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Follicular development and oocyte maturation in hypogonadotrophic women employing recombinant follicle-stimulating hormone: the role of oestradiol

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Both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are required for follicle development and oestrogen production. Moreover, under normal conditions a close association between dominant follicle size and serum and intrafollicular oestradiol levels is observed. With the recent availability of human recombinant FSH (recFSH), it was possible for the first time to study effects of FSH alone, in the complete absence of endogenous or exogenous LH, on ovarian function. Recent studies applying recFSH in hypogonadotrophic women have shown convincingly that normal growth of follicles up to the preovulatory stage occurs despite extremely low oestradiol levels, in keeping with previous observations using exogenous gonadotrophins in women incapable of synthesizing oestradiol due to steroid enzyme abnormalities. Insufficient data are presently available in humans to conclude whether or not oocyte quality is compromised under these circumstances. It should, however, be realized that sufficient oestradiol levels are required for fertilization *in vivo*. Therefore LH, or human chorionic gonadotrophin (HCG), should be added to stimulation protocols in hypogonadotrophic individuals. These observations may also be relevant for monitoring of ovarian response during recFSH therapy, especially when combined with gonadotrophin-releasing hormone agonists for ovarian hyperstimulation for in-vitro fertilization.

Key words: follicular development/hypogonadotrophic women/oestradiol/recombinant FSH

The two-cell, two-gonadotrophin concept

It has long been recognized that the gonadotrophic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) operate in synergy to ensure ovarian follicle development and secretion of adequate amounts of oestradiol (Table I). Androgen production by theca cells is a function of LH, whereas aromatization of these androgens to oestradiol by granulosa cells is controlled by FSH. Experiments in hypophysectomized rats and mice showed that injection of LH combined with FSH was far more effective in stimulating augmented oestradiol production and a subsequent increase in uterine weight as compared to FSH alone (Fevold, 1941; Greep *et al.,* 1942;

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Eshkol and Lunenfeld, 1967). In fact, a three-fold increase in plasma oestradiol was obtained when FSH was supplemented with just a minor amount of human chorionic gonadotrophin (HCG) (Mannaerts *et al.*, 1991). When a fixed amount of FSH was supplemented with increasing doses of HCG, ovarian weight was observed to increase in a dose-dependent fashion (Mannaerts *et al.*, 1994). Finally, an amenorrhoeic woman (due to pituitary irradiation) receiving human pituitary FSH alone, showed no oestradiol response and ovulation was absent. When FSH was administered together with LH in the subsequent cycle, a reduced amount of FSH was effective in promoting augmented urinary oestradiol output and ovulation (Berger and Taymor, 1971).

Table I. The two-cell, two-gonadotrophin concept

LH stimulates androgen production by theca cells.
FSH stimulates the conversion of androstenedione to oestradiol by granulosa cells.
FSH and LH operate in synergy to ensure the secretion of adequate amounts of oestradiol by growing ovarian follicles.

LH = luteinizing hormone.
FSH = follicle-stimulating hormone.

Table II. Classical concepts regarding follicle growth and concomitant oestradiol secretion

Under normal conditions growth of the dominant follicle is closely associated with rising oestradiol concentrations in serum and follicle fluid.
Increased intrafollicular oestradiol concentrations are required for normal follicle development.

In-vitro studies using cells isolated from ovarian follicles have demonstrated indeed that theca cells are the source of follicular androgens—predominantly androstenedione—whereas cultured granulosa cells are only capable of producing oestradiol when androgens are added to the medium (Ryan *et al.*, 1968; Tsang *et al.*, 1980). Immunocytochemistry (allowing direct visualization of the distribution of the studied enzyme in tissue), as well as Northern blot analysis of RNA, has shown that the cytochrome $P450_{C17}$ enzyme (responsible of the conversion of progestins to androgens) is restricted to the theca cell layer, in keeping with the notion that theca cells are the major site of intra-ovarian androgen biosynthesis. Moreover, appreciable quantities of the cytochrome $P450_{AROMATASE}$ enzyme as well as RNA could be demonstrated in the granulosa cells of dominant follicles (Sasano *et al.*, 1989; Doody *et al.*, 1990; Suzuki *et al.*, 1993), in line with observed correlations between follicle size and intrafollicular oestradiol concentrations (van Dessel *et al.*, 1996a) (for review see Fauser and van Heusden, 1997) (Table II).

