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Reduced in-vitro fertilization of human oocytes from patients with raised basal luteinizing hormone levels during the follicular phase

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Summary. A series of 62 women were managed in the University of Western Australia/PIVET Laboratory in-vitro fertilization programme. In 60 of them follicle growth was stimulated with clomiphene citrate with or without additional human menopausal gonadotrophin (hMG) and in two with hMG alone. Follicles were aspirated at laparoscopy following an hCG trigger injection and occasionally following a spontaneous luteinizing hormone (LH) surge. Oocytes were inseminated with $0{\cdot}5\times10^5$–10^5 sperm/ml 3–6 h later. A significant reduction ($P<0{\cdot}001$) in the fertilization rate of mature oocytes was observed in those patients whose basal serum LH values were >1 SD above the mean. Fifty-nine women subsequently had embryo transfer and of 10 clinical pregnancies, none occurred in those with elevated LH values. Reduced fertilization may be a reflection of premature oocyte maturation or ageing. This may have clinical implications in the management of some patients with unexplained infertility.

During the monitoring of follicular development for in-vitro fertilization, clinical attention is focused on the physical enlargement of individual follicles detected by ultrasound and increasing steroidogenic activity, measured by serum oestradiol-17β (Yovich *et al.* 1984*a*). The growth phase is also a period of intense metabolic activity and cytoplasmic development within the oocyte (Tsafriri 1978). Indeed, many of the proteins and much of the RNA required during early embryonic cleavage stages are thought to be generated in the oocyte both before and following the initiation of meiosis (Biggers & Powers 1979; Motlik *et al.* 1980). Since steroid hormone production within the follicle is under the control of gonadotrophins, any alterations in the balance of the control mechanisms governing follicle growth may also be expected to indirectly affect oocyte function. There is some evidence, for instance in sheep, that impaired fertilization and embryo growth may result from atypical gonadotrophin levels (Moor & Trounson 1977).

Luteinizing hormone (LH) has several important roles in follicle development. The more important are those associated with the mid-cycle surge. These include initiating the mechanism leading to follicle rupture (Strickland & Beers 1979), stimulating luteal progesterone secretion (Katz 1981), activating the oocyte to resume meiosis (Jagiello *et al.* 1975) and promoting the expansion of the cumulus oophorus and its detachment from the granulosa layer of the follicle wall (Dekel *et al.* 1979). During the follicle growth phase, LH also appears to be important in promoting the synthesis of androgens by thecal cells, which, under the influence of follicle

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stimulating hormone (FSH), are later converted to oestradiol-17β in the granulosa cells (Katz 1981). During this period serum levels of LH are normally low (Baird *et al.* 1976).

Abnormal LH secretion in women has been reported in association with polycystic ovarian disease where FSH is normal and basal LH is usually elevated (Baird *et al.* 1976). During the intensive monitoring of women treated for infertility by in-vitro fertilization and ovulation induction we have observed elevated basal LH levels in patients without additional stigmata of polycystic ovarian disease and who had ovaries of normal size and appearance at laparoscopy. Patients with polycystic ovarian disease are known to have reduced or absent ovulation and although many conceive following ovulation induction, the fertilization potential of oocytes derived from patients with elevated serum LH has not been fully investigated.

The development of in-vitro fertilization, embryo culture and embryo transfer procedures have provided an opportunity to assess gamete quality and the fertilization potential from patients with various clinical and hormonal states. The results presented in this report suggest that oocytes obtained from the pre-ovulatory follicles of women with elevated LH levels have impaired fertilization ability and that those embryos which do occur are less likely to implant successfully.

