

# Letters to the Editor

## Progesterone elevation on the day of hCG: methodological issues

Sir,

Two recent interesting and learned articles in *Human Reproduction Update* concerning elevations of progesterone (P) in the follicular phase of controlled ovarian stimulation (Venetis *et al.*, 2007; Bosch, 2008) address a number of issues which deserve further comment. The comments relate to technical issues (steroid hormone measurement and study design) as well as biological/biochemical concepts.

Both articles addressed specific critical concentration points of P in the follicular phase, and Bosch indicated the imprecise nature of selecting a single value (0.9 ng/ml). If we first set aside the debate over specific concentrations of circulating P demonstrating negative clinical consequences, presumably through advancing endometrial development, we must consider whether the assays used are reliable at the values observed. Most commercial assays were developed to determine the standard clinical question for the clinical biochemistry laboratory—whether a patient has ovulated or not. This requires an operating concentration sensitivity some 5–10-fold higher than those of the follicular phase. Correspondingly, little development has been undertaken for these automated and semi-automated platforms to examine the more subtle changes taking place prior to ovulation. This matter was addressed in preparation for the study by Adonakis *et al.* (1998), and there are important lessons to be learned which I would like to discuss below.

Three commercial, non-extraction assays were compared for routine use in the assisted reproduction programme, and these were compared with a validated in-house assay deploying petroleum-ether extraction for removal of more hydrophilic interfering factors. Table I shows the results of performance evaluation of the four assays tested with a pool of follicular phase samples. The in-house assay demonstrated a recovery rate in the range of 90% at concentrations between 0.5 and 1.5 ng/ml (1.5 and 5.0 nmol/l), which was significantly more precise than the commercial assays which varied from 0 to ~80% (assay D). This latter value was achieved by the fluorimetric assay, which showed greater potential for measurements at these concentration ranges than the two colourimetric enzyme assays. The variation of control samples (both intra- and inter-assay) in this concentration range was poorer than those generally acknowledged for commercial assays, which are usually tested at concentration values seen in the luteal phase. However, the most disturbing characteristics of the comparisons were identified when tested with sequential individual follicular phase samples. Under these circumstances, two of the assays, the fluorimetric and one of the colourimetric assays, showed mean concentration values leading to the day of HCG administration which were superimposable. However, a correlation test

( $r = 0.58$ ) revealed that there was little agreement between the tests for individual samples. The samples in the critical concentration range (evaluated to be in the region of 5.0 nmol/l) showed more samples with discordant evaluation ('elevated' in one assay and 'normal' in the other,  $n = 28$ ) than showed concordant results (elevated in both,  $n = 9$ ). The discrepancies were deemed to be patient-specific matrix effects, which may be related to other steroidal factors, such as 17- $\alpha$  hydroxyprogesterone, mineralocorticosteroids and glucocorticosteroids which are present in high concentrations in serum, and whose interference will be more marked when the P concentrations are lower (the follicular phase) due to reduced competitive binding to the antibody. Such interference roles would be reduced by differential extraction procedures. The actual assay used for the study described by Adonakis *et al.* was that showing greater parallels with the in-house extraction assay.

A more recent survey of routine assay systems performing clinical tests on serum P concentrations (Coucke *et al.*, 2007) showed high bias evaluations and a lack of linearity in a limited number of samples whose concentrations were <1.0 nmol/l (absolute reference value), indicating that efficacy and reliability have improved little if at all in this domain. Greater reliability (Coefficient of variation <20%) was seen in samples >6 nmol/l for most (but not all) of the methods examined, but variation between individual samples was not assessed in this study at any concentration.

However, the point of debate here is that evaluation of P concentrations in samples obtained prior to HCG in controlled ovarian stimulation are subject to extensive methodological errors, such that a consensus of valid biological relevance will be difficult to achieve, until the methods are validated in more detail, and show greater consistency between them. It is therefore no surprise that a meta-analysis could determine no clinical relevance to P elevations in the non-luteinized situation.