Until recently, it has been difficult to address the two-cell, two-gonadotrophin concept in the human since clinical conditions where endogenous LH and FSH are completely abolished are rare. In addition, all urinary gonadotrophin preparations available so far contained LH in variable quantities in addition to FSH. However, recombinant DNA technology has allowed production of pure human recombinant FSH (recFSH) (Keene *et al.*, 1989), completely devoid of LH activity. Since 1990, this compound has been available for preclinical studies in the human. Several years ago, the first pregnancies and births were reported following the administration of recFSH for both induction of ovulation (Donderwinkel *et al.*, 1992) and ovarian stimulation in in-vitro fertilization (IVF) (DeVroey *et al.*, 1992; Germond *et al.*, 1992), and results of large scale clinical IVF studies (Out *et al.*, 1995; Recombinant Human FSH Study Group, 1995) have recently been published.

Why is the two-cell, two-gonadotrophin concept clinically relevant?

For the first time a recFSH preparation completely devoid of LH activity will be available for routine clinical use. Reduced stimulation of theca cell androgen production may affect FSH-induced oestradiol synthesis and follicle development. When hypogonadotrophic females (or males) are treated with recFSH for stimulation of gonadal function, the quantity of exogenous LH (or HCG) needed to allow for normal steroid biosynthesis should be established. If recFSH is to be combined with gonadotrophin-releasing hormone (GnRH) agonists during ovarian stimulation for IVF, it should be determined which lower limit of endogenous serum LH concentrations still allows for adequate oestradiol biosynthesis. Moreover, it should be investigated in greater detail whether reduced late-follicular phase oestrogen concentrations affects oocyte quality (chances of fertilization *in vitro*) and embryo implantation. Ovarian response during the administration of exogenous gonadotrophins is monitored by ultrasound (focusing on number and size of ovarian follicles) together with serum oestradiol assays. It should be established whether the relationship between follicle size and corresponding serum oestradiol concentrations changes when recFSH is used instead of urinary gonadotrophin preparations (containing variable quantities of LH activity). This seems to be particularly relevant in cases where endogenous LH secretion is relatively low, i.e. ovarian hyperstimulation by combined FSH and GnRH agonist medication for IVF.

Exogenous FSH administration in hypogonadotrophic women

Purified urinary FSH

Several studies have been undertaken to compare effects of purified urinary FSH versus HMG (containing equal amounts of FSH and LH bioactivity) preparations, in hypogonadotrophic individuals (due to isolated gonadotrophin deficiency, panhypopituitarism or hypophysectomy) or during long-term suppression of endogenous gonadotrophin secretion by GnRH agonist medication. A randomized, cross-over comparison in 10 women with complete gonadotrophin deficiency (Couzinet *et al.*, 1988) showed greatly reduced maximum serum oestradiol concentrations (760 versus 2474 pmol/l) in cases where women were treated with purified FSH. Furthermore, plasma androgen concentrations did not change during FSH administration. Similar results were obtained when nine patients with isolated gonadotrophin deficiency were treated with HMG (first cycle) followed by purified urinary FSH (second cycle) using the same stimulation protocol for ovulation induction (Shoham *et al.*, 1991). Significantly more ampoules of FSH resulted in a lower number of dominant follicles, lower serum oestradiol (377 versus 1477 pmol/l) concentrations and reduced endometrial thickness. This suggests that the co-administration of LH is required during FSH therapy of World Health Organization (WHO) group I anovulation for optimal stimulation of follicle development and oestradiol synthesis (Loumaye *et al.*, 1995).

