The effect of gonadotrophins with differing LH/FSH ratios on the secretion of the various species of inhibin in women receiving IVF

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We have measured secretory patterns of inhibin A, B, total α inhibin, pro-αC inhibin and oestradiol in women following pituitary suppression who were randomised into two groups to receive either urinary gonadotrophin (25:75 IU/ampoule of luteinizing hormone (LH) and follicle stimulating hormone (FSH; Normegon; n = 11) or recombinant (r)FSH (75 IU/ampoule of FSH alone, n = 16). The women were of similar age (~33 years) and length of infertility (~4 years) and had a normal endocrine evaluation. Plasma FSH, LH, oestradiol, inhibin A, B, pro-αC and total α inhibin were measured by immunoassay prior to and following gonadotrophin stimulation. Immunoactive FSH, LH and oestradiol blood concentrations following pituitary down regulation were similar in the two groups being <2.0, <3.6 IU/l and <82 pmol/l respectively. The units of FSH given (2230 versus 2764 IU; Normegon versus rFSH), duration of treatment (9.1 versus 9.4 days) and number of follicles of ≥14mm on the day of human chorionic gonadotrophin (HCG) administration (17 versus 14) were also similar. Inhibin A or B concentrations rose similarly during Normegon or rFSH administration, peaking at days 9–11. Total α and pro-αC inhibin concentrations were lower (P < 0.05) in the rFSH group during days 10 and 11 of treatment being 18.9/15.9 ng/ml (Normegon) and 4.6/2.8 ng/ml (rFSH) for total α inhibin and 8.5/6.8 ng/ml (Normegon) and 2.8/1.6 ng/ml (rFSH) for pro-αC inhibin on day 10. Overall, higher total α inhibin concentrations were associated with more mature follicles and oocytes, greater fertilization rates and better quality embryos. We conclude that inhibin A and B secretion was similar in both groups and is primarily controlled by FSH, whereas total α inhibin and pro-αC increased preferentially in the Normegon group over the rFSH group, indicating that they are, in part, stimulated by LH.

Key words: Gonal-F/inhibin/Normegon/Puregon/recombinant FSH

Introduction

Inhibins are heterodimeric glycoproteins consisting of αβA (inhibin A) and αβB (inhibin B) subunits which are secreted mainly by the ovaries in women. Inhibin B is known to be a selective suppressor of pituitary follicle stimulating hormone (FSH) secretion and inhibin A and B have local paracrine actions in the gonads (Knight, 1996; Hayes et al., 1998; Lockwood et al., 1997; Lahlou et al., 1999). Most of the data indicate that only the dimeric forms of inhibin are physiologically important in controlling FSH secretion and ‘fine-tuning’ the hypothalamic–gonadal axis (Lockwood et al., 1998). Their role in growth and development during human ovulation, conception and pregnancy has recently been reviewed (Lockwood et al., 1998).

The α subunit is synthesised in excess over the β subunits and several monomeric forms are present in high concentrations in serum and ovarian follicular fluid (Knight, 1996). Moreover, there is some evidence that monomeric inhibin α subunits are bioactive in that they may modulate follicular function at the local level by inhibiting the binding of FSH to its receptors on granulosa cells (Schneyer et al., 1991), may reduce bovine oocyte developmental competence in vitro (Silva et al., 1999) and may have a role in the pathogenesis of adrenal tumours (Munro et al., 1999). The α subunit is also present in precursor forms in combination with the β subunits (β₁ or β₂) to form pro-αC–containing inhibins.

