New approaches to ovarian stimulation

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Suppression of endogenous hormone production by gonadotrophin-releasing hormone (GnRH) agonists followed by controlled ovarian hyperstimulation (COH) with human gonadotrophins, especially the so-called ‘long protocol’ has developed from second-line into first-line therapy. Due to this attitude premature luteinization can be safely avoided, enhancing therapeutic efficacy. Recombinant preparations of human follicle stimulating hormone (FSH) have been proven to be effective within COH according to the long protocol. The high purity of these compounds may have clinical advantages. GnRH antagonists could be successfully introduced in COH protocols. Also, daily injections in the midcycle phase according to the ‘Lübeck protocol’, as single or only dual administrations around day 9 seem to abolish any premature LH rises. Due to their different pharmacological mode of action, based on a classic competitive receptor blockage GnRH antagonists avoid any flare-up period and allow ovarian stimulation to start within the spontaneous cycle. Pregnancy rates are comparable to those after long protocol stimulation. Combination of softer stimulation regimes like clomiphene citrate and low dose HMG with midcycle administration of GnRH antagonists may be the way to a cheap, safe and efficient ovarian stimulation. It seems to be high time for modest forms of ovarian stimulation, lowering burden and risk for our patients.

Key words: clomiphene citrate/GnRH agonists/GnRH antagonists/soft stimulation

Introduction

The use of gonadotrophin-releasing hormone (GnRH) agonists for ovarian stimulation marks the beginning of modern management in assisted reproduction techniques (ART). Premature surges of luteinizing hormone (LH) had been found to be responsible for the reduced effectiveness of ovarian stimulation by human menopausal gonadotrophin (HMG) in an in-vitro fertilization (IVF) programme. At the same time, they negatively affected oocyte and embryo quality and due to this, the pregnancy rates obtained (Stanger and Yovich, 1985; Loumaye, 1990). The introduction of agonist treatment has remedied most of these difficulties and
drawbacks, and the rate of stimulated cycles which must be terminated has been brought down to <2%. Ovulation induction is now able to be planned so that the psychological pressure on patients and physicians has been eased to some extent. Suppression of endogenous hormone production by GnRH analogues followed by HMG stimulation has developed from second-line into first-line therapy. Different treatment schedules are applied today, including the so-called ‘long protocol’, which aims at complete pituitary suppression, and the ‘short’ and ‘ultrashort’ protocols, in which the initial ‘flare-up’ of gonadotrophins is used for ovarian stimulation (Loumaye et al., 1988; Macnamee et al., 1989). Among these protocols the ‘long protocol’ is generally the most effective and is currently the most frequently used. In Germany for instance, >70% of all stimulated cycles in ART are carried out according to this protocol (Deutsches IVF Register, 1996).

The long protocol

The long protocol has become the standard method in most major centres. This protocol aims for the complete desensitization of the pituitary gland before the start of stimulation with human gonadotrophins (urinary or recombinant). For this purpose, the GnRH agonist can be administered as a monthly depot preparation in form of a s.c. (e.g. 3.6 mg goserelin) or i.m. (e.g. 3.2 mg triptorelin) formulation in the midluteal (days 21–22) or early follicular phase (day 1). The advantage of starting medication in the midluteal phase is that the flare-up of gonadotrophins coincides with the physiological rise of follicle stimulating hormone (FSH) and LH at that stage of the menstrual cycle. However, this medication schedule may accidently clash with an existing pregnancy at a very early stage. No teratogenic side-effects of GnRH agonists were reported until Ron-El et al. (1990).

