Oestradiol and immunoreactive inhibin-like secretory patterns following controlled ovarian hyperstimulation with urinary (Metrodin) or recombinant follicle stimulating hormone (Puregon)


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Inhibin (and its α-subunit) may be of particular value as a marker for follicular development in in-vitro fertilization (IVF) in comparison with the classic follicle stimulating hormone (FSH)-dependent marker oestradiol in patients following pituitary desensitization and treatment with recombinant FSH (rFSH). This preparation lacks luteinizing hormone (LH), which is essential for thecal cell androgen secretion and thus oestradiol production. Our study has assessed oestradiol and immunoreactive inhibin-like secretion following ovarian stimulation with rFSH or a purified urinary FSH preparation (Metrodin) (uFSH). A randomized, assessor-blind study was initiated using patients receiving a single treatment cycle of IVF (using fresh embryos) following pituitary desensitization with intranasal buserelin (500 μg daily) and the i.m. injection of either rFSH (n = 38) or uFSH (n = 17). Ovarian ultrasound examinations were performed and bloods (10 ml) collected prior to FSH treatment and every 1–2 days until ovulation induction with human chorionic gonadotrophin. LH and FSH concentrations were measured by an immunoradiometric assay, and inhibin-like immunoreactivity by a radioimmunoassay and an enzyme-linked immunosorbent assay, both with α-subunit specificity. Oestradiol concentration was measured with a coated tube radioimmunoassay. Following desensitization, basal LH, FSH and oestradiol concentrations were measured, as was that of immunoreactive inhibin. Following treatment with either rFSH or uFSH, LH concentrations remained low while FSH concentrations rose to a plateau of 5.6–6.7 IU/l in both groups. In contrast, the concentration of oestradiol was higher (P < 0.05) with rFSH than with uFSH in the last four days of treatment, a pattern that was repeated for inhibin-like immunoreactivity. The change in oestradiol and inhibin concentrations during treatment was ~2-fold higher with rFSH. The total number of follicles obtained with rFSH was similar to that with uFSH. However, the number of follicles with a diameter of ≥15 mm was higher in the rFSH group, and there was a concomitant increase in the number of oocytes recovered. Oestradiol concentration and inhibin-like immunoreactivity (determined by either method) were associated with total follicle number and number of follicles ≥15 mm in diameter, as well as with each other (P < 0.001). When ovarian hormone output was normalized per follicle produced, oestradiol output was higher for rFSH than for uFSH (P = 0.04). Inhibin output was clearly higher using rFSH than uFSH. There were seven pregnancies (one miscarriage) with rFSH and two with uFSH. Despite similar concentrations of FSH in patients, rFSH (Puregon) appears to be more potent in vivo in terms of follicular number, ovarian hormone secretion (both concentration and output/follicle) and oocyte recovery. In both groups, LH concentrations of ~1.3 IU/l were sufficient to support oestradiol secretion similar to that normally found in IVF programmes using human menopausal gonadotrophin preparations containing large amounts of LH. Despite the known problems of specificity with the assay of inhibin, its measurement was of similar value to oestradiol as a marker of follicular development.

Key words: FSH/inhibin/IVF/recombinant proteins

Introduction

The gonadotrophins that are currently used for ovarian stimulation are preparations from the urine of post-menopausal women and consist of a mixture of luteinizing hormone (LH) and follicle stimulating hormone (FSH) either in equal proportions (Humegon and Pergonal) or with a reduced (Normegon) or very low (<1 IU/l; Orgofol, Metrodin and Metrodin-HP) LH content. With the exception of Metrodin-HP, these preparations contain contaminating urinary proteins (Morse et al., 1988; Rodgers et al., 1992, 1995; Stokman et al., 1993; Giudice et al., 1994), are composed of different populations of FSH glycoforms (Lambert et al., 1995) and may have some variability in the biological and immunoreactive content (Rodgers et al., 1992, 1995).

