Oocyte recovery and fertilization rates in women at various times after the administration of hCG

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Summary. Volunteer women requesting laparoscopic sterilization were subjected to a fixed schedule of ovulation induction and oocyte recovery. Follicle aspiration was carried out in four groups: those to whom hCG was not administered and 12, 24 or 36 h respectively after the administration of hCG. For each group oocytes were cultured *in vitro* for 42 h, 30 h, 18 h and 6 h respectively, before insemination with donor spermatozoa. Oocyte recovery rates improved with longer hCG-to-recovery intervals (36% with no hCG to 81% 36 h after hCG). Although there was a slight reduction in fertilization rates when oocytes were not exposed to hCG in the follicle, normal cleavage was noted in more than 50% of oocytes in all four groups. It therefore appears that the final maturation stages of the human oocyte are not dependent on the midcycle gonadotrophin surge, provided the oocyte is matured *in vitro* before insemination. However, it was also evident that the fertilization rates were reduced when oocytes were removed from less mature follicles, as reflected by high androstenedione/ oestradiol ratios.

Introduction

Although it has been known for some time that mammalian oocytes can undergo meiotic maturation when removed from antral follicles and cultured *in vitro* (Pincus & Enzmann, 1935; Edwards, 1965), such oocytes apparently have a limited capacity for normal fertilization and a very low frequency of preimplantation development (Cross & Brinster, 1970; Moor & Trounson, 1977). It has been suggested that there is deficient cytoplasmic maturation in such spontaneously maturing oocytes, although nuclear maturation appears normal (Thibault, 1977). The stage at which human oocytes acquire such maturity and capacity for normal development has not been clearly defined and in particular the relevance of the preovulatory LH surge to oocyte maturation has not been clarified. Trounson, Leeton & Wood (1982) have reported that the oocytes of sheep must remain within the follicle after LH or hCG for an obligatory phase of 6–12 h before removal, if normal embryo development is to be achieved. We have tried to determine whether such an obligatory phase exists in women and, if so, could the period of time be defined. Besides considering more basic questions of oocyte maturation, this study aimed to determine whether flexibility was possible, within the context of clinical in-vitro fertilization programmes, in the timing of oocyte recovery after hCG administration.

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Materials and Methods

This study was carried out within guidelines suggested by the MRC and the RCOG and with the approval of the local Reproductive Medicine Ethical Committee.

Ovulation induction. All patients were volunteers who had attended the gynaecological clinics, requesting laparoscopic sterilization. After this consultation, they were asked if they would be prepared to donate oocytes for research purposes. If their response was favourable the implications of involvement in the study were explained in detail and the patients were asked to discuss this with their partners. All patients (and partners) signed a consent form which stated that they could withdraw from the study at any time without prejudice.

Patients using oral contraception were requested to stop this and use another form of contraception. They were asked to contact the clinic with the date of their first spontaneous menstruation. They were then sent a further list of instructions which included directions to take oral norethisterone (Primolut N: Schering, Burgess Hill, West Sussex, UK), 5 mg twice daily starting on Day 21 of that cycle and to continue this for 7–14 days. The timing of onset of the next withdrawal bleeding was then adjusted (it usually occurred 2–3 days after cessation of norethisterone treatment), so that the subsequent fixed schedule of ovulation induction and follicle aspiration could be arranged to the convenience of the patient and the hospital. This fixed schedule, which involves the oral administration of clomiphene citrate (Clomid: Merrell, Slough, U.K.) 150 mg daily from Days 7 to 11 of the treatment cycle (Day 1 being first day after cessation of norethisterone, regardless of onset of bleeding), has been described in detail (Templeton *et al.*, 1984). For this study the adjustments described by Messinis, Templeton, Angell & Aitken (1985) were used and hCG (Pregnyl: Organon, Harden, U.K.) 4500 i.u. was given intramuscularly on Day 16.