Recombinant gonadotrophins

Recent work in biotechnology has made it possible to produce a recombinant preparation of human FSH in vitro. Since FSH needs to be glycosylated to be biologically active, recombinant FSH is produced by genetically engineered Chinese hamster ovary (CHO) cells, in which the genes coding for the α- and β subunits are inserted (Chappel et al., 1992). Pharmaceutical preparations of urinary HMG contain a mixture of FSH and LH and are of low specific activity (~100–150 IU FSH/mg protein). More than 95% of the protein content consists of non-specific co-purified urinary proteins. RecFSH is totally free of any LH activity and is of extreme high purity (mean specific activity 10 000 IU/mg of protein). It is debatable whether this high purity may have clinical advantages, as the discussed improved batch-to-batch consistency due to permanent control of the FSH isoform profile, contrasts with that of the current HMG and urinary hFSH preparations (Rector et al., 1993). Recent case reports suggesting that co-purified urinary proteins could lead to adverse immune reaction are a little puzzling, as
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urinary preparations have been thought to be absolutely safe for >20 years (Li and Hindle, 1993).

Due to its high purity, recFSH shows a bioavailability of 75% after s.c. administration as well as after i.m. injection. This decreases the patient’s burden to some extent (Loumaye et al., 1995). It could be demonstrated that (with the exception of WHO I patients) in spite of down-regulation of the pituitary gland by a GnRH agonist, endogenous LH secretion is sufficient to allow satisfactory ovarian stimulation by pure recFSH. A meta-analysis of eight studies of 592 patients undergoing controlled ovarian hyperstimulation (COH) with recFSH, revealed significantly higher pregnancy rates in comparison with the use of HMG (Daya, 1995). Compared with highly purified urinary FSH, recFSH showed no differences regarding follicular maturation in the long agonistic protocol (Recombinant Human FSH Study Group, 1995).

Obstacles

Ovarian stimulation using human urinary or recombinant gonadotrophins in combination with GnRH analogues has been proven to be highly efficient in ART (Smitz et al., 1993; Strowitzki et al., 1996). The long protocol synchronizes follicle maturation and allows the selection of a higher number of follicles or oocytes for IVF than the other protocols. With regard to pregnancy rates, retrospective studies have shown a significant benefit to the patients treated according to the long protocol compared with those treated by ‘flare protocol’ (De Mouzon et al., 1988). Pregnancy rates were also higher after long-protocol stimulation in prospective studies, but these differences were not significant (Tan et al., 1990).

However, the ‘long protocol’ has the disadvantages of a long treatment period until desensitization occurs as well as relatively high costs due to an increased requirement for HMG (Smitz et al., 1992). On the other hand excessive ovarian stimulation with the recovery of 30 or more oocytes is seldom seen, and large numbers of follicles and aspirated oocytes are almost always regarded as the criteria of success (Balen, 1995). It is debatable whether this is still acceptable with the advent of intracytoplasmic sperm injection (ICSI) with its high fertilization outcome independent of sperm morphology (Küpker et al., 1995a,b, 1996). The question arises, of whether we can avoid the complexities and costs of prolonged pharmaceutically-driven treatments (Edwards et al., 1996). A reduction in the amount of gonadotrophins used and in the number of mature oocytes (metaphase II) could be the goal to aim for, reducing burden and risk for the patient as well as financial costs. For this the introduction of GnRH antagonists into ovarian stimulation protocols opens a new path.

GnRH antagonists

In parallel with the development of GnRH agonists, other analogues were synthesized which also bind to the pituitary GnRH receptors, but which are not...
functional in inducing the release of gonadotrophins. These compounds are far more complex than GnRH agonists with modifications in the molecular structure not only at positions 6 and 10, but also at positions 1, 2, 3 and 8. In comparison with the GnRH agonists, the pharmacological mechanism by which GnRH antagonists suppress the liberation of gonadotrophins is completely different. While the agonists act on chronic administration through down-regulation of receptors and desensitization of the gonadotrophic cells, the antagonists bind competitively to the receptors and thereby prevent the endogenous GnRH from exerting its stimulatory effects on the pituitary cells so avoiding any ‘flare-up’. Within hours, the secretion of gonadotrophins is reduced. This mechanism of action is dependent on the equilibrium between endogenous GnRH and the applied antagonist. This antagonistic effect is highly dose-dependent in contrast with the agonists (Felberbaum et al., 1995).