The relatively poor pregnancy rates associated with assisted reproduction procedures have triggered a drive for improvements in the treatment of infertility. One possibility lies in the use of highly pure recombinant hormones which will allow for the modification of clinical strategies and a tighter control of follicular maturation in in-vitro fertilization (IVF) protocols (Hillier, 1994). Recombinant FSH (rFSH) preparations are currently being assessed in assisted reproduction (Out et al., 1995; Recombinant Human FSH Study Group, 1995) and clearly offer advantages over most urinary counterparts because they contain no contaminating proteins, can be self-administered and should be easier to control with regard to
both the quality and quantity of hormone from one batch to another.

In assessing the development of follicular growth in ovulation induction cycles, it is standard practice to combine the ultrasound examination of follicular number and size with a hormonal marker, such as oestradiol. The development of inhibin assays, despite some problems with their specificity, has led to the identification of this FSH-dependent ovarian product as a further potential marker for follicular development (McLachlan et al., 1986a; Hughes et al., 1990; Matson et al., 1991; Buckler et al., 1992). The usefulness of inhibin-like immunoreactivity rather than oestradiol as a more predictive marker for follicular maturation has yet to be investigated in women being treated with rFSH following pituitary desensitization. In such patients, inhibin and its subunit may be of particular relevance because the rFSH preparation, Puregon, has no intrinsic or added LH, the hormone essential for thecal and androgen secretion and thus the production of the classic FSH-dependent marker of follicular growth, oestradiol. Therefore our study was designed to compare the secretory patterns of inhibin-like immunoreactivity and oestradiol following the administration of either rFSH (Puregon) or the urinary preparation, Metrodin. The hormones were assessed following pituitary desensitization and were compared for follicular number and size and for the number of oocytes.

**Materials and methods**

**Patients**

Women (n = 55, age range 25–35, mean 32 years), mostly with tubal infertility, were entered into the IVF programme at St Mary's Hospital, Manchester, UK, as part of a larger clinical trial (Out et al., 1995) on the efficacy of rFSH (Puregon; Organon Laboratories, Cambridge, UK). The cause of infertility in the patients had to be potentially remediable by IVF, and the patients were allowed a maximum of three previous IVF, gamete intra-Fallopian transfer or zygote intra-Fallopian transfer attempts in which oocytes were collected at least once. In addition, their ovulatory cycles were normal hormonally (mean length 24–35 days), they had to be in good physical and mental health and had been willing to give written informed consent to the study which was approved by the hospital ethics committee. The patients were administered the intranasal gonadotrophin-releasing hormone (GnRH) agonist buserelin daily (500 µg; Suprefact; Hoechst UK Ltd, Hounslow, UK). Blood (10 ml) was collected at 2–3 day intervals for 8–10 days until the oestradiol and LH concentrations in the serum were <150 pmol/l and <2.0 IU/l respectively. An ovarian ultrasound examination was then performed to count the follicular number and size prior to the commencement of FSH therapy (n = 38 with rFSH (Puregon); n = 17 with uFSH (Metrodin; Serono Laboratories, Welwyn Garden City, UK)) to promote follicular maturation. Both preparations were prepared as ampoules containing 75 IU FSH, as assessed against the same in-vivo bioassay. Patients received two to three i.m. injections daily. Blood sampling (10 ml) and ovarian ultrasound examination were performed at regular intervals (1–2 days for the first week of treatment; daily for the last week), until a number of follicles >17 mm in diameter were clearly detectable. Human chorionic gonadotrophin (HCG; 10 000 IU; Profasi; Serono Laboratories, Woking, UK) was given i.m. in the evening after 10.30 p.m. Oocyte recovery was performed 36 h later using an ultrasound-guided needle. Following aspiration, all the eggs were incubated in Medi-cult IVF culture medium (Imperial Laboratories, Andover, UK) for 24 h prior to insemination.