Endogenous release of pituitary LH is not completely suppressed in cases of GnRH agonist down-regulation of pituitary function. However, it seems that long-term GnRH agonist administration suppresses endogenous LH secretion to such an extent that ovarian oestradiol secretion following FSH administration is severely compromised. Nine women with chronic anovulation due to polycystic ovary syndrome (PCOS) were down-regulated by GnRH agonist for up to three cycles of FSH therapy (Remorgida *et al.*, 1989). Although follicle growth *per se* was unaffected, maximum oestradiol concentrations were clearly decreased during subsequent cycles (from approximately 2200, to 1000, to 600 pmol/l), and pregnancy rates during subsequent cycles were low.

Recombinant FSH

Our group has described the first woman suffering from congenital isolated gonadotrophin deficiency, who received recFSH as part of a phase I clinical trial to assess safety and pharmacokinetic properties of recFSH (Schoot *et al.*, 1992). Hypogonadotrophic subjects were selected

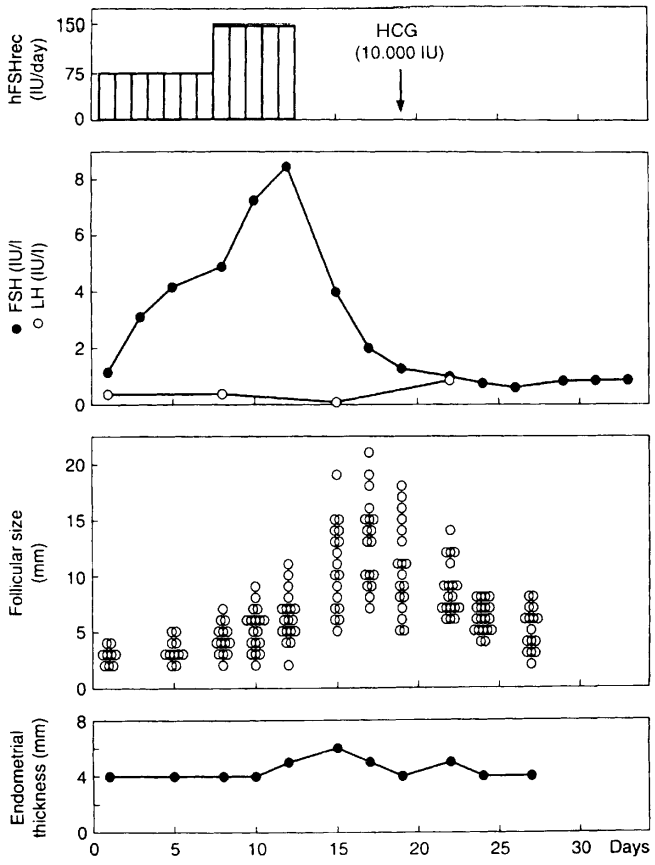

Figure 1. Recombinant follicle stimulating hormone (hFSHrec) daily i.m. doses, serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations, diameter of ovarian follicles and endometrial thickness as assessed by transvaginal ultrasound in a single patient suffering from congenital isolated gonadotrophin deficiency. HCG = human chorionic gonadotrophin. (Reproduced with permission from The Endocrine Society; from Schoot *et al.*, 1992).

for this first human study to exclude the possibility of inducing ovarian failure in case anti-FSH antibody formation (and cross-reactivity with endogenous FSH) would be elicited by the administration of recFSH. This appeared to be a unique 'model' to assess the significance of LH for normal oestradiol biosynthesis, since for the first time effects of FSH alone could be studied in the complete absence of endogenous and exogenous LH. RecFSH was administered daily by i.m. injections of one ampoule followed by two ampoules per day, which resulted in a clear rise in serum FSH concentrations from 1.2 to 8.5 IU/l. However, LH concentrations did not change with serum concentrations ranging between 0.09 and 0.38 IU/l. Serum oestradiol concentrations showed a minimal but steady increase with maximum concentrations (236 pmol/l) still within the range of normal early follicular phase concentrations (see Figures 1 and 2). The observed minimal increase in endometrial thickness further