While the first generation of GnRH antagonists showed allergic side-effects due to an induced histamine release which hampered the clinical development of these compounds, modern GnRH antagonists such as Ganirelix (Organon, Oss, Netherlands) or Cetrorelix (ASTA-Medica, Frankfurt am Main, Germany) seem to have solved these problems and thus may become available medically in the near future. Both of them have been used in our department (Hahn et al., 1985; Reissmann et al., 1995) (Table I).

**GnRH antagonists within COH**

Dittkoff et al. (1991) showed that a GnRH antagonist applied for a short period is capable of suppressing the ovulation-inducing midcycle LH peak. Nal-Glu (50 µg per kg body weight per day for 4 days) was administered in the midcycle phase. The LH peak failed to occur, oestradiol production came to halt and follicular growth was interrupted. After discontinuing the antagonists, gonadal function normalized within days. Apparently, antagonists neither deplete the FSH and LH stores of gonadotrophic cells nor inhibit gonadotrophin synthesis.

Transferring these results into an ovarian stimulation protocol using HMG to avoid the onset of premature luteinization, the premature LH surge also seems to be abolished by daily administration of the modern GnRH antagonist Cetrorelix from day 7 onward until ovulation induction (Figure 1), as well as by single or dual administration around day 9. In this protocol the antagonist is injected at the time when the oestradiol concentration reaches 150–200 pg/ml and the follicle size is >14 mm, which usually is the case on day 9 of the cycle (Olivennes et al., 1994; Leroy et al., 1994). Up to now, >730 patients have been treated by
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Figure 1. Ovarian stimulation with human menopausal gonadotrophin (HMG) and concomitant midcycle administration of the gonadotrophin-releasing hormone (GnRH) antagonist Cetrorelix: multiple dose application – the ‘Lübeck protocol’.

these protocols and both have been proven to be safe and effective. The discussion about the advantages and disadvantages of the two possible methods of administration is still going on, although we would like to favour the multiple dose application because of its stability, whilst preserving the advantages of the ‘long’ agonistic protocol we are accustomed to.

Two subsequent open phase II studies have been carried at our centre to elucidate the question of the dosage necessary for sufficient suppression of the pituitary gland at this critical moment of ovarian stimulation. Three dosages were administered in accordance to the multiple-dose protocol. Hormone profiles, the number of oocytes retrieved, fertilization rates and the consumption of HMG were compared.

A total of 35 patients, all suffering from tubal infertility alone with no other infertility factor, were treated as follows: starting on cycle day 2 stimulation with two ampoules of HMG daily. From cycle day 7 until induction of ovulation, 12 patients were treated with 3 mg Cetrorelix s.c./day. As no premature LH surge was observed, 12 patients received 1 mg Cetrorelix/day, and another 11 patients 0.5 mg Cetrorelix/day. On day 5, the dose of HMG was adjusted according to the individual ovarian response of patient to the stimulation, as assessed by oestradiol values and follicle measurement. This treatment was continued until induction of ovulation with 10 000 IU HCG i.m., given when the leading follicle reached a diameter of 18–20 mm as measured by transvaginal ultrasound, and when oestradiol values indicated a satisfactory follicular response.

No premature LH surges were observed. All cycles could be evaluated. Progress in the three dosage groups of FSH and LH was quite similar, with a profound suppression of LH and a less-pronounced suppression of FSH, the latter observation probably due to the longer plasma half-life of injected FSH.
In the case of oestradiol there was a distinctly greater increase in concentration in the group treated with 0.5 mg Cetrorelix/day, reaching an average maximum of 2165 pg/ml on cycle day 10, compared with 852 pg/ml in the 3 mg group and 1023 pg/ml in the 1 mg group. The results were not significant; the differences seem to indicate a slightly more sensitive reaction to the stimulation with HMG in the group treated with the lowest dosage of antagonist.