**Hormone assays**

Oestradiol concentration was measured with the DPC coat-a-count assay (Diagnostic Products Corporation, Los Angeles, CA, USA) by following the standard protocol outlined by the manufacturer. The sensitivity of the assay (defined as the lowest kit standard concentration, provided a >2 SD difference in counts from the zero standard was consistently recorded) was 73 pmol/l. The intra- and interassay coefficients of variation (CV) were <7 (determined from duplicate samples) and <8% (determined from four quality control sera containing 120–300 pmol/l and oestradiol) respectively.

LH was measured with the LH Miacclidean immunoradiometric assay (IRMA) kit (Serono Diagnostics Ltd, Woking, UK) according to the standard protocol. The sensitivity of the assay, as defined above, was 0.5 IU/l relative to the pituitary reference preparation 68/40 (NIBSC, Potters Bar, UK). The intra-assay CV at <2 IU/l was <11%, and was 2–10% at ≥2 IU/l. The interassay CV based on four quality control sera at 1.2–40.2 IU/l was <13.5%, and the specificity quoted by the manufacturer was <0.1% cross-reactivity for FSH/thyroid-stimulating hormone (TSH) and <0.03% for HCG. LH was measured with an in-house IRMA kit in routine use for measuring clinical samples in the Department of Clinical Biochemistry, Hope Hospital, Salford, UK. It employed a 125I-labelled mouse anti- intact human FSH monoclonal antibody (Serotec Ltd, Oxford, UK; stated by the manufacturer to have no cross-reactivity with β-FSH or any other gonadotrophin) and a solid phase-bound (Dynospheres, Dyno Particles, Lillestrom, Norway) sheep anti-intact human FSH polyclonal antibody (Scottish Antibody Production Unit, Karluke, UK). The standard curve was constructed using a human pituitary FSH secondary standard (Porton Products, Porton Down, UK) standardized against the pituitary reference preparation 78/549 (NIBSC). A concentration of 0.8 IU/l gave a >2 SD difference in counts between itself and the zero standard. An intra-assay CV of <10% was observed across the range 0.8–40.0 IU/l and the inter-assay CV based on four quality control sera of 1.7–16.4 IU/l was <8%. The cross-reactivity with LH, TSH and HCG was <0.1% (data derived from the National External Quality Assurance Scheme, NEQAS, Edinburgh, UK) and the assay was performed within acceptable limits proposed by NEQAS.

Inhibin and its subunit were measured using the inhibin radiomunoassay (RIA-inh; McLachlan et al., 1986b) and an enzyme-linked immunosorbent assay (ELISA; E-inh; Medgenix Diagnostics, Fleurus, Belgium). Both assays employ antibodies directed to epitopes on the α-subunit of inhibin and cross-react with the free α-inhibin subunit and some inhibin precursor peptides (Schneyer et al., 1990), both of which circulate in human serum. Because of this, the term immunoreactive inhibin-like material (or immunoreactive inhibin) is used to differentiate between that measured by these assays and the more specific two-site assays for dimeric inhibin (Groome et al., 1994; Muttukrishna et al., 1994). It is important to note that in the absence of an International Reference Preparation for human inhibin, the units defined in the two assays are not equivalent. The inhibin radioimmunoassay employed an antibody raised against 31 kDa bovine inhibin and has been validated in a variety of clinical studies (reviewed in Burger, 1993, 1994). Its sensitivity is 58 U/l and the intra- and interassay CV calculated from repeated serum measurements of women undergoing IVF procedures were <8%. The specificity was <0.1% cross-reactivity for human LH, FSH, free gonadotrophin α-subunit, HCG and insulin-like growth factor (IGF)-1 (McLachlan et al., 1986b). The Medgenix ELISA (Poncelet and Franchimont, 1994) was used in accordance with the manufacturer's instructions.
and was standardized against the Medical Research Council porcine standard code 86/690. The limit of detection was 100 U/l and the intra- and interassay CV of four quality control sera (1900–9000 U/l) were <6 and <10% respectively. Supra-physiological concentrations of FSH, LH, HCG, human seminal inhibin-like peptide, activin A and IGF-I were without effect on the assay at 2.5 U/ml.