Inhibin A and B are differentially secreted across the menstrual cycle (Groome et al., 1994). Inhibin A is thought to be a marker of the maturity of the dominant follicle (Lockwood et al., 1996) whereas inhibin B is the predominant form in the smaller antral follicles and may be an indicator of ovarian reserve (Lockwood et al., 1996; Seifer et al., 1997).
With regard to the gonadotrophic control of inhibin secretion, early studies were compromised by the use of impure gonadotrophin preparations often in combination with the use of unspecified assays of inhibin which detected precursors and free subunits in addition to dimeric forms (Buckler et al., 1992; Mitchell et al., 1996). Only recently with the advent of recombinant (r)FSH and luteinizing hormone (LH) preparations and the development of the specific assays for the various species of inhibin has an investigation of the control of inhibin secretion been possible. Stimulation with rFSH in IVF cycles produces large increases in inhibin A, B, pro-αC and the α subunit which are correlated with the number of follicles developing (Lockwood et al., 1996).

In our experimental model we have examined the role of LH in the control of secretion of the inhibin family of glycoproteins by first inhibiting endogenous pituitary gonadotrophin secretion with buserelin and then treating patients with either pure rFSH alone or urinary gonadotrophin containing FSH and LH. Secretory profiles of immunoactive inhibin A,B (inh A,B), total α inhibin (inh α), pro-αC and oestradiol were compared in the two groups of women. Some of these results have been presented in a preliminary form elsewhere (Fawzy et al., 1998).

Materials and methods

Women presenting with unexplained or tubal infertility at the Human Assisted Reproduction Unit in Ireland (HARI), Rotunda Hospital, Dublin were recruited to take part in this study. The patients were endocrine normal and had regular menses. Ethical Committee approval and specific consent to participate were obtained. The study was a randomised, prospective, assessor-blind, comparative study. We examined the secretory profiles of inh A,B, total inh α, pro-αC and oestradiol in women following pituitary down-regulation with buserelin (long protocol) (Kondaveeti-Gordon et al., 1996) who were randomised to receive either Normegon (25:75 IU/ampoule of LH:FSH; n = 11; Organon, Cambridge, UK), or rFSH (75 IU/ampoule of FSH alone: Puregon and Gonal-F; rFSH; Serono, Welwyn Garden City, Herts, UK). Puregon and Gonal-F are similar both with respect to their physiochemical and biological characterisation and clinical efficacy (Brinsden et al., 2000; Horsman et al., 2000). Furthermore in this study no differences were observed in response to the two preparations (Puregon, n = 10 patients and Gonal-F, n = 6 patients) and therefore the data were combined into a single rFSH group (n = 16).

This was the first IVF cycle the women had undertaken. They were of similar age mean (range) 33 (24–39) years and length of infertility mean (range) 4 (2–8) years (Table I). IVF treatment was as described previously (Harrison et al., 1992) except they were randomised to receive Normegon or rFSH. A randomisation code for choice of gonadotrophin therapy, blind to the clinician, was computed by the pharmacist. Blood was taken prior to down regulation (pretreatment), after down regulation (day 0) and on days 5, 8, 9, 10 and 11. All post-treatment samples were taken 24 h post gonadotrophin injection. In addition, in five women from each group blood samples were taken 3 h post gonadotrophin injection on day 8 for immunoactive LH analysis. The blood was allowed to clot, serum separated and stored at −80°C until hormone assay. Inhibin A (Groome et al., 1994, Muttukrishna et al., 1994), inhibin B (Groome et al., 1996), pro-αC inhibin (Groome et al., 1995), total α inhibin (Knight et al., 1989, 1990), FSH, LH and oestradiol were measured by immunoassay.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normegon</th>
<th>rFSH</th>
</tr>
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<tbody>
<tr>
<td>Age years mean (range)</td>
<td>31.5 (26–35)</td>
<td>33.6 (24–39)</td>
</tr>
<tr>
<td>Cause of infertility:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubal</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Unexplained</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Primary infertility (%)</td>
<td>55</td>
<td>63</td>
</tr>
<tr>
<td>Duration of infertility years mean (range)</td>
<td>4.5 (3–7)</td>
<td>3.9 (2–8)</td>
</tr>
<tr>
<td>LH (IU/L)*</td>
<td>2.0 ± 1.1</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>FSH (IU/L)*</td>
<td>3.6 ± 1.0</td>
<td>3.5 ± 1.2</td>
</tr>
<tr>
<td>Oestradiol (pmol/l)*</td>
<td>66 ± 21</td>
<td>82 ± 19</td>
</tr>
</tbody>
</table>