Patients and methods

This study was based on 62 consecutive aspirations carried out between November 1982 and May 1983 at the PIVET Laboratory/University of Western Australia in-vitro fertilization programme (Yovich *et al.* 1984*a*). Of the 62 patients admitted to the study 55 had absent, blocked or non-functional fallopian tubes (one had defined polycystic ovarian disease), three had endometriosis, and four had oligospermic husbands. Procedures for follicle stimulation and patient management were similar to those described elsewhere (Stanger & Yovich 1984; Yovich *et al.* 1984*a*, *b*). Briefly, follicle growth was stimulated with clomiphene citrate (Clomid, Merrell Dow Pharmaceutical Inc., Cincinatti, Ohio, USA), 150 mg given daily on days 2 to 6 of the menstrual cycle, in 60 patients and 11 of them received additional maintenance of follicular growth with three ampoules of human menopausal gonadotrophin (hMG) (Pergonal, I.F. Serono, Rome, Italy) administered daily between days 6 and 11. Generally, supplementation was given to those patients who had previously demonstrated a poor response to clomiphene citrate (fewer than two mature oocytes recovered at laparoscopy). The two remaining patients had follicle development stimulated solely with hMG (three ampoules daily) administered between days 3 to 12. Follicle growth was monitored from day 8 to the day before aspiration by combined early morning ultrasound tracking of follicle diameter (mean of three diameters), cervical score and hormonal assays (oestradiol-17β, progesterone and LH) from serum collected before the hMG injection. As an indication of follicle maturity, the mean number and size of follicles present and the serum oestradiol-17β concentration on the day of either ovulation induction or beginning of the LH surge were recorded in relation to the patient's mean LH level.

The patients were admitted for ovulation induction when the average size of the leading follicles was ≥18 mm and the serum oestradiol-17β concentration was about 1500 pmol/l per major follicle. Before the intramuscular injection of 5000 units of human chorionic gonadotrophin (hCG), a blood sample was taken to ensure LH levels were still within the basal range. In 49 patients not demonstrating a surge in LH, laparoscopy was timed at 36 h after the hCG injection. In 11 patients whose serum LH had risen to levels greater than 2 SD above the mean basal level at the time of hCG, laparoscopy was between 30 and 34 h after the hCG injection and the two patients whose routine morning sample indicated the early stage of a spontaneous LH surge, were laparoscoped 33–36 h after estimated onset without hCG administration. Patients who were considered to be in an advanced stage of spontaneous LH surge were not included in this study.

Fertilization in vitro and embryo development

Oocytes were collected by laparoscopic follicle aspiration into modified Tyrode's medium (Whittingham 1971; Quinn *et al.* 1984) containing 15% heat-inactivated maternal serum. The oocyte enclosed in the cumulus mass was incubated for 3–6 h in 5% CO_2, 5% O_2, and 90% N_2 before the addition of spermatozoa at a concentration of between $0{\cdot}5\times10^5$ and 10^5 spermatozoa/ml. Fertilization was recognized

16–20 h later by finding two pronuclei in the cytoplasm of the oocyte and usually a second polar body in the perivitelline space. Embryos and oocytes without pronuclei were returned to 1 ml of fresh culture medium and incubated overnight before embryo transfer 40–48 h after aspiration. Immature oocytes identified at the time of ovum collection with a small incompletely expanded cumulus oophorous enclosed by a tight and dense coronal cell layer were excluded from this study. No attempt was made to re-inseminate unfertilized oocytes. In this series, cleavage was not observed in any embryo failing to demonstrate pronuclei on the day after insemination.

Hormone assessment

LH was assessed in the serum of all patients within 2 h of collection (08.00 hours) by radioimmunoassay. LH was isolated by rabbit anti-human LH antiserum, separated by goat anti-rabbit α-globulin antiserum, and compared to a standard curve of human LH (0–200 i.u./l) standardized against the WHO pituitary reference preparation (IRP 68/80) (Diagnostic Products Corporation, Los Angeles, California). The limit of this assay is reported to be 2 i.u./l. The intra-assay and inter-assay coefficients of variation were <3·6% and 8·3% respectively. Cross-reactivity with FSH was <4·5% and with TSH <3·7%. Progesterone was also estimated in the same serum sample using solid phase radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, California).