Statistical methods
The data were split according to whether patients had been treated with rFSH or uFSH. All hormone data were normalized relative to the day of HCG injection (day 0) and are expressed as mean ± SD. Data from rFSH and uFSH groups were compared at given time-points by an unpaired Student’s t-test. Ovarian hormone outputs per follicle produced were compared in the two treatment groups by performing a paired Student’s t-test over days −4 to 0. Correlations were performed using Pearson’s correlation coefficient. Significance was defined as \( P < 0.05 \).

Results

Hormone changes
Following pituitary desensitization and immediately prior to the commencement of treatment with FSH, serum LH and FSH concentrations in all patients \((n = 55)\) were 1.3 ± 0.6 and 2.7 ± 1.0 IU/l respectively. Oestradiol concentrations of 106 ± 37 pmol/l confirmed a successful desensitization protocol. Inhibin-like immunoreactivity levels were 62.0 ± 17.6 (RIA-inh) and 700.0 ± 300.0 U/l (E-inh). Treatments with rFSH \((2151 ± 583 \text{ IU, } n = 38)\) and uFSH \((2466 ± 865 \text{ IU, } n = 17)\) were of similar duration \((12.0 ± 2.7 \text{ versus } 12.5 ± 2.5 \text{ days respectively})\) and failed to alter the LH concentration, while the FSH concentration rose to a plateau of 5.6–6.7 IU/l between days −9 and +1 (Figure 1). There were no differences in the FSH concentrations between the two treatment groups. In contrast, oestradiol concentrations over days −4 to +1 were higher \((P < 0.05)\) with rFSH \((7428 ± 4074 \text{ pmol/l})\) than with uFSH \((4811 ± 941)\) (Figure 2). This pattern was repeated for RIA-inh \((5905 ± 3129 \text{ IU/l})\) and E-inh \((23900 ± 9500 \text{ U/l})\) (Figure 2). The change in oestradiol and RIA/E-inh levels from days −9 to 0 was 2-fold higher in the rFSH group; oestradiol/RIA-inh and E-inh were all highly correlated with each other both within treatment groups and using all patients \((P < 0.001, r > 0.5)\).

Follicular development
The total number of follicles in the rFSH group rose from 1.1 ± 1.0 (day −9) to 9.6 ± 4.3 (day 0). A similar pattern was observed for uFSH \((1.0 ± 0.9 \text{ at day } −9 \text{ to } 7.6 ± 3.9 \text{ at day } 0)\) (Figure 1). While the total numbers of follicles at day 0 were similar, the number of follicles with a diameter of ≥15 mm was higher with rFSH \((6.2 ± 2.3 \text{ versus } 4.6 ± 2.1; P < 0.01)\). There was also a concomitant increase in the number of oocytes recovered \((7.2 ± 4.6 \text{ rFSH versus } 3.3 ± 2.4 \text{ uFSH}; P < 0.01)\). Oestradiol and immunoreactive inhibin levels were all correlated with the total number of follicles and the number of follicles ≥15 mm in diameter \((P < 0.001)\). There was no evidence that inhibin was correlated with these parameters to a higher degree than oestradiol. However, when the ovarian hormone output was normalized per follicle produced over days −4 to 0 (Figure 3), the oestradiol output was higher for rFSH \((365 ± 128 \text{ pmol/l/follicle})\) than for uFSH \((292 ± 85 \text{ pmol/l/follicle}; P = 0.04)\), a pattern that was repeated with more discrimination (i.e. a higher level of significance) for immunoreactive inhibin output after rFSH \((392 ± 45 \text{ RIA-inh and } 1393 ± 167 \text{ E-inh})\) compared with uFSH \((307 ± 42, P = 0.001)\) RIA-inh and 1099 ± 269, \(P = 0.008\) E-inh). There were seven pregnancies (one miscarriage) with rFSH and two with uFSH.
Figure 3. Ovarian hormone output per total number of follicles produced during treatment between days -4 and 0, normalized relative to the day of ovulation induction with human chorionic gonadotrophin (HCG), recombinant follicle stimulating hormone (FSH; ○) or urinary FSH (C). Data are mean RIA-inhibin/follicle (A), E-inhibin/follicle (B) and oestradiol/follicle (C).