Twenty-five patients completed the treatment cycle. Two treatment cycles in the rFSH-alone group were abandoned due to poor ovarian stimulation. The duration of stimulation was similar in both groups (mean; range 9.1; 8–11 days for Normegon and 9.4; 7–11 days for rFSH) as were the units of FSH administered (2230; 1350–3600 IU) for Normegon and 2764 (1575–4050) IU for rFSH. The number of follicles ≥14 mm in diameter collected from each group was similar 17; (5–24) for Normegon and 14; (6–27) for rFSH.

Oestradiol assay

Serum oestradiol concentrations were measured by Delfia kit (Wallac, Milton Keynes, UK) according to the manufacturer’s protocol. The assay has a limit of detection of 50 pmol/l and intra- and inter-variation of <10 and <13% respectively over the range 100–1200 pmol/l.

Inhibin A assay

Inhibin A was measured by ELISA using a commercial kit (Serotech, Kidlington, Oxford, UK). The assay has minimal cross reaction with inhibin B, pro-αC or activins. The limit of detection is 4 pg/ml and intra- and inter-assay variation was <6 and <16% respectively over the working range 4–500 pg/ml.

Inhibin B assay

Inhibin B was measured by ELISA using a commercial kit (Serotech, Kidlington, Oxford, UK). The assay has minimal cross reaction with inhibin pro-αC or activins, and ~ 1% cross reaction with inhibin A. The limit of detection is 16 pg/ml and intra- and inter-assay variation was <6 and <13% respectively over the working range 16–1000 pg/ml.

Inhibin pro-αC assay

Inhibin pro-αC was measured by ELISA using a commercial kit (Serotech, Kidlington, Oxford, UK). The assay has less than 0.1% crossreactivity with inhibin-A, inhibin-B, activin-A, activin-B and follistatin. The limit of detection is 2 pg/ml and intra- and inter-assay variation was <10 and <15% respectively over the working range 2–200 pg/ml.

Total α inhibin

Total α inhibin was measured by an α subunit-directed inhibin radioimmunoassay (Knight et al., 1989; Muttukrishna et al., 1994). The detection limit of the assay was 2ng/ml and the mean intra- and inter-assay coefficients of variation (CV) were 7 and 12% respectively over the working range 2–12.5 ng/ml.

FSH and LH immunoassays

Serum samples were assayed for FSH and LH using the appropriate Delfia time-resolved fluorescence immunoassay kits following the
Figure 1. Circulating concentrations of inhibin A (pg/ml, upper panel), inhibin B (pg/ml, lower panel) during treatment of women with Normegon (●) (n = 11) and recombinant FSH (□) (n = 14). Results are presented as mean ± SD.

Table II. Hormone production calculated as the secretion per follicle ≥14 mm.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Normegon</th>
<th>rFSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol (pmol/l)</td>
<td>330 ± 120</td>
<td>360 ± 110</td>
</tr>
<tr>
<td>Total inhibin (ng/ml)</td>
<td>0.9 ± 0.6</td>
<td>0.5 ± 0.3*</td>
</tr>
<tr>
<td>Pro-αC inhibin (ng/ml)</td>
<td>0.42 ± 0.24</td>
<td>0.26 ± 0.12*</td>
</tr>
<tr>
<td>Inhibin A (pg/ml)</td>
<td>110 ± 50</td>
<td>90 ± 40</td>
</tr>
<tr>
<td>Inhibin B (pg/ml)</td>
<td>90 ± 20</td>
<td>110 ± 60</td>
</tr>
</tbody>
</table>

*indicates P < 0.05 compared to Normegon. Results are presented as mean ± SD.

manufacturer’s instructions. The limit of detection of the serum assay was 1 IU/l (FSH) and 0.6 IU/l (LH) and the intra and inter-assay CV’s were <6 and <15% respectively over the range 1–250 IU/l.