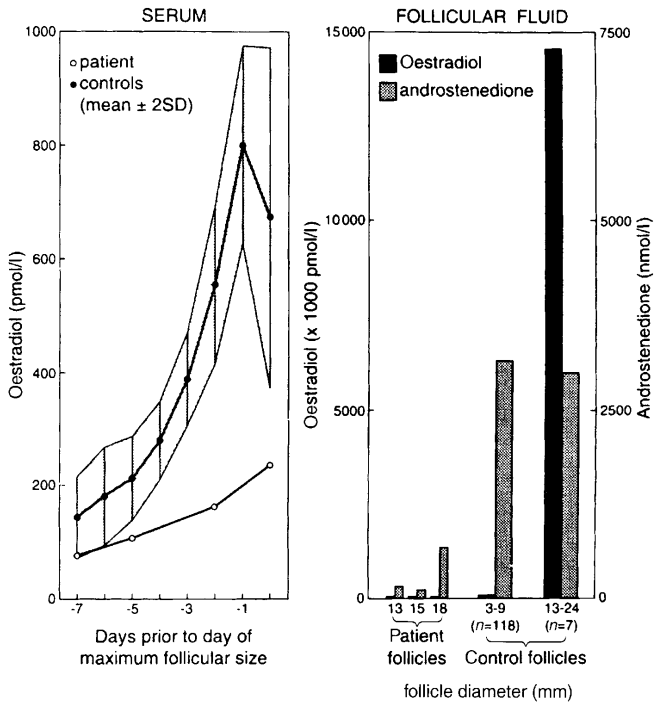

Figure 2. Serum oestradiol concentrations and follicle fluid oestradiol and androstenedione concentrations (three follicles of 13, 15 and 18 mm diameter) during recombinant FSH administration in the same woman with isolated gonadotrophin deficiency and control values in regularly cycling women. (Reproduced with permission from The Endocrine Society; from Schoot *et al.*, 1992).

suggested exposure to minimal amounts of oestradiols. For the first time, conclusive evidence could be provided demonstrating that the two-cell, two-gonadotrophin concept is indeed operative in the human. Despite low oestradiol synthesis, multiple follicles increased in size up to a diameter of 22 mm in this woman (Table II). Three follicles (between 13 and 18 mm in size) were punctured, revealing intrafollicular oestradiol concentrations 1000-fold lower as compared to follicles of similar size under normal conditions. Similar observations were subsequently reported in two (Shoham *et al.*, 1993) and seven (Schoot *et al.*, 1994) hypogonadotrophic women. In the latter study, the possibility of a granulosa cell defect *per se* could be ruled out in these women, since a normal rise in immunoreactive inhibin was observed in the majority of volunteers following recFSH administration (Schoot *et al.*, 1994). Unfortunately, oocyte quality could not be assessed in these studies.

In all subjects a minor but significant increase in serum oestradiol concentrations was found following recFSH administration. This can be explained in several ways. Firstly, since adrenal androgen production was normal in some women, circulating androgens could provide some substrate for conversion to oestradiol (Ben-Chetrit *et al.*, 1996). This observation is supported by findings in GnRH agonist down-regulated monkeys (Karnitis *et al.*, 1994), where recFSH administration combined with dexamethasone (suppressing adrenal androgen output) resulted in lower oestradiol concentrations as compared to recFSH alone. This possibility, however, seems less likely in the human, since a direct relationship between serum androgens and oestradiol concentrations was absent in hypogonadotrophic women receiving recFSH (Schoot *et al.*, 1994). Secondly, granulosa cells stimulated by FSH produce peptides (like insulin-like growth factor, or inhibin) which could in turn stimulate Leydig cell androgen production by intra-ovarian paracrine up-regulation (Magoffin *et al.*, 1990; Smyth *et al.*, 1993). Thirdly, low concentrations of androgens could be produced constitutively by theca cells, allowing for some substrate to be converted to oestradiol.