The biological considerations require consideration of a combination of established and contentious matters pertaining to ovarian biochemistry. It is established that FSH acts on granulosa cells, promoting cell division and steroid biosynthesis which terminates at the production of P. Further metabolism (to androgens) requires the intervention of thecal cells under the influence of LH—the 2-cell, 2-gonadotrophin hypothesis (Armstrong *et al.*, 1978; Moon *et al.*, 1978). It is a predictable physiological phenomenon, therefore, that an ovary with a large number of growing follicles, stimulated by high FSH concentrations, will produce and secrete more P than a single follicle in the normal mid-follicular phase, with its declining FSH concentrations. In the non-luteinized environment, LH can only act to reduce circulating P, by promoting its conversion to androgens, which are then further metabolized to estrogens by the granulosa cells.

**Table 1.** Evaluation of three commercial assays for progesterone concentrations in pooled follicular phase samples.

Assay	Recovery % (range)	Coefficient of variation (intra-assay) (%)	Coefficient of variation (inter-assay) (%)
In house	90 (70–120)	13	15
Assay C <sup>a</sup>	0 (–15 to 45)	(18)	(25)
Assay I	55 (15–90)	11	18
Assay D	80 (45–115)	10	15

The reference sample concentration was estimated to be 2.5 nmol/l (by the in-house assay), and the added value for recovery testing was 2.8 nmol/l. <sup>a</sup>Data for assay C constituted from three test evaluations only.

There is strong circumstantial evidence supporting a clinical consequence of this biological concept in reports from the large prospective randomized study comparing recombinant FSH (r-hFSH) with urinary derived FSH, containing LH-like activity (Andersen *et al.*, 2006). In this study, young normogonadotrophic patients were treated with moderate to high dose FSH maintained into the mid-late follicular phase combined with potent endogenous gonadotropin suppression using daily injected GnRH agonist. The authors used a concentration of 4 nmol/l as the critical value for defining 'elevated P' concentrations at the end of the follicular phase, and they showed that elevated P was associated with high ovarian responses (indicated by oocyte yield) in both treatment groups. There was a higher incidence of elevated P in patients treated with r-hFSH, as would be expected where there is reduced further metabolism of P, in the absence of LH drive to the thecal cells. The r-hFSH treatment group showed a higher incidence of elevated circulating P (24 versus 11.8%), and also a reduced implantation rate. This latter observation suggests that there can be clinical consequences of a pre-hCG rise in P in what should be 'ideal' (high responding) patients.

It can be debated that the reported incidences of 24% of cases with elevated P following r-hFSH and 11.8% with urinary-derived FSH may be higher than expected due to the specific protocol employed, using a highly suppressive agonist treatment. In this regard, the use of a less aggressive agonist treatment (such as intranasal Nafarelin: Lockwood *et al.*, 1995), which has been shown to reduce the demand for FSH injections may also reduce the incidence/degree of profound LH suppression, and the consequences regarding the levels of circulating P would be interesting.

There has been much debate over the last few years related to the requirement for LH activity in the stimulant used in controlled stimulation (Fleming *et al.*, 2000; Westergaard *et al.*, 2001; Humaidan *et al.*, 2004; Marrs *et al.*, 2004), and it has proven difficult to determine patients where a specific requirement is mandatory (Anderson *et al.*, 2008). It is possible that the observations above may explain the recorded consequences of excessive LH suppression during COS with high-dose FSH protocols and aggressive endogenous gonadotrophin suppression in high responding women. The nature of the LH activity required to avoid this consequence, may be a matter of conjecture, but it is clear that thecal cells are very sensitive to LH, and there is currently no indication that HCG with its longer half-life should show any advantage over pure r-hLH.

In a final point, it is pleasing that both papers have questioned the use of the term 'premature luteinization' in the circumstances of control with GnRH agonists. This phenomenon is clearly not luteinization.

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Richard Fleming<sup>1</sup>

Glasgow Centre for Reproductive Medicine, Glasgow, UK

<sup>1</sup>Correspondence address. E-mail: richard.fleming@grcm.co.uk

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