Classification of embryo quality

This was performed 44 h post-insemination as per established criteria (Plachot et al., 1987).

Statistical analysis

Unless otherwise stated all data are presented as the mean ± SD. For the purposes of calculation, values falling below the limit of detection of the hormone assays were assigned a value halfway between zero and the limit of detection. The data were analysed using GraphPad Instat version 3.01 for Windows 95, (GraphPad Software; San Diego, California, USA). Log transformation of hormone data was used to normalise the distribution. Unpaired two-tailed tests were used to compare normally distributed continuous variables. Fisher’s exact tests were used to compare categorical outcomes and when appropriate, Spearman’s rank correlation coefficient (r) was calculated.

Results

Concentrations of LH, FSH and oestradiol in women following pituitary down regulation were similar in the two groups as were the units of FSH given, duration of treatment and number of follicles of ≥14 mm on the day of HCG (Table I). FSH concentrations were similar throughout Normegon or rFSH administration, rose for the first 5 treatment days and peaked on days 9–11. Thus, FSH concentrations in the Normegon group rose from 3.6 ± 1.0 IU/l after down regulation to 11.6 ± 1.6 IU/l (day 5) and to 16.2 ± 7.3 IU/l (day 10) whilst in the rFSH group concentrations rose from 3.5 ± 1.2 IU/l after down regulation to 12.6 ± 2.4 IU/l (day 5) and to 21.4 ± 7.1 IU/l (day 10). Immunoreactive LH concentrations were similarly low (<1.6 IU/l) in both groups throughout treatment due to the short half-life and consequent clearance in the 24 h
post injection period. In five patients from each group blood samples were taken for LH analysis 3 h after gonadotrophin injection on day 8. In this small subset of patients LH was higher (P = 0.001) in the Normegon group (2.0 ± 0.7 IU/l) than in the rFSH (1.0 ± 0.2 IU/l) group.

Inhibin A and inhibin B concentrations also increased during both Normegon and rFSH administration, again reaching a peak on days 9–11 (Figure 1). However, their concentrations were highly variable being 1517 ± 652 pg/ml (Normegon) versus 1009 ± 408 pg/ml (rFSH) for inhibin A and 1908 ± 917 pg/ml (Normegon) versus 941 ± 418 pg/ml (rFSH) for inhibin B respectively on day 10 of treatment (Figure 1). In contrast, total α and pro-αC concentrations were lower (P < 0.05) in the rFSH group during the final days of gonadotrophin stimulation being 18.9 ± 15.9 ng/ml (Normegon) versus 4.6 ± 2.8 ng/ml (rFSH) for total α inhibin and 8.5 ± 6.8 ng/ml (Normegon) and 2.8 ± 1.6 ng/ml (rFSH) for pro-αC inhibin on day 10 (Figure 2). The corresponding results for oestradiol were 5570 ± 3108 pmol/l and 3381 ± 1046 pmol/l (Figure 2). The hormone production per follicle ≥14mm is given in Table II and as can be seen the concentrations of inhibin α and pro-αC per follicle are lower in the rFSH group compared with the Normegon group in contrast to oestradiol, and inhibin A and B.

In view of the dissociation between total α inhibin secretory patterns observed in the Normegon and rFSH groups, an analysis of the relationship between this parameter and clinical correlates of IVF outcome were performed. Total α inhibin concentrations on days 9–11 were positively correlated with the number of mature follicles (r = 0.85; P < 0.0001), mature oocytes (r = 0.80; P < 0.0001) and fertilization rates (r = 0.4; P = 0.03). Moreover, if the patients were divided on the basis of having a total α inhibin concentration of <4 or ≥4 ng/ml it can be seen that the latter group had more follicles of ≥14mm, more mature oocytes, greater fertilization rates but required less gonadotrophin (Table III). Furthermore, the number of good (grade 1) embryos found in the higher total α inhibin group was 58% compared to 6% in the low total α inhibin group (Table IV), in which only two patients had received Normegon.

