Characteristics of populations of granulosa cells from individual follicles in women undergoing 'coasting' during controlled ovarian stimulation (COS) for IVF

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BACKGROUND: The aim of this study was to evaluate the functional characteristics of granulosa cell populations of individual follicles of women undergoing controlled ovarian stimulation (COS) for IVF/ICSI in whom gonadotrophin had been withheld ('coasted') for the prevention of OHSS. METHODS: Follicular fluid and granulosa cells were isolated from 224 individual follicles in 41 women who had been coasted and from 257 individual follicles in 50 women who had a 'normal' response to COS. Cells were cultured at 10 000 cells per well, to evaluate progesterone secretion. Follicular fluid was assayed for progesterone and estradiol (E_2). RESULTS: No significant differences were observed between the two groups with respect to granulosa cell number or follicular fluid progesterone and E_2 and follicle size, the retrieval of an oocyte and the subsequent fertilization of the oocyte. However, the granulosa cells derived from the coasted group showed a higher rate of progesterone secretion per cell at 72 h which was sustained for longer. Differences were also seen at 72 and 120 h of culture with a loss of correlation between progesterone secretion and follicle diameter in the coasted group. CONCLUSIONS: Our findings suggest that coasting has an effect on the functional capacity of the granulosa cells and the duration of their function. It is likely that in women at risk of OHSS who are not coasted, the granulosa cells have the capacity to produce significantly more chemical mediators per cell and for a more prolonged period of time.

Key words: coasting/controlled ovarian stimulation/granulosa cells/progesterone

Introduction

Ovarian hyperstimulation syndrome (OHSS) is a potentially severe, iatrogenic complication of supra-physiological ovarian stimulation which has a serious impact on the patient's health and may cause severe morbidity and even mortality. Although the pathophysiology of this syndrome has not been fully elucidated, it seems likely that the release of chemical mediators or precursors secreted by the ovaries under the control of HCG plays a key role in triggering this syndrome. The exact factors are unknown, but increasing evidence supports the role of cytokines and vasoactive substances (Mathur *et al.*, 1997).

Since OHSS only occurs following HCG administration, cancellation of the cycle is the best method of preventing OHSS. Other strategies have included, pre-ovulatory aspiration of some follicles (Amit *et al.*, 1993), the peri-ovulatory administration of a GnRH agonist instead of HCG, cryopreservation of all embryos (Wada *et al.*, 1993; Titinen *et al.*,

1995) and i.v. administration of albumin (Asch *et al.*, 1993). Coasting, in which gonadotrophins are withheld and HCG administration delayed to allow serum estradiol (E_2) levels to decrease to so-called 'safer' levels, has been shown to be an effective strategy in preventing severe OHSS without compromising the cycle outcome (Sher *et al.*, 1995; Benadiva *et al.*, 1997; Al-Shawaf *et al.*, 2001). The hypothesis in this strategy is that coasting diminishes the functional granulosa cell cohort available for luteinization by inhibiting granulosa cell proliferation and ultimately leading to progressive granulosa cell apoptosis. Thus, there is a gradual decline in circulating levels of serum E_2 and, probably more importantly, a reduction in the chemical mediators or precursors that trigger OHSS.

The aim of this study was to evaluate the granulosa cell population in individual follicles with respect to cell number and *in vitro* and *in vivo* steroidogenesis in women undergoing controlled ovarian stimulation (COS) who have been coasted for the prevention of OHSS.

Table I. Patient characteristics of women who were coasted and 'normal' responders

	Coasted group	Control group	P
Number	41	50	
Mean age ($\pm$ SD)(years)	31.0 $\pm$ 3.2	32.6 $\pm$ 4.2	0.3
Mean BMI ($\pm$ SD)(kg/m^2)	24.0 $\pm$ 3.9	25.1 $\pm$ 4.5	0.3
Mean FSH ($\pm$ SD)(U/l)	6.1 $\pm$ 1.8	7.0 $\pm$ 1.7	0.2
Mean LH ($\pm$ SD)(U/l)	6.7 $\pm$ 4.4	5.3 $\pm$ 2.4	0.2
Mean duration of subfertility ($\pm$ SD)(years)	5.4 $\pm$ 3.0	4.7 $\pm$ 2.3	0.2
Median cycle number (IQR)	1 (1–2)	1 (1–2)	0.6
Aetiology			
Tubal	13	23	
Male	13	13	
Unexplained	8	12	
Donor	1	2	
Polycystic ovary syndrome (PCOS)	5	0	
Pre-chemotherapy	1	0	

Materials and methods

Patients

Ninety-one women were studied in total. All patients were less than 40 years of age (range 24–39) with various infertility diagnoses (tubal, male, unexplained and an ovum donor) excluding endometriosis (Table I).

Coasted group

Forty-one women undergoing COS for IVF/ICSI were identified as being at risk of developing OHSS during the study period. In accordance with the clinical protocol, women were defined as being at risk if >20 follicles were observed in both ovaries by transvaginal ultrasound. The serum E_2 level was determined and gonadotrophins were withheld and HCG delayed if the serum levels were >13 000 pmol/l. Daily serum E_2 levels were determined together with alternate day transvaginal scans until the serum E_2 levels had fallen below 10 000 pmol/l. Protocol violators were not included in the study group (Table II).