The fertilization rates of the recovered oocytes were 45.3% in the 3 mg group, 53.2% in the 1 mg group and 67.7% in the 0.5 mg group. In the 3 mg group, 106 oocytes were recovered and 30 embryos were obtained, 36.7% of them being excellent according to morphological microscopic criteria (Staessen et al., 1989). In the 1 mg group, 94 oocytes were collected and 28 embryos obtained, 53.6% being excellent. In the 0.5 mg group, 127 oocytes were recovered and 27 embryos were obtained, 37% of them being excellent (Table II).

The average number of HMG ampoules was 30 in the 3 mg group, 27 in the 1 mg group and 26 in the 0.5 mg group. These differences were not significant, but have to be compared with the higher amount of ampoules used in an agonistic ‘long protocol’ (Felberbaum et al., 1996).

Subsequent studies using 0.5 mg Cetrorelix/day as well as 0.25 mg Cetrorelix/day or 0.1 mg Cetrorelix/day proved the efficacy and safety of 0.25 mg Cetrorelix/day in avoiding premature LH surges. With doses of 0.1 mg Cetrorelix/day, premature LH surges could be observed (Albano et al., 1997). In these studies, the use of ICSI for the treatment of male subfertility of the husband was permitted, leading to fertilization rates within the range to be expected after normal oocyte maturation. Unfortunately, in these studies a median number of 33 ampoules of HMG was needed per patient, showing no important difference in consumption compared with long-protocol cycles, although a large individual variation was noted within the group (16–70 ampoules) (Albano et al., 1996, 1997) (Table III).

So far, 238 patients have been treated with Cetrorelix in phase II studies; 134 patients according to the single/dual-dose protocol and 104 patients according to the multiple dose protocol. In all, 69 pregnancies have been achieved, giving a pregnancy rate of 29%. As the minimal effective daily multiple dosage, 0.25 mg Cetrorelix/day is now being used in a multicentric phase III study. Overall, 658

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**Table II.** Recovered oocytes, fertilization rate, number and quality of embryos after controlled ovarian stimulation with human menopausal gonadotrophin (HMG) and concomitant gonadotrophin-relasing hormone (GnRH) antagonist treatment in different dosages

<table>
<thead>
<tr>
<th>Dose of GnRH antagonist (mg)</th>
<th>3</th>
<th>1</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. oocytes</td>
<td>106</td>
<td>94</td>
<td>127</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>45.3</td>
<td>53.2</td>
<td>67.7</td>
</tr>
<tr>
<td>No. embryos</td>
<td>30</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Excellent embryos (%)</td>
<td>36.7</td>
<td>53.6</td>
<td>37.0</td>
</tr>
</tbody>
</table>
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Table III. Ovarian stimulation with human menopausal gonadotrophin (HMG) and cetrorelix (3, 1, 0.5 and 0.25 mg)

<table>
<thead>
<tr>
<th>HMG (mg)</th>
<th>3b</th>
<th>1b</th>
<th>0.5b</th>
<th>0.5a</th>
<th>0.25c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>27</td>
<td>26</td>
<td>33</td>
<td>33</td>
</tr>
</tbody>
</table>

Taken from Albano et al. (1996).

bTaken from Felberbaum et al. (1996).

cTaken from Albano et al. (1997).

Figure 2. Ovarian stimulation with human menopausal gonadotrophin (HMG) and concomitant midcycle administration of the gonadotrophin-releasing hormone (GnRH) antagonist Cetrorelix at different dosages (3, 1, 0.5 and 0.25 mg/day) Cetrorelix plasma concentrations (ng/ml).

Patients have been treated with Cetrorelix and HMG for ovarian stimulation in phase II and phase III studies. In all, 171 clinical pregnancies have been obtained, reflecting a pregnancy rate of 26%. The abortion rate was 23%. Currently, Ganirelax is being used in a phase II dose-finding study.