Discussion

Our experimental design was chosen to allow us to probe the role of LH in the secretion of the various species of immunoreactive inhibin by comparing their secretory patterns in patients treated either with rFSH, containing no LH, or a urinary gonadotrophin preparation, Normegon, containing both FSH and LH, but no added HCG.

We found immunoactive LH concentrations of ~1.6 IU/l (Normegon) and ~1.1 IU/l (rFSH) 24 h post gonadotrophin injection. These concentrations of LH would not reflect peak values as the blood samples were taken 24 h post injection and LH has a short initial half-life of ~1 h and a terminal half-life of 10–12 h (le Cotonnec et al., 1998). However, in those samples taken 3 h post gonadotrophin injection from five women from each group on day 8, immunoactive LH was higher in the Normegon group (2.0 ± 0.7 IU/l) than in the rFSH group (1.0 ± 0.2 IU/l). Oestradiol secretion was not different in the two groups although it tended to be lower in the rFSH group suggesting that in some of these patients there may have been insufficient endogenous LH to sustain oestradiol secretion at the concentrations seen with patients treated with Normegon. Our data are in general compatible with previous observations on the threshold concentrations of LH required to sustain normal concentrations of oestradiol secretion in stimulated cycles ie ≥1–2 IU/l (Chappel and Howles, 1991; Schoot et al., 1992; Mitchell et al., 1996; Fleming et al., 1998). It is clear that, although FSH may be the only factor required to induce follicular growth in the human, LH or a product derived from its action may assist in producing fully mature follicles and oocytes capable of fertilization (Schoot et al., 1992; Shoham et al., 1993; Balasch et al., 1995).

We have shown that inhibin A and inhibin B increase progressively in the blood with exogenous gonadotrophin treatment and were similar in both the Normegon and rFSH groups, indicating that they are primarily controlled by FSH. Serum concentrations of inhibin α and pro-αC increased preferentially in the Normegon group in contrast to the rFSH group, indicating that they are primarily stimulated by LH. Moreover, total inhibin and pro-αC concentrations, calculated

<table>
<thead>
<tr>
<th>Parameter</th>
<th>α inhibin (&lt;4 ng/ml)</th>
<th>α inhibin (≥4 ng/ml)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of patient (years)</td>
<td>33 ± 1.9 (31–37)</td>
<td>33 ± 2.9 (26–37)</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>4 ± 1.6 (3–8)</td>
<td>5 ± 1.5 (3–8)</td>
<td>NS</td>
</tr>
<tr>
<td>Length of stimulation (days)</td>
<td>10 ± 0.7 (8–10)</td>
<td>9 ± 1.3 (7–11)</td>
<td>NS</td>
</tr>
<tr>
<td>Dose of gonadotrophin (ampoules)</td>
<td>41 ± 10 (18–48)</td>
<td>32 ± 9 (21–48)</td>
<td>0.03</td>
</tr>
<tr>
<td>Follicles ≥14mm</td>
<td>10 ± 3 (5–16)</td>
<td>18 ± 4 (12–27)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Mature oocytes</td>
<td>8 ± 4 (2–14)</td>
<td>16 ± 4.4 (9–24)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Fertilized oocytes</td>
<td>4 ± 2.6 (0–9)</td>
<td>7 ± 3 (2–15)</td>
<td>0.01</td>
</tr>
<tr>
<td>Cleaved oocytes</td>
<td>3 ± 2.6 (0–9)</td>
<td>3 ± 1 (1–5)</td>
<td>NS</td>
</tr>
<tr>
<td>Embryos transferred</td>
<td>2 ± 1 (0–3)</td>
<td>3 ± 0.6 (1–3)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Results presented as mean ± SD (range). P < 0.05 is significant, NS = non-significant.
as the secretion per follicle $\geq$14mm, was such that the output per follicle was significantly decreased in the rFSH group.

