follicles of different maturation stages. Increasing estradiol in response to high doses of FSH may be due to increases within smaller follicles or to recruitment of additional, less mature, follicles. The trend to lower serum levels of ovarian hormones in low responders and in older women is consistent with other studies (Scott, 1996; Hall et al., 1999; Seifer et al., 1999). For example, Seifer et al. reported that age was associated with declining ovarian reserve, decreased serum inhibin-B, increased requirement for FSH, lower success rate and fewer oocytes retrieved (Seifer et al., 1999).

We did not observe a difference in serum progesterone between normal and low responders. Other reports describing the relationship of serum progesterone and responses to gonadotrophin are inconsistent. Lindheim et al. found that low serum progesterone responses to hCG were associated with poor estradiol responders and suggested that this reflected inadequate steroidogenesis (Lindheim et al., 1999). Women with premature luteinization, defined by serum progesterone >1.1 ng/ml on the day of hCG administration, required a higher number of FSH ampoules to attain an adequate estradiol response (Ubaldi et al., 1996). Likewise, premature luteinization, defined by a serum progesterone/estradiol ratio >1 on the day of hCG administration, was associated with low estradiol responses and ovarian reserve (Younis et al., 2001). Follicular fluid hormone levels were not measured in these studies, and thus there is no information on the activity of maturing follicles. Differences in conclusions among studies may reflect different types of information obtained by assessing serum or follicular fluid, differences in definition of low and normal responses, and differences in study design and the day of serum sampling.

Thus, it appears that rather than reduced responsiveness, follicles of older ‘low responders’ are maturing and differentiating at a faster rate than those of normal responders. The results are consistent with previous reports of accelerated luteinization of follicles in non-stimulated ovarian cycles of older women (Klein et al., 1996a; Klein and Soules, 1998). Greater luteinization could be due to higher levels of FSH, higher levels of LH associated with higher doses of FSH, enhanced responses to low levels of LH as cells acquire LH receptors (Filicori et al., 1999, 2001), or to a reduced ability of oocytes to inhibit luteinization (Vanderhyden and Macdonald, 1998) prior to oocyte retrieval.

The results are consistent with heterogeneous aetiologies of low responses. Tanbo et al. reported two types of low responses based on clomiphene citrate responses (Tanbo et al., 1990). Hanoch et al. also differentiated low responses by age and noted that young low responders had better pregnancy rates (Hanoch et al., 1998). Low estradiol responses were also differentiated by day 3 LH (Noci et al., 1998). FSH receptor polymorphism at position 680 was associated with different requirements for FSH during stimulation, suggesting partial hormone resistance in some women (Perez Mayorga et al., 2000). Low responses have also been associated with decreased blood flow measured by Doppler ultrasonography (Pellicer et al., 1994). We showed that some low responders had ovarian antibodies (Meyer et al., 1990). In this study, we found that low responders differentiated by the presence or absence of ovarian antibodies differed in age and hormone profiles. Because of the heterogeneity of low gonadotrophin responses, it has been suggested that pre-cycle screening to differentiate potential responses may improve treatment outcomes (Surrey and Schoolcraft, 2000).

Although the primary objective of this research study was to obtain information on potential mechanisms of low responses to FSH, there are implications for clinical practice. The lack of correlation of FSH dose with peak serum estradiol, the correlation with follicular fluid markers of luteinization and the lack of difference in oocytes retrieved suggest that higher FSH doses are not required for older low responders. This is consistent with other studies (Tinkanen et al., 1999; Out et al., 2000). Premature luteinization associated with ageing may result in over-ripe or poorer quality oocytes (Eichenlaub-Ritter, 1998; Sauer, 1998). It has been reported that monitoring inhibin-B in addition to estradiol to determine the timing of hCG improves prediction of a successful outcome compared with estradiol monitoring alone (Seki et al., 1997). This would be supported by this study since serum inhibin-B was correlated with the follicular response to gonadotrophin. Since the rate of follicle growth is slower in response to recombinant FSH which lacks trace LH (Filicori et al., 1999, 2001), it has been suggested that outcomes for low responders may be improved by using recombinant FSH or a combination of recombinant FSH and controlled levels of trace LH, although treatments may need to be individualized (Pellicer et al., 1998; Howles, 2000; Surrey and Schoolcraft, 2000; Fawzy et al., 2001).

The significant differences among low responders with ovarian antibodies suggest that treatment responses to standard hormone therapy may be less predictable in low responders with ovarian antibodies. Thus, individuals with ovarian antibodies may benefit from closer pre-cycle screening and monitoring during treatment for markers of follicular growth. In some cases, hormone stimulation may overcome the autoimmune reaction and recruit some healthy follicles. However, there is evidence that treatment outcomes associated with ovarian autoimmunity are poor (Geva et al., 1999; Ivanova et al., 1999; Horejsi et al., 2000; Luborsky and Pong, 2000). Treatment with low dose immunosuppression has been successful (Luborsky et al., 1990; Geva et al., 1999), but treatment protocols have not been standardized in controlled trials.

In summary, mature follicles of low responders appear to be more luteinized than follicles of normal responders. In the absence of ovarian antibodies, low responses are associated with higher chronological and somatic age and accelerated luteinization of mature follicles, rather than diminished responsiveness. However, the follicular environment of
low responders with ovarian autoantibodies differs from low responders without autoantibodies, suggesting different mechanisms for low responses in the presence of ovarian autoimmunity. In addition, use of ovarian antibody testing provides an additional tool for predicting response to gonadotrophin and may contribute to more individualized regimens for controlled ovarian stimulation.

Acknowledgements

This study was supported in part by an educational grant from Serono Laboratories, Inc. (Norwell, MA, USA). This work was presented in part at the Second International Conference on Experimental and Clinical Reproductive Immunobiology (2000) and the annual meeting of the Society Study of Reproduction (2000).

References


Submitted on March 4, 2002; accepted on May 23, 2002.