Laparoscopy was carried out using a standard technique, with CO_2 for intraperitoneal gas insufflation. All follicles $\geqslant 16$ mm were aspirated. With the aid of microscopy, the oocyte was identified in the follicular fluid and placed in culture medium (described below). The appearance of the cumulus, corona and follicle cells was noted. The volume of follicular fluid was measured, and aliquants were set aside for subsequent assay of oestradiol and androstenedione (Templeton *et al.*, 1984).

Timing of hCG administration and oocyte recovery. Each patient was allocated to one of four groups, depending on the interval between hCG administration and follicle aspiration. The first group had follicle aspiration on Day 16 of the fixed schedule without hCG administration (Group 1). In the remaining three groups follicle aspiration was carried out 12, 24 or 36 h after hCG (Groups 2, 3 and 4 respectively). Venous blood was withdrawn from each patient on admission to hospital (Day 15) and again immediately before hCG (or oocyte recovery in Group 1) for assay of plasma LH (Djahanbakhch, McNeilly, Hobson & Templeton, 1981).

In-vitro oocyte culture. Oocytes were cultured *in vitro* before insemination for various times, i.e. 42, 30, 18 and 6 h for Groups 1, 2, 3 and 4 respectively. This was based on the optimal hCG-to-recovery interval of 36 h, plus the 6-h pre-fertilization incubation of the oocyte suggested by Trounson *et al.* (1982). Insemination and culture techniques have been described previously (Angell, Aitken, van Look, Lumsden & Templeton, 1983; Templeton *et al.*, 1984). The culture medium was Earles medium (Flow Laboratories, Stirling, U.K.) with sodium pyruvate and 8% heat-inactivated serum from the patient. The medium was bicarbonate-buffered and culture took place in a CO_2 incubator at 37°C. Insemination was carried out using donor spermatozoa of proven fertility prepared as previously described and at a concentration of 2×10^5 spermatozoa/ml.

After insemination, fertilization was confirmed by the appearance of pronuclei (when visible), and then signs of cleavage at the normal rate. After fertilization was confirmed, the oocytes were transferred to culture medium containing 15% patient's serum. In most cases, development was stopped between 4 and 8 cells by addition of colcemid in an attempt to obtain preparations for

chromosome analysis (Angell *et al.*, 1983). These results will be reported in detail elsewhere. Development was sometimes allowed to proceed to the morula or blastocyst stage.

Results

The number of patients initially recruited into each group, and the reasons for and number of exclusions are shown in Table 1. For the purposes of this study, any patient who had ovulated, or had a spontaneous LH surge before hCG administration, or had poor follicular response (no follicles $\geqslant 16$ mm) was excluded. The number of follicles $\geqslant 16$ mm aspirated in each group is also shown in Table 1 as is the average number of follicles per patient. This was similar ($1 \cdot 8$–$2 \cdot 0$) in all four groups.

Table 1. Numbers of patients recruited, reasons for exclusions and number of follicles in each group

	Group 1 (no hCG)	Group 2 (12 h after hCG)	Group 3 (24 h after hCG)	Group 4 (36 h after hCG)
No. of patients recruited	26	17	9	13
Excluded for:				
Ovulated	1	0	1	3
Endogenous LH rise	2	1	1	0
No follicles $\geqslant 16$ mm ($2 \cdot 0$ ml)	4	1	2	2
No. of patients in study	19	15	5	8
Follicles aspirated	36	27	10	16
Follicles/patient	$1 \cdot 9$	$1 \cdot 8$	$2 \cdot 0$	$2 \cdot 0$

There was no difference in the mean $\pm$ s.e.m. follicular volume amongst the four groups: $5 \cdot 44 \pm 0 \cdot 49$ ml in Group 1, $5 \cdot 93 \pm 0 \cdot 61$ ml in Group 2, $5 \cdot 97 \pm 1 \cdot 50$ ml in Group 3, and $5 \cdot 82 \pm 0 \cdot 67$ ml in Group 4.