Analysis of data

LH and progesterone data in the study were drawn from all daily estimations obtained before either the hCG injection, where no endogenous surge of LH was observed, or before a detectable rise in LH associated with the onset of the surge. Care was taken not to include any estimations associated with the LH surge. In the 49 patients whose LH was at basal levels before the hCG injection, this was uncomplicated. In the 13 patients whose LH had risen before admission, the value for that day was omitted from the calculations. The effect of the type of follicle stimulation on the daily serum LH concentration and the change in concentration with time were both examied by one-way analysis of variance of the raw LH data (Zar 1974). An overall group mean and standard deviation (SD) were then calculated from the data pool. For each patient, mean follicular phase LH and progesterone levels were obtained. Based on this, each patient was allocated to one of the following three categories: group 1, LH level <1 SD below the mean value; group 2, LH level within 1 SD of the mean; and group 3, LH level >1 SD above the mean.

Individual fertilization rates and cleavage rates were calculated within each group and the totals compared by χ^2 analysis using a standard 2×2 contingency table.

The rates in groups 1 and 2 were first tested for homogeneity before being combined and compared with the rate in group 3 (Zar 1974).

Results

The overall mean follicular phase serum LH value of the 62 patients was 13·9 i.u./l (SD 6·69) (Fig. 1). There was no significant difference ($P>0·20$) in the mean LH level between those patients receiving clomiphene citrate (14·2 i.u./l) and those receiving clomiphene citrate and hMG (13·8 i.u./l) (Fig. 2). In both groups, the LH concentration fell significantly ($P<0·05$) between days 8 and 12 of the follicular phase (Fig. 2). Nine patients had a mean LH level >1 SD above the mean.

The proportion of mature oocytes which fertilized and the subsequent proportion of fertilized ova which proceeded to cleaving embryos for the three groups are shown in Table 1. In all, 153 mature oocytes were collected providing an average yield of 2·5 oocytes per patient. The fertilization rate was >84% for the two groups whose follicular phase serum LH level was not >1 SD above the mean. However, the fertilization rate in the nine patients in group 3 (LH level >1 SD above the mean) was significantly reduced ($P<0·001$). In addition, fewer of the fertilized oocytes proceeded to cleavage ($P<0·01$).

Of the 62 women admitted for follicle aspiration, 59 subsequently had embryo transfer. Two of the three patients who failed to produce any fertilized oocytes came from group 3. Ten patients have subsequently had pregnancies confirmed by rising β-hCG levels and positive ultrasound confirmation of an intrauterine gestational sac.

Little difference existed in the maturity of the follicles generated in the three groups as indi-

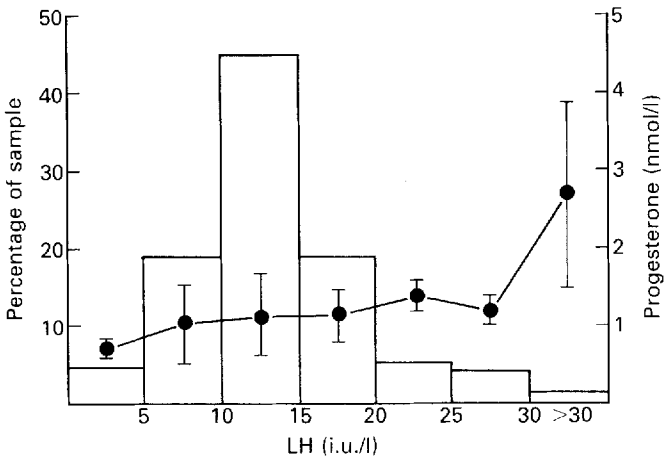

Fig. 1. Distribution of individual mean serum LH concentration (histogram) and the corresponding mean progesterone concentration (●) in 62 patients admitted for in-vitro fertilization. Both hormones were averaged over the whole follicle growing phase before the mid-cycle LH surge. Vertical bars show the SEM values.

cated by the number and diameter of the follicles and their oestrogen production (Table 2). The higher oestrogen and progesterone levels noted in group 3 were attributed to the administration of hMG since proportionately more patients in group 3 required this supplementation. The relation between serum LH and progesterone levels during the phase of maximum follicle growth is shown in Fig. 1. The progesterone level remained low at all LH levels except in those patients whose LH exceeded 30 i.u./l. Applying the same criteria as with LH levels (i.e. mean±1 SD), six patients had a mean progesterone level >1·84 nmol/l (1 SD above the mean) throughout the follicular phase. Of these, four had mean LH levels ≤20·6 i.u./l and their oocytes fertilized and developed normally (Table 3). One of the patients conceived following embryo transfer. However, in seven patients whose LH level was elevated without a concurrent increase in progesterone, the fertilization rate of oocytes was reduced to 57%. Where both hormone levels were raised (two patients only), only one of eight ova was fertilized. Overall there was no significant difference in the fertilization rate between the groups with high or low progesterone levels ($0{\cdot}05<P<0{\cdot}1$).