Discussion

Classically, oestradiol secretion has been used as a hormonal marker of follicular development in IVF programmes. However, its use may have its problems because the concentrations of this hormone are dependent on the availability of LH to provide an androgen precursor pool for FSH-stimulated oestradiol production. This situation may prevail when LH-free preparations are used in combination with full down-regulation protocols, during which LH concentrations may fall below a threshold value sufficient to drive androgen synthesis within the theca interna. As earlier work has indicated that immunoreactive inhibin-like material has potential as a marker of follicular development (McLachlan et al., 1986a; Hughes et al., 1990; Matson et al., 1991; Buckler et al., 1992; Burger, 1993), because its production is FSH dependent, our study examined the relative merits of oestradiol and inhibin as markers of follicular number and size in patients treated with rFSH following pituitary down-regulation.

In our study, oestradiol concentrations increased markedly during multi-follicular development, even with the use of LH-free rFSH. One problem with the study was that the buserelin failed to completely suppress LH and FSH secretion and the residual LH concentration of 1.3 IU/l was sufficient to support the secretion of oestradiol. Both oestradiol and inhibin-like immunoreactivity (measured by two different methods) concentrations were highly correlated with follicular number, suggesting that both were good follicular markers. However, the combined output of inhibin and its subunit per follicle was slightly more discriminating than oestradiol in revealing differences in the potencies of rFSH and uFSH. Our data on oestradiol secretion confirm those of Devroey et al. (1994), who reported that Puregon induced elevated oestradiol secretion in a cohort of patients down-regulated with a variety of GnRH agonist protocols and in whom LH concentrations were >1.2 IU/l. In contrast, Schoot et al. (1994), who used Puregon to induce multi-follicular growth in five hypogonadotrophic hypogonadal women, found increased immunoreactive inhibin secretion as treatment progressed, whilst oestradiol concentrations did not exceed those found in the early follicular phase of the normal menstrual cycle. In that study the concentrations of LH were <0.37 IU/l using the Delfia assay. Multi-follicular development and low oestradiol outputs have also been reported in primates treated with another rFSH preparation after pituitary desensitization with a GnRH antagonist (Karnitis et al., 1994). Furthermore, another study showed that the use of menopausal gonadotrophin (urinary LH and FSH combined) resulted in similar follicular growth but high oestradiol secretion. These data support the classic two-cell theory that FSH-stimulated oestradiol secretion is dependent on the production of ovarian androgen precursors from the theca interna, which is LH dependent. However, the maturing follicle appears to need only FSH for its growth (Thompson et al., 1995). Taking all of these studies together, the threshold amount of LH required for normal oestradiol secretion is likely to be between 0.3 and 1.0 IU/L. Concentrations of LH lower than the threshold value may become more common following the higher degrees of pituitary desensitization that can be achieved using GnRH antagonist analogues, e.g. Nal-Glu (Paulson et al., 1994; Minaretzis et al., 1995) or Cetrorelix (Olivennes et al., 1994; Sommer et al., 1994). For example, Nal-Glu desensitization lowered LH and oestradiol concentrations to a greater degree than the GnRH agonist leuprolide acetate, but resulted in a greater number of mature oocytes and embryos of better quality after treatment with uFSH despite the higher suppression (Minaretzis et al., 1995). It may be that the use of inhibin-like peptides as markers of follicular development will be of more value when using rFSH in combination with some of the down-regulation protocols outlined above, e.g. those in which levels are suppressed below the threshold for oestradiol secretion, rather than that employed here.