The oocyte recovery rates per follicle aspirated are shown in Table 2. Recovery became progressively easier with longer hCG-to-recovery intervals. The recovery rate in patients in Groups 1 and 2 (36% and 44% respectively) was significantly lower than in Group 4 (81%). This latter recovery rate is only slightly less than that seen in therapeutic in-vitro fertilization programmes. Oocytes recovered from patients in Groups 1 and 2 showed little or no cumulus expansion, and in several cases were identified with difficulty in sheets of undissociated follicle cells.

Fertilization and cleavage rates amongst recovered oocytes are also shown in Table 2. Because of the surrounding follicle cells, there was difficulty in identifying pronuclei in some oocytes,

Table 2. Oocyte recovery and cleavage rates in the four groups

	Group 1	Group 2	Group 3	Group 4
No. of follicles aspirated	36	27	10	16
No. of oocytes identified (%)	13 (36)	12 (44)	5 (50)	13 (81)
No. of oocytes which cleaved (%)	7 (54)	8 (67)	4 (80)	10 (77)

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particularly those in Groups 1 and 2. However, whenever two pronuclei were confirmed, cleavage continued at the normal rate. Normal cleavage was noted in more than 50% of oocytes in all four groups. There was slight reduction in Group 1, but this was not statistically significant when compared with Groups 2, 3 and 4. Thus, 7 of 13 oocytes that had not been exposed to hCG but were allowed to mature *in vitro* showed evidence of early cleavage and, of these, two were allowed to proceed to the blastocyst and one to the morula stage. Of the remaining 4 eggs, DNA microfluorometry or direct chromosome analysis confirmed a diploid number of chromosome sets.
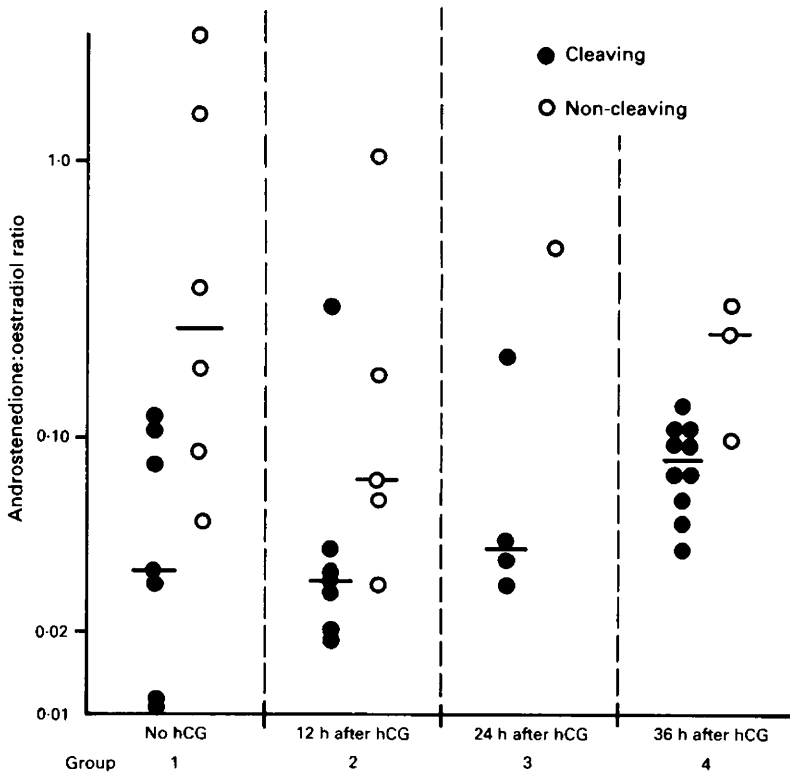

Fig. 1. Comparison of individual results in the four study groups of androstenedione:oestradiol ratios in the follicular fluid.