The reduced fertilization rate in group 3 was analysed against the follicle-stimulation regimen and ovulation-induction trigger (Table 4). There was no significant association with the follicle-stimulation regimen but none of the women had a spontaneous LH surge. However, the observation did not reach significance in this small series (χ^2, v 1:1·5, $P>0{\cdot}01$). None of the 10 pregnancies occurred in group 3, nine occurred in women who had hCG alone and the tenth had an LH surge with hCG augmentation.

Discussion

The average range of basal serum LH estimated in this study agrees well with previous reports (Kerin *et al.* 1981; Djahanbakhch *et al.* 1981). The administration of hMG, which contains equal amounts of FSH and LH, to sustain follicle growth made no significant difference in the amount of LH detectable in serum when compared to the group given clomiphene citrate only. In unstimulated cycles, LH is generally observed to remain relatively unchanged from day 1 (Faiman *et al.* 1976). Irrespective of the type of stimulation, the LH values in this group of patients fell significantly ($P<0{\cdot}05$) from a mean of 17·5 i.u./l on day 8 to 11·6 i.u./l on day 12 (Fig. 2). The decrease most likely reflects a downturn in gonadotrophin secretion following both the period of sustained clomiphene citrate exposure and the rising oestradiol-17β levels. The effect of clomiphene citrate on LH secretion has been reported to be less than on FSH during the early part of the follicular phase (Vandenburg & Yen 1973) with an increase in responsiveness as the stage of the cycle advances. The

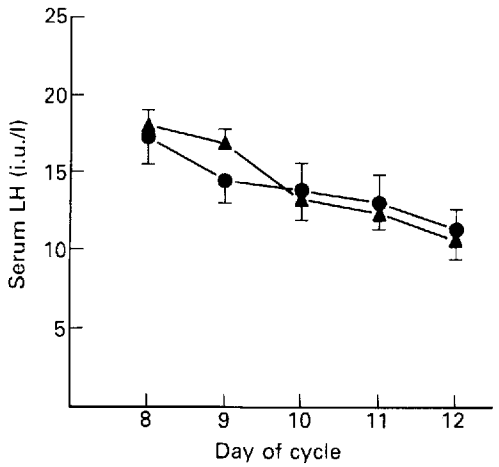

Fig. 2. Mean serum LH levels between days 8 and 12 of the menstrual cycle in women stimulated with either clomiphene citrate (●) or clomiphene citrate plus hMG (▲). Vertical bars show the SEM values.

patients described here all received clomiphene citrate from days 2 to 6 and it is therefore unknown if the pattern of LH secretion would be similar in a regimen where clomiphene citrate was given later.

The elevated basal follicular LH value found in eight patients in group 3 was somewhat unexpected since seven of them were included in the programme because of a known tubal disorder and one patient because her husband had moderate oligospermia. Only one patient in this group was known to have elevated basal LH levels consistent with a clinical diagnosis of polycystic ovarian disease. In the investigations of patients for selection for in-vitro fertilization, we have not routinely monitored gonadotrophin levels unless a co-existing ovulation disorder was indicated from the menstrual history, hence we do not have prior information about LH levels in this group.