Plasma concentrations of Cetrorelix have been shown to be dose-dependent with regard to both the concentration after first administration and the maximal concentration to be measured. With 0.25 mg Cetrorelix/day as the minimal effective dose, no plasma concentrations of Cetrorelix can be detected at the day of embryo transfer (Figure 2). Thus, there are no residual medication effects after embryo transfer with a possible negative impact on the implantation process. Concentrations of Cetrorelix in follicular fluid were also dose-dependent.

Based on the mechanism of competitive binding, it is possible to modulate the degree of hormone suppression by the dose of antagonist administered. This preservation of the pituitary response due to competitive mechanisms could be clearly demonstrated by using a GnRH test during GnRH antagonist treatment. Three hours before injecting HCG to stimulate ovulation, 25 μg of GnRH was...
administered in patients treated with 1 mg Cetrorelix per day or 3 mg Cetrorelix per day. Blood samples for LH measurement were taken before and 30 min after GnRH treatment. The mean increase was 10 mIU/ml for the 3 mg group, while the average maximum concentration of serum LH in the 1 mg group was ~32.5 mIU/ml (Figure 3). These results are significant (Felberbaum et al., 1995). They could open new paths in the treatment of patients at higher risk of developing ovarian hyperstimulation syndrome (OHSS). This approach would minimize deleterious effects of HCG administration in some cases. Ovulation induction should be possible using GnRH agonists or native GnRH itself under antagonistic treatment. This could help to lower the incidence rate of early onset OHSS (Shalev et al., 1994).

**Future perspectives of GnRH antagonists in COH**

Cetrorelix has been proven to be highly effective in ovarian stimulation with HMG as well in the form of multiple dose applications and single dose protocols. However, the combination of GnRH antagonists and recombinant FSH remains a challenge.

Cetrorelix and Ganirelrix are being tested at the present time within the frame of clinical phase II studies in combination with recombinant FSH. In contrast to urinary compounds, these preparations are free of LH activity. Their effectiveness in ovarian stimulation using the long protocol has been proven (Recombinant FSH Study Group, 1995). Even after down-regulation of the pituitary gland, endogenous LH secretion seems to be sufficient for normal ovarian sexual steroid...
biosynthesis. However, extreme suppression of LH secretion by high doses of GnRH antagonists could cause problems according to the two-cell/two-gonadotrophin hypothesis of follicular oestrogen production (Adashi, 1994). It could cause a situation very similar to that found in WHO group I infertile patients. Ovarian stimulation with pure FSH, depleted of any LH activity, could induce follicular growth in the absence of any oestrogen secretion, as has been described for these patients (Loumaye et al., 1994). Further research is needed to find the minimal effective dose, which inhibits any premature surge or tonic elevation of LH concentration within the follicular phase, while offering sufficient LH for normal oestradiol production.