Finally, as a measure of the maturity of the follicles at the time of oocyte recovery, we measured oestradiol and androstenedione concentrations and calculated the androstenedione to oestradiol ratio (McNatty, Smith, Makris, Osathanondh & Ryan, 1979). A ratio of around 0·1 or less is said to reflect a mature follicle (Thebault, Testart, Castanier & Frydman, 1982). We used this ratio rather than the measured concentration of hormone because of the marked decrease in concentration of oestradiol and androstenedione that takes place after the administration of hCG (Templeton, 1985). The results are shown in Fig. 1. The androstenedione/oestradiol ratios were noticeably lower in follicles which yielded cleaving oocytes than in those yielding oocytes that did not cleave. When there were sufficient numbers to perform statistical analysis (Groups 1 and 2), the difference was significant (Wilcoxon Rank Sum Test, $P < 0{\cdot}05$). When all four groups were studied together (Fig. 2) there was again a significant difference (Wilcoxon Rank Sum Test, $P < 0{\cdot}001$).
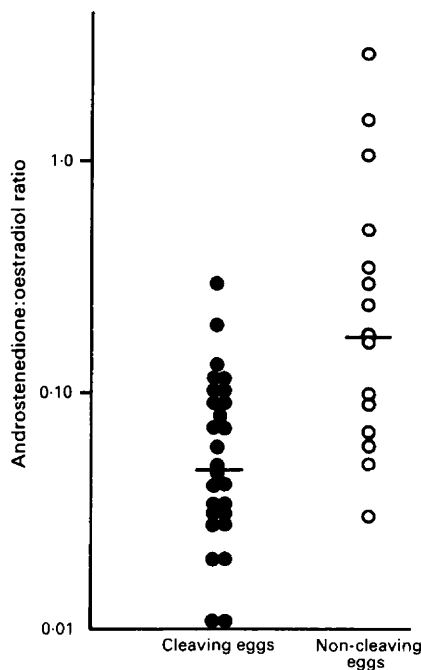
Fig. 2. Androstenedione:oestradiol ratios in fluids of follicles that gave rise to cleaving or non-cleaving oocytes.

Discussion

A study such as this would only be possible within the context of a donor oocyte programme. Any manoeuvre that might risk the already precarious outcome in an embryo transfer programme could not be justified on medical and ethical grounds. Our first problem was to establish a fixed schedule of ovulation induction and follicle aspiration which would provide the best results in terms of follicular development and oocyte availability, but cause minimal inconvenience to the volunteer patients and to hospital schedules. The schedule described here has been developed over a period of 3 years, and is believed to be optimal (Templeton *et al.*, 1984; Messinis *et al.*, 1985). Other approaches to achieving a supply of mature human oocytes have been described in the context of different research objectives (Braude *et al.*, 1984).

In this study 28% of 65 patients recruited did not provide suitable oocytes, either because of inadequate follicular development or because they had already ovulated or started an endogenous LH surge. In the remainder, about 2 follicles per patient were obtained and this is only slightly less than that obtained with similar ovulation induction regimens, but within the context of embryo transfer programmes (Quigley, Maklad & Wolf, 1983; Marrs, Vargyas, Shangold & Yee, 1984).

The oocyte recovery rates in therapeutic programmes is very high (Johnston, 1985; Steptoe, 1985), but then follicle aspiration is done in the optimal window just before ovulation (24 h after onset of the LH surge in urine and 36 h after an injection of hCG). The present study confirms that the laparoscopic oocyte recovery rate decreases significantly when recovery is attempted less than 24 h after the administration of hCG. The recovery rates when no hCG is given is only 36%, and this would be unacceptable within the context of current therapeutic regimens. Many of the oocytes recovered at this stage were identified only with difficulty in sheets of undissociated follicle cells, and their features could only be discerned after culture for about 24 h. However, the study has

shown clearly that when these oocytes are identified and subjected to maturation *in vitro* before insemination, their fertilization and cleavage rates are not significantly less than those of oocytes that had 36 h of intrafollicular exposure to hCG. Indeed, oocytes recovered from follicles before exposure to the midcycle LH surge or to the administration of hCG can be fertilized after maturation culture *in vitro* and in this way differ from sheep oocytes (Trounson, 1982) which require an obligatory phase of 6–12 h in the follicle if normal fertilization is to be achieved. When cleavage occurred with the no hCG group (Group 1), it was at the normal rate and we could confirm that blastocyst formation was possible. Furthermore, all embryos in this group were shown by karyotyping or analysis of DNA content (Angell *et al.*, 1983) to be of diploid constituency. Besides the 3 embryos that proceeded as far as the blastocyst or morula stage, all others were stopped at the 4- to 8-cell stage in an attempt to carry out chromosome analysis.