The data suggest that there may be a relatively small group of patients who have LH values in excess of the normal range during stimulated cycles. It would appear that oocytes from these patients have a reduced ability to be fertilized. The correlation between high basal levels of LH and reduced fertility became apparent when two patients with very high LH values (>30i.u./l) and elevated progesterone levels (Table 3) failed to generate the anticipated number of embryos even though eight oocytes were collected; the cumulus masses at collection were fully expanded but dark in appearance. The majority of the oocytes, when examined 16 h after insemination, did not demonstrate two pronuclei and a normally light-brown translucent cytoplasm. The majority of the ova, whilst appearing spherical in shape, were darker in colour although showing no clear signs of degeneration. In some oocytes, a polar body was visible. None of the oocytes contained a germinal vesicle, indicating the resumption of meiosis had been initiated. Furthermore, the quality of the few embryos which were generated were clearly inferior to those routinely developed in the programme displaying excessive fragmentation and asymmetrical cleavage. These features suggested that the oocytes were in the early stages of atresia.

The reduced fertilization cannot be attributed to poor follicular development since all patients in group 3 had follicle growth and oestradiol-17β production indistinguishable from that in the other two groups (Table 2). The progesterone levels appeared similar in all groups (Table 2) and overall were independent of LH con-

Table 1. The relation between basal luteinizing hormone (LH) concentrations and the fertilization of human ova *in vitro*

Group	Basal serum LH (i.u./l)	Number of patients	Pronuclear ova/ inseminated ova		Cleaving embryos/ fertilized ova		Number pregnant
			n/n	(%)	*n/n*	(%)	
1	<7·3 (mean−1 SD)	8	15/17	(88)	14/15	(93)	2
2	7·3–20·6 (mean±1 SD)	45	96/107	(90)	87/96	(91)	8
3	>20·6 (mean+1 SD)	9	13/29	(45)	8/13	(61)	0
Total	—	62	124/153	(81)	109/124	(88)	10

Table 2. Hormonal and follicle characteristics on the day of hCG administration in relation to mean basal luteinizing hormone (LH) levels

Characteristic	Basal LH concentration (i.u./l)		
	<7·3	7·3–20·6	>20·6
Number of patients	8	45	9
Follicles >15 mm in diameter			
Mean number	2·2 (0·36)	2·5 (0·37)	2·7 (0·43)
Mean diameter (mm)	19·2 (0·24)	19·3 (0·57)	18·9 (0·21)
Mean oestradiol-17β (nmol/l)	3·1 (0·99)	3·7 (0·28)	4·5 (0·88)
Mean progesterone (nmol/l)	0·8 (0·23)	0·9 (0·25)	1·6 (0·69)

SEM values are shown in parenthesis.

centration, except where the latter was grossly elevated (Fig. 1).

In attempting to explain the reduced fertilization rate of oocytes obtained from patients with elevated serum LH levels two possibilities were considered likely. The first was that LH acted directly to initiate the resumption of meiosis at a premature stage of follicle development resulting in aged oocytes at aspiration. The second was that levels of steroid hormone produced by the growing antral follicle were sufficiently altered to interfere with the normal development of the oocyte. At present neither possibility can readily be assessed.

Even though LH is the hormone responsible for triggering the resumption of meiosis in the oocyte, it is only known to do so during the surge via an indirect action by increasing intrafollicular cyclic AMP levels (Hunzicker-Dunn *et al.* 1979). Two mechanisms have been proposed. One is the removal of a low-molecular-weight inhibitor of oocyte maturation (OMI) thought to be produced by the granulosa tissue (Channing 1979) and which is lost following LH stimulation (Tsafriri & Linder 1978). More recently, it has been suggested that reduced oocyte fertilization occurs when high levels of OMI persist in the antral fluid after LH activation of ovulation, presumably where the follicle is immature (Channing *et al.* 1983). If this is the case, could the reverse also occur when sustained LH exposure during the follicle growth phase leads to a premature loss of OMI in mature follicles without initiating ovulation? The other possible mechanism involves disruption of the intracellular coupling between the oocyte and the coronal cells during cumulus expansion. While the relationship between cumulus expansion, cellular

Table 3. Relation between serum luteinizing hormone (LH) and progesterone and the fertilization rate of human ova *in vitro*

Progesterone	Basal LH concentration (i.u./l)				Total	
	≤20·6†		>20·6			
	No. of patients	Fertilization rate* *n/N* (%; range)	No. of patients	Fertilization rate *n/N* (%; range)	No. of patients	Fertilization rate *n/N*
≤1·84‡	49	100/113 (88; 0–100)	7	12/21 (57; 0–75)	56	112/134
>1·84	4	11/11 (100; —)	2	1/8 (12; —)	6	12/19
Total	53	111/124	9	13/29	62	124/153

*The fertilization rate is the number of mature ova with pronuclei (*n*) divided by the number of mature ova inseminated (*N*).