It seems that follicle growth characteristics are not compromised by low oestradiol output in these hypogonadotrophic women (Table III). This is also supported by observations in women with steroid enzyme defects (for review see Fauser and Hsueh, 1995). However, the number of subjects studied was small, which does not allow definitive conclusions regarding the effects of low granulosa cell oestradiol production on follicle growth dynamics. This observation disputes an important physiological concept, since numerous in-vivo and in-vitro animal studies have shown that increased intrafollicular oestradiol production is mandatory for growth of the follicle (Hsueh *et al.*, 1984). These animal studies suggested that oestradiol plays an important role in granulosa cell proliferation, aromatase enzyme induction, increase in oestradiol and FSH receptors, and induction of LH receptors on granulosa cells. Moreover, oestradiol inhibits androgen production by adjacent theca cells, and inhibits apoptotic changes of ovarian follicles (Fauser and van Heusden, 1997). We have developed a system where effects of FSH alone on oestradiol production and follicle growth dynamics can be studied independently. Complete suppression of endogenous LH and FSH can be achieved by the administration of classical high-dose (containing 50 μg ethinyloestradiol) combined steroid contraceptive pills (van Heusden *et al.*, 1994; Mannaerts *et al.*, 1996). Follicle growth can be studied following the administration of FSH preparations with different potencies and with variable quantities of LH in these women.

Recently, two female patients have been described with inactivating mutations of the LH receptor (Latronico *et al.*, 1996; Toledo *et al.*, 1996). Both individuals presented with anovulation and very low oestradiol concentrations. These patients might represent a rare but unique opportunity to

study effects of absent theca cell stimulation on follicle development.

Recently published case histories showed normal follicle growth, oestradiol production (maximum concentrations of 333 and 780 pmol/l, respectively) and subsequent pregnancies following the administration of recFSH supplemented with 75 IU/day recombinant LH in hypogonadotrophic women (Hull *et al.,* 1994; Kousta *et al.,* 1996). As compared with recFSH alone, maximum oestradiol concentrations were increased 2–3-fold (Hull *et al.,* 1994). In addition, normal gonadal function and steroid synthesis was induced following recFSH plus HCG administration in a hypogonadal male with pituitary insufficiency (Kliesch *et al.,* 1995).

Table III. New observations related to the two-cell, two-gonadotrophin concept

LH-induced androgen synthesis by theca cells is not required for normal follicle development.
FSH-induced follicle development in the human is not driven by high intrafollicular oestradiol concentrations. Instead, other non-steroidal mediators may be involved.

LH = luteinizing hormone.
FSH = follicle stimulating hormone.

Exogenous FSH plus GnRH agonist administration and controlled ovarian hyperstimulation for IVF

Purified urinary FSH

Since a purified urinary FSH preparation became available over a decade ago, several studies have been undertaken to compare effects of FSH versus HMG both for treatment of anovulatory patients and for stimulating development of multiple follicles for IVF. Treatment of IVF patients seems of special interest in this respect, since endogenous LH secretion is suppressed (for prevention of premature LH surges) by the concomitant administration of GnRH agonists. It has been investigated whether ovarian response and oestradiol synthesis is affected by the use of preparations containing different quantities of LH-like activity in women. A meta-analysis of eight randomized trials of purified urinary FSH versus HMG for IVF, with or without GnRH agonists, revealed >50% improvement in clinical pregnancy rates for FSH (Daya *et al.,* 1995). As expected, better treatment outcome following FSH alone was more prominent in studies not employing GnRH agonist comedication presumably due to harmful effects of high LH activity in women with intact endogenous LH release receiving HMG.

This analysis supports the notion that remaining endogenous LH following pituitary down-regulation is sufficient to allow for normal ovarian response following FSH stimulation. Unfortunately, potential differences in serum oestradiol concentrations were not assessed in this meta analysis. Although some individual studies reported significantly lower late-follicular phase concentrations (up to 50% reduction) of serum oestradiol in cases where urinary FSH was combined with GnRH agonist (Howles *et al.,* 1994; Hull *et al.,* 1994), it is generally concluded that exogenous LH is not required for ovarian stimulation and that low concentrations of LH in conjunction with FSH are sufficient for adequate ovarian response (Chappel and Howles, 1991).