**Simple ovarian stimulation**

Ovarian stimulation with clomiphene citrate alone or in combination with HMG is very simple and was the standard procedure in the early period of IVF. Nevertheless it has fallen out of use in conventional IVF, due to its low efficiency (Mettler et al., 1984; Abdalla et al., 1987, 1990). On the other hand, IVF seems to have become easier to obtain since the onset of ICSI, even when only one or two oocytes can be retrieved. Pregnancies after ICSI of a single oocyte during unstimulated cycles have been reported (Paulson et al., 1994; Norman et al., 1995). In contrast, the impact of routinely administered protocols for ovarian stimulation e.g. the so-called ‘long protocol’ seems to be very heavy, with high financial costs and risks for the patient, some of them even life threatening, e.g. OHSS (Asch et al., 1993; Bauer and Diedrich, 1996). It seems to be high time for more modest forms of ovarian stimulation; a limited number of Graafian follicles should suffice. The feasibility of ovarian stimulation with clomiphene citrate for ICSI has been demonstrated very recently. A total of 15 nulliparous women (aged 30 ± 4 years) with normal ovulatory cycles and no infertility factors other than subfertility in the husband were treated in their first ICSI trial with 100 mg of clomiphene citrate per day from days 3-7 of the spontaneous menstrual cycle. From day 7 of the cycle onwards, blood samples were drawn each day for measurement of oestradiol and LH concentrations. HCG (10 000 IU) was administered when a follicular diameter of at least 18 mm was achieved in a minimum of two follicles (as measured by transvaginal ultrasound), and serum oestradiol concentrations indicated adequate follicular maturation. Oocyte retrieval was performed transvaginally under ultrasound guidance without anaesthesia. A two-channel 12 gauge aspiration needle was used (Cook Inc Ltd, Eight Mile Plains, Australia), allowing constant suction to be applied while flushing the follicles. After sperm preparation by ‘mini-swim-up technique’ (Al-Hasani et al., 1995), removal of the cumulus, ICSI, cultivation of the injected oocytes and embryo transfer 48 h later were performed as usual (Küpker et al., 1995b). For luteal phase support, 5000 IU of HCG were administered i.m. on days 2 and 5 and another 2500 IU of HCG on day 8 after oocyte retrieval.

In 10 patients, satisfactory follicular development was observed and oocyte
retrieval performed. Five cycles had to be cancelled, four due to insufficient response and one due to a premature LH surge. A total of 17 cumulus–oocyte complexes were retrieved in eight patients. In two patients, no oocytes could be aspirated despite careful flushing of the follicles. After removal of the cumulus, all 17 oocytes were found to be in metaphase II with an extruded first polar body. No oocyte was lost due to damage by ICSI. All 17 oocytes proved to be successfully fertilized developing to the 2 pronuclear (2PN) stage; 16 embryos were obtained and transferred, 38% of them being estimated as excellent (Grade I) according to microscopic morphological criteria and 62% of good quality (Grade II) (Staessen et al., 1989). One ongoing pregnancy was achieved.

Ovarian stimulation by oral administration of clomiphene citrate is extremely simple. The quality, maturity and fertilization rates of the oocytes obtained were excellent. All 17 oocytes obtained were in metaphase II and all of them could be fertilized by ICSI, resulting in a fertilization rate of 100%. All 2PN oocytes in culture cleaved to the embryonic stage. Embryo quality was satisfactory. Financial costs for the ongoing pregnancy achieved were about 40 times less than a successful treatment carried out in accordance with a ‘long protocol’. On the other hand, the number of retrieved oocytes per oocyte retrieval (1.7) may be too low and the cancellation rate of cycles too high for the procedure to be considered efficient. The combination of clomiphene citrate with HMG would probably lead to a higher incidence of premature LH surges. Nevertheless ‘soft protocols’ such as clomiphene citrate alone or in combination with HMG seem to offer promising future perspectives when GnRH antagonists become available for administration over a short period in the midcycle thus avoiding any premature LH surges (Cassidenti et al., 1992; Diedrich et al., 1994; Olivennes et al., 1995;
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Felberbaum et al., 1996). The first feasibility study regarding the combination of a midcycle administration of GnRH antagonists with clomiphene citrate and HMG or recFSH has gained the approval by the ethical board and has already begun. Clomiphene citrate (100 mg) is administered orally from cycle days 2–8. From cycle day 6 onwards, three ampoules of HMG or 225 IU recFSH are administered, overlapping with clomiphene citrate for 3 days. Cetrorelix in its minimal effective dose of 0.25 mg/day is given from cycle day 6 onwards until ovulation induction by 10 000 IU HCG occurs (Figure 4). Although the results of this ‘soft’ protocol are awaited, we are convinced that this could be the way to cheap, safe and efficient ovarian stimulation, lowering burden and risks for our patients.

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