Clearly, it has not been possible to determine the full developmental capacity of these oocytes (as we were not in a position to replace them) but certain conclusions about human oocyte maturation are justified. Firstly, sperm penetration of the zona pellucida, and the subsequent steps of sperm head decondensation and male pronucleus formation, appear to be independent of the midcycle gonadotrophin surge. It would appear that spermatozoa have limited ability to penetrate immature zonae (Overstreet & Hembree, 1976; Edwards, Bavister & Steptoe, 1969) and that both FSH and LH increase sperm penetrability (Soupart & Morgenstern, 1973). Nishimoto *et al.* (1982) have shown that the number of oocytes penetrated by spermatozoa and showing an enlarged sperm head or male pronucleus increases during the follicular phase. Oocytes recovered around midcycle showed a 3-fold increase in sperm penetrability. Although we have been able to show that sperm penetration is not dependent on the midcycle gonadotrophin surge, provided the oocyte is matured *in vitro* before insemination, it would appear that the fertilization and cleavage rates were reduced when oocytes were removed from less mature follicles as reflected by high androstenedione: oestradiol ratios. The process of nuclear (meiotic) maturation in the human oocyte is remarkably consistent both *in vitro* and *in vivo* after hCG or an endogenous LH surge. Germinal vesicle breakdown occurs around 24 h after the ovulatory stimulus or removal from the follicle and the egg is arrested in metaphase II at around 32–36 h. Ovulation occurs 2–8 h after this (Edwards, 1973; Shea, Baker & Latour, 1975; Seibel, Shine, Smith & Taymor, 1982; Testart, Thebault & Frydman, 1982). Presumably, any attempts to fertilize the egg before the completion of this process would be in vain and, in this respect, possibly the delay in timing insemination of up to 42 h in this study was crucial. Trounson (1982) has reported problems with sheep and human oocytes for which early insemination is associated with abnormal fertilization and subsequent fragmentation of the embryo. It would therefore seem that early diploid cleavage and hence sperm penetration and pronuclear formation can be achieved in human oocytes that have not been exposed to the LH surge or hCG in the follicle. However, cytoplasmic maturation could not be fully assessed in this study and such assessment would depend on replacement of cleaving embryos in a recipient uterus, a step which we were not in a position to take and which, even in optimal circumstances, carries a low success rate in women.

Schroeder & Eppig (1984) have shown that mouse oocytes at the germinal vesicle stage can undergo spontaneous maturation independent of gonadotrophin and steroids and normal developmental capacity was proved by the birth of live offspring. Schroeder & Eppig (1984) argued that the success was dependent on the culture medium and particularly on the addition of mouse serum. However, the time course of events in the mouse is considerably shorter than in women and sheep, and further study in this area is needed. We achieved in-vitro maturation only when oocytes were removed from mature follicles, and when the androstenedione:oestradiol ratio was more than 0·1, successful cleavage was seldom achieved. Almost certainly, oocytes removed before this stage will require further support if in-vitro maturation is to be achieved, and in this respect the addition of gonadotrophin and steroid to the culture medium would appear to be critical. Staigmiller & Moor (1984) have demonstrated the importance not only of these factors, but also of the support of adjacent follicle cells in the culture medium. Studies along these lines will have considerable benefit

in clinical terms, particularly if robust in-vitro maturation systems could be developed which could obviate the need for the intensive midcycle monitoring that is currently required in embryo transfer programmes. However, bearing in mind the low recovery rate of immature oocytes using standard techniques, it may be necessary to develop new methods of follicle aspiration.

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