Recombinant FSH

Two, large multi-centre randomized trials have been published recently comparing the effects of recFSH preparations versus urinary FSH for IVF. These studies, both involving GnRH agonist co-administration, showed no difference for one preparation (Recombinant Human FSH Study Group, 1995) and an indication for improved clinical outcome (Out *et al.,* 1995) following administration of the other recFSH preparation. Late follicular phase oestradiol serum concentrations were reported to be lower in one (Recombinant Human FSH Study Group, 1995) and higher in the other (Out *et al.,* 1995) study during recFSH administration. However, differences between follicle number and size were not discussed in this regard and therefore no conclusions can be drawn concerning possible changes in the relationship between follicle development and oestradiol output in patients with low LH treated with recFSH. A non-randomized pilot study evaluated the efficacy of five different regimens for GnRH agonist medication in association with recFSH for IVF (DeVroey *et al.,* 1994). Although, late follicular phase LH concentrations were lower in some group (i.m. or s.c. triptorelin, as compared to intranasal buserelin) serum oestradiol concentrations as well as number of oocytes retrieved and fertilized were not compromised despite serum LH concentrations as low as 1.2 IU/l.

Is there a lower limit for endogenous serum LH allowing for sufficient oestradiol biosynthesis?

Studies applying recFSH administration in hypogonadotrophic females indicate that serum LH concentrations <0.5 IU/l severely compromise FSH-induced oestradiol biosynthesis. Extremely low LH concentrations allow for insufficient theca cell derived androgen substrate for conversion to oestradiols by FSH-stimulated granulosa cell

aromatase activity. In contrast, studies in IVF patients (where exogenous FSH is combined with GnRH agonists) suggest that low endogenous LH concentrations (as low as 1.2 IU/l immunoreactive LH) are sufficient to allow for normal follicle growth, oocyte quality and subsequent pregnancies (Coelingh Bennink *et al.* 1994). However, other studies did show clearly reduced late-follicular phase oestradiol concentrations comparing purified urinary FSH versus HMG preparations combined with GnRH agonists for IVF. It should be stressed that assessment of immunoreactive LH in these patients should be interpreted with caution, since the LH isohormone profile may alter following GnRH agonist administration resulting in differences in biopotency not reflected in immunoassays. Secondly, immunoreactive LH may not adequately reflect LH-like activity where HMG preparations are used, since HCG is added to these preparations (Stokman *et al.*, 1993; Rodgers *et al.*, 1994). Low endogenous LH concentrations combined with a pure FSH preparation may not be detrimental for clinical outcome of IVF. However, several studies reported clearly reduced late-follicular phase serum oestradiol concentrations as compared with HMG.

Table IV. Clinical significance of reduced follicle-stimulating hormone (FSH)-stimulated oestradiol biosynthesis, due to diminished availability of androgen substrate

rec FSH, completely devoid of LH activity, is now available for routine clinical use.
Exogenous LH is not needed for follicle development, where endogenous LH concentrations are within the normal range.
Exogenous LH (or HCG) should be added when hypogonadotrophic subjects are treated with recFSH.
In the case of combined recFSH and GnRH agonist medication for IVF, low endogenous LH concentrations may not allow for normal oestra diol, biosynthesis. This does not appear to be harmful for treatment out come, but should be taken into consideration when serum oestradiol assays are used for ovarian response monitoring.

RecFSH = recombinant follicle stimulating hormone.
LH = luteinizing hormone.
HCG = human chorionic gonadotrophin.
GnRH = gonadotrophin-releasing hormone
IVF = in-vitro fertilization.