The regulatory mechanisms that give rise to differential granulosa cell expression of inhibin $\alpha$ and inhibin/activin $\beta_A$ and $\beta_B$ subunits during follicle development in primates are poorly defined but are likely to involve an interplay between gonadotrophins and locally-produced factors including steroids and peptide growth factors (Knight, 1996). Expression of $\beta_B$ mRNA is predominant in preantral and small antral follicles which express relatively little $\beta_A$ and $\alpha$ subunit; this implies that activin B and inhibin B are preferentially synthesized in small follicles (Schwall et al., 1990; Roberts et al., 1993). Pre-ovulatory follicle growth is associated with increased expression of $\beta_A$ and $\alpha$ subunit and decreased expression of $\beta_B$ subunit (Schwall et al., 1990; Roberts et al., 1993), consistent with the finding of increased circulating concentrations of inhibin A, total $\alpha$ inhibin but not inhibin B during the spontaneous follicular phase (Groome et al., 1994, 1996; Muttukrishna et al., 1994) most likely in response to the changing concentrations of FSH taking place during the latter stages of the follicular phase of the cycle. Furthermore, the observation that serum inhibin B concentrations rise in parallel with inhibin A during FSH-induced multiple follicular development in down-regulated, LH-deficient treatment cycles (Lockwood et al., 1996; present study) further supports a role for FSH in promoting expression of $\alpha$ and $\beta$ subunits in the human follicle (Eramaa et al., 1994). However, in late-stage preovulatory follicles and granulosa-lutein cells which have acquired LH receptors, expression of $\alpha$ and $\beta_A$ subunit mRNA (but not $\beta_B$ mRNA) and secretion of inhibin A and total $\alpha$ inhibin are positively regulated in vitro by LH (Hillier et al., 1989, 1991; Eramaa et al., 1994, 1995). Indeed, granulosa-lutein cells of the primate corpus luteum secrete high concentrations of inhibin $\alpha$ subunit and inhibin A in an LH/HCG-responsive manner (Webley et al., 1991; Fraser et al., 1995). Thus, under conditions of combined FSH and LH drive, as in the case of our patients treated with Normegon, the $\alpha$ subunit is synthesized and secreted in even greater excess due to the direct effect of LH.

One other major observation emerged from our study, that is the correlation of increased concentrations of total $\alpha$ inhibin with a variety of clinical parameters likely to be associated with a positive outcome of treatment. This observation supports earlier studies using the classical Melbourne radioimmunoassay which indicated that total inhibin secretion in the last days of stimulation treatment might be a good predictor of IVF clinical outcomes (McLachlan et al., 1986; Hughes et al., 1990; Matson et al., 1991; Buckler et al., 1992; Mitchell et al., 1996).

However, this appears incompatible with other evidence indicating that inhibin $\alpha$ subunit can compete with FSH for binding to its receptor (Schneyer et al., 1991) and reduce poor development of bovine oocytes to form blastocysts (Silva et al., 1999). It should be noted, however, that while the number of mature oocytes recovered per subject in the present study was positively linked to their serum inhibin $\alpha$ concentrations, the percentage developing to the $\geq$4-cell stage tended to be lower in the group with high inhibin $\alpha$ concentrations (60.5 versus 77.7%, NS). Moreover, developmental competence was only recorded up to the $\geq$4 cell stage in the present study, rather than the blastocyst stage as in Silva et al. (1999).

We conclude that inhibin A and inhibin B secretion was similar in both groups and is primarily controlled by FSH whereas inhibin $\alpha$ and pro-$\alpha$C secretion was lower in the rFSH group and are primarily stimulated by LH. Moreover, total $\alpha$ inhibin secretion in the final days of treatment was positively correlated with a number of clinical outcomes likely to influence IVF outcome.

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Gonadotrophin LH/FSH ratio and secretion of inhibin


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