†Value represents the mean+1 SD serum LH concentration.

‡Value represents the mean+1 SD serum progesterone concentration.

Table 4. Stimulation and ovulation regimens of patients with normal and elevated basal serum luteinizing hormone (LH) concentration

Basal LH concentration (i.u./l)	Stimulation regimen			Ovulation regimen		
	Clomiphene	Clomiphene +hMG	hMG	LH Surge	LH Surge +hCG	hCG
≤20·6	43	8	2	2	11	40
>20·6	6	3	0	0	0	9
Total	49	11	2	2	11	49

communication and oocyte meiotic maturation is unclear in women, in the mouse, cumulus expansion and initiation of oocyte maturation appears linked and controlled by both LH and FSH (Eppig 1981, 1982*a*, *b*). It is suggested that elevated LH levels during follicle growth may trigger meiotic activity in the oocyte either by affecting OMI levels or by altering the cumulus–coronal cell–oocyte axis without a concommittant induction of luteinization or ovulation. In our study the net effect would therefore be that some oocytes may spontaneously resume meiosis well before the administration of hCG such that by the time of aspiration the oocyte has passed beyond its fertilizable life.This might be assessed by the examination of oocytes collected from growing follicles not stimulated with hCG.

Since LH is thought to stimulate steroidogenic activity in the thecal tissue of the growing follicle (Katz 1981), sustained exposure to elevated LH levels could potentially result in an altered hormonal environment within the antrum. This may be the more relevant hypothesis since, in isolated mouse follicle experiments, high progesterone levels induce oocyte atresia (Tyler & Collins 1980) and increase the number of meiotically inactive oocytes (Tyler *et al.* 1980; Smith & Tenny 1980). Reduced fertilization rates of ovine oocytes and abnormal embryo development *in vivo* after follicle growth *in vitro* has been shown in the presence of elevated gonadotrophin (Moor & Trounson 1977) and steroid levels (Moor *et al.* 1980).

When the relation between basal serum LH, progesterone and the fertilization of human ova was examined, no evidence was obtained to support a primary role of progesterone in reduced fertilization of human ova *in vitro* (Table 3). This of course does not negate its involvement since a compounding influence with LH may exist as illustrated by reduced penetration when both factors were elevated. As indicated by the error bars in Fig. 1, progesterone levels of >1·84 nmol/l may be found in patients with very low as well as normal LH values. However, markedly elevated progesterone values (>2·8 nmol/l) were only found where the mean LH concentration exceeded 30 i.u./l.

While care needs to be exercised in interpreting results obtained by association rather than by controlled experimentation, the data presented here suggest that as a result of excessive LH secretion, the capacity of the oocyte to be fertilized and develop normally may be diminished and as such warrants further investigation. At a clinical level, the presence of basal LH values >1 SD above the mean may represent a potential cause of infertility. This is apparent despite adequate follicle growth according to follicle size and oestradiol-17β production. While a value of 20·6 i.u./l was chosen to represent a division between groups 2 and 3, the numerical value is of less importance than the distribution of patients relative to the mean. This may vary with the type of assay and between laboratories. Even within an elevated group, variation in the fertilization rate may represent differences in the relative sensitivity of the follicles to LH and possibly in the level of biologically active gonadotrophin. Efforts to reduce basal LH levels, for instance by the use of LHRH antagonists (Flemming *et al.* 1984), may prove to be beneficial if FSH can be given to support follicle growth. Elevated LH and progesterone levels are classically associated with polycystic ovaries and anovulation. Although all patients in this series, except one, had regular menses and no other stigmata of polycystic ovarian disease, those patients with elevated LH may reflect one edge of a much broader spectrum. Levels of FSH and androgens such as testosterone were not estimated in these samples but would be of interest in this regard.

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