When gonadotrophin preparations are applied for treatment of hypogonadotrophic patients—usually presenting with serum LH concentrations <0.5 IU/l—FSH-induced oestradiol production and clinical outcome will be severely compromised. Exogenous LH should therefore be added (Table IV). Exogenous LH is not needed for stimulation of ovarian function under circumstances where some endogenous LH is secreted, even when pituitary LH release is down-regulated in IVF patients by the co-administration of GnRH agonists. This conclusion will certainly also hold when in the near future only late follicular phase augmented LH concentrations will be suppressed in IVF patients using GnRH antagonists. However, with the use of high doses of new generation GnRH antagonists, LH concentrations may be suppressed to extremely low concentrations insufficient to maintain oestradiol synthesis by theca cells.

During gonadotrophin treatment of clomiphene-citrate resistant anovulatory patients, endogenous LH concentrations are usually within the normal range which will allow for sufficient availability of androgen substrate for conversion to oestradiol. Despite immunoreactive and bioactive FSH serum concentrations closely resembling normal conditions when gonadotrophin preparations are administered in a decremental dose regimen (van Dessel *et al.*, 1996b), serum oestradiol concentrations were shown to be highly variable (Schoot *et al.*, 1995) indicating differences in ovarian responsiveness of these patients.

Is treatment outcome compromised because recFSH-stimulated follicle development coincides with lower late-follicular phase oestradiol concentrations?

In contrast to previous belief, it has now been convincingly demonstrated that increased oestradiol biosynthesis is not required for follicle growth in the human. It may be postulated that growth of the follicle is driven by other (as yet unidentified) non-steroidal intra-ovarian factors (Fauser, 1996; Fauser and van Heusden, 1997). At this stage it is uncertain whether low late-follicular phase oestradiol secretions have a bearing on human oocyte quality (as discussed by Tesarik and Zelinski-Wooten in this mini symposium). However, it should be emphasized that oestradiol plays a crucial role in normal reproductive function at various levels (Wang and Greenwald, 1993). Following induction of ovulation in anovulatory patients, exposure to normal oestradiol concentrations is important for production of adequate amounts of cervical mucus as well as for endometrial receptivity. If late-follicular phase oestradiol concentrations are reduced below the physiological range during FSH treatment of hypogonadotrophic anovulatory patients, chances for in-vivo fertilization and subsequent pregnancy will certainly be compromised. Oestradiol concentrations may or may not be reduced following FSH and GnRH agonist medication as compared with HMG for IVF.

Lower oestradiol concentrations may improve treatment outcome in IVF patients, since detrimental effects of high oestradiol concentrations on endometrial receptivity have been described, resulting in lower implantation rates (Simon *et al.*, 1995; Pellicer *et al.*, 1996). It should be

realized, however, that these concentrations are still in the supraphysiological range.

Should an altered relationship between follicle size and corresponding oestradiol concentrations affect clinical monitoring of ovarian response following recFSH administration?

Monitoring of ovarian response following the administration of exogenous gonadotrophins can be performed by ultrasound and serum oestradiol assays (Schoot and Fauser, 1992). The aims of ovarian monitoring are to assess the effective FSH dose, duration of FSH therapy and timing of HCG, prevention of ovarian hyperstimulation, and, in case of ovulation induction, prevention of multiple pregnancies. Relationships between oestradiol concentrations and number and size of follicles have been studied (Hull *et al.*, 1986; Ellenbogen *et al.*, 1996), and it was shown that oestradiol production is the net result of all developing follicles, which is in sharp contrast to normal conditions. However, there is still debate whether determination of serum oestradiol concentrations adds to efficacy and safety of gonadotrophin therapy (Wikland *et al.*, 1994; Ellenbogen *et al.*, 1996). It has become clear for ovulation induction that high oestradiol concentrations are associated with increased chances for ovarian hyperstimulation, regardless of ultrasound findings (Schoot *et al.*, 1995; Ellenbogen *et al.*, 1996).

As discussed previously, recFSH-induced growth of medium sized and large follicles for IVF may coincide with reduced oestradiol biosynthesis. This phenomenon should be recognized, to avoid misinterpretation (and potential harmful continuation of exogenous FSH) of relatively low oestradiol serum concentrations in IVF monitoring.

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