Problems with the specificity of α-inhibin subunit-specific immunoassays such as those used here have been highlighted (Schneyer et al., 1990) and are indicated by quantitatively non-parallel changes in the α-subunit-specific and dimeric inhibin across the normal menstrual cycle (Muttukrishna et al., 1994). When standardized identically, α-inhibin immunoreactivity is secreted in higher amounts (~10-fold) than dimeric inhibin immunoreactivity in the follicular phase, and the two parameters are only weakly correlated. Both rise at mid-cycle, but the relative increase for dimeric inhibin (20-fold) is greater than that of α-inhibin (4-fold), which in turn is similar to a 5-fold increase in oestradiol concentrations. These data suggest that the new highly specific assays for dimeric inhibin may be even more discriminating than those used in our study, and the situation will be improved further once kits are routinely standardized with pure recombinant inhibin. However, despite the difficulties with the specificity of the measurement of inhibin in our study, it is clearly a good alternative to oestradiol as a marker of follicular development. Further, as the ELISA kit used only takes 3 h to perform, it is quite practical to use
it in monitoring folliculogenesis, although it costs considerably more than comparable measurements of oestradiol.

Another feature of our study was that, in comparison with uFSH, rFSH stimulated the production of a higher number of oocytes and follicles \( \geq 15 \) mm in diameter as well as higher serum concentrations of oestradiol and immunoreactive inhibin discussed above, despite the similar serum FSH concentrations in the two groups. Taken together, these data suggest that rFSH provides a more potent stimulus to the ovary than the urinary counterpart. Indeed, rFSH also produced a higher mean number of oocytes and good embryos than Metrodin in the larger study of 1000 IVF cycles of which our patients formed a subset (Out et al., 1995). Interestingly, rFSH has different physico-chemical properties to Metrodin in that it has more basic glycoforms (Lambert et al., 1995) and is retained to a higher degree on concanavalin-A lectin affinity columns than uFSH (Harris et al., 1995), indicating that its carbohydrate chains are less sialylated and branched than those of the natural molecules. Moreover, 36% of its mass is carbohydrate compared with 28% of endogenous FSH (Storring, 1992). Unit for unit (based on the in-vivo biopotency), these less acidic glycoforms appear to endow rFSH with greater in-vitro steroidogenic biopotency than uFSH (Lambert et al., 1995), although such comparisons are difficult because of lack of information concerning the absolute mass of FSH present in the ampoules of these preparations. What is clear is that both of them are less acidic and contain less complex carbohydrate structures than the forms circulating during the follicular phase (Padmanabhan et al., 1988; Papandreou et al., 1993). Taken together, these data highlight the fact that differences in the carbohydrate sub-structure of exogenous FSH preparations may modify their biopotencies in vivo, which in the case of rFSH may be associated with the decreased charge and complexity of the hormone. This is surprising because current dogma suggests that more acidic species have a longer half-life which is believed to be the primary determinant of biological activity in vivo (Wilson et al., 1990). However, this assumption may be too simplistic because Metrodin-HP, which is far more acidic than Metrodin (Lambert et al., 1995), displays similar in-vivo pharmacokinetics (Le Cotonnec et al., 1993) and produced similar numbers of oocytes and pregnancy rates in a recent trial (Wikland et al., 1994). The relationship between the carbohydrate component of the gonadotrophins and their in-vivo activity remains unclear, although the understanding of it will underpin future developments of ‘designer’ recombinant species.

In conclusion, rFSH (Puregon) appeared to be more potent in vivo in terms of producing more follicles of a greater size, higher ovarian hormone secretion (both serum concentration and output/follicle) and oocyte recovery. Pituitary desensitization with buserelin decreased LH to 1.3 IU/L, which nevertheless was sufficient to support high levels of oestradiol secretion with both preparations. Overall, inhibin-like immunoreactivity was as good a marker of follicular development as oestradiol, and may become the biochemical indicator of choice in IVF cycles using down-regulation protocols which suppress LH concentrations below that required for normal oestradiol secretion.

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References
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