Control group—'normal' responders

Fifty women undergoing COS for IVF/ICSI were recruited to the study prior to oocyte retrieval, on the day of HCG administration,

if a 'normal' response was observed on ultrasound. A 'normal' response was defined as more than six follicles >14 mm in diameter but <20 follicles in total after no more than 13 days of gonadotrophin stimulation (Table II).

Written consent was obtained from each patient prior to commencing the study which was approved by the East London and The City Health Authority Research Ethics Committee on February 18, 1999 (Study number: P/98/222).

All women underwent a long GnRH agonist stimulation protocol commencing in the mid-luteal phase. Multiple follicular development was stimulated with s.c. injections of FSH [Puregon (Organon, Cambridge, UK), Metrodin (Serono, Welwyn Garden City, UK) or HMG (Menogon containing both FSH and LH (Ferring, Datchet, UK)] and monitored by transvaginal ultrasound measurements. When a minimum of three follicles had reached a diameter of at least 18 mm, 10 000 IU of HCG (Profasi; Serono) was administered s.c. Oocyte retrieval was performed 36 h later under ultrasound guidance and i.v. sedation.

For routine IVF, oocytes were inseminated at 40 h post-HCG administration and were checked the following day for the appearance of normal fertilization by the presence of two pronuclei. Where ICSI was necessary, the cumulus cells were digested 1 h after oocyte retrieval and maturity assessed. All metaphase II oocytes were injected and checked the following day for the presence of two pronuclei (2PN).

Isolation and culture of granulosa cells from individual follicles

Individual follicles of varying size (total of 4–6 follicles) were identified prior to aspiration and the mean diameter was measured in two dimensions by transvaginal ultrasound (Hitachi, model EUB-525, Northants, UK). The first follicle was never used for analysis as it had been shown to contain large numbers of vaginal epithelial cells. Subsequent follicles were used, if the size was appropriate, in order of being the next easiest follicle to aspirate. Following measurement, the follicle was gently pierced using a double lumen needle (Cook, Herts, UK) and aspirated, allowing the follicle to collapse slowly around the needle. The follicle was then flushed with 4.5 ml of heparinized saline (3 × 1.5 ml automated flushes) and aspirated again to maximize the number of luteinized granulosa cells collected. All follicles were flushed with the same volume of saline. All measurements and aspirations were performed by the same operator (A.J.T.). Heavily blood-stained aspirates were

Table II. Details of ovarian stimulation and clinical outcome in 'normal' responders ($n = 50$) compared with women who were coasted ($n = 41$) [mean $\pm$ SD, median (IQR)]

	Coasted group	Control group	P
Days of stimulation	10.3 $\pm$ 1.6	11.8 $\pm$ 1.3	<0.0001
No. of units of gonadotrophin	1800 (1463–2250)	2325 (1781–3188)	0.0008
Day of coasting	11 (10–12)	–	
E_2 on day coasted (pmol/l)	>13 200	–	
FSH on day coasted (U/l)	13.5 $\pm$ 6.0	–	
No. of days coasted	3 (3–5) (range 2–7)	–	
Day of HCG	14 (14–16)	13 (12–13)	<0.0001
E_2 on day of HCG (pmol/l)	6690 $\pm$ 3500	–	
FSH on day of HCG (U/l)	4.1 $\pm$ 1.9	–	
E_2 on day of oocyte retrieval (pmol/l)	4790 $\pm$ 1970	3920 $\pm$ 2550	0.1
No. of follicles ≤14 mm	10 (5.5–14)	7 (5–10)	0.03
No. of follicles ≥15mm	20 (15–28)	7 (6–9)	<0.0001
No. of oocytes	13 (9.5–17.5)	11 (9–15)	0.1
No. of 2PN	6 (3–8)	6 (4–8)	0.7
Fertilization (%)	224/491 (45.6)	298/555 (53.7)	0.01
No. of embryos transferred	2 (2–2)	2 (2–2)	0.9
Pregnancy rate (%)	11/38 (28.9)	18/49 (36.7)	0.5
Implantation rate (%)	14/69 (20.3)	20/104 (19.2)	0.9

discarded. A record was also kept of the volume of fluid aspirated from each individual follicle. Following examination of the follicular fluid from the single follicle by the embryologist, the fluid was placed into a sterile tube and the oocyte number (if present) recorded on the tube. If an oocyte was not retrieved, the follicle was flushed again with a further 3 ml of heparinized saline and if an oocyte was still not obtained, this follicle was recorded as having no oocyte retrieved. The additional 3 ml of flush was discarded and not used for analysis. If there was any doubt regarding the origin of the oocyte due to cross-follicle contamination, a different follicle was aspirated for the purposes of the study.

The follicular fluids were taken to the laboratory immediately after aspiration for isolation of the granulosa cells. Each tube was centrifuged at 250 g. for 10 min and the follicular fluid supernatant frozen at $-20°C$ for later hormone analysis. The pellet was resuspended in 4 ml of medium [RPMI-1640 with glutamine and $NaHCO_3$ (Sigma, Poole, UK) supplemented with 10% fetal calf serum (Gibco-BRL, Paisley, Renfrewshire) and 1% antibiotic containing 10 000 µg/ml penicillin G sodium, 25 µg/ml streptomycin (Gibco-BRL)], and centrifuged at 250 g for 10 min. The pellet was again resuspended in 5 ml of medium and then incubated at 37°C for 30 min with 0.2% hyaluronidase (80 IU/ml, Medicult, UK) for cell dispersion.

Following further centrifugation at 250 g for 10 min, the pellet was resuspended with 2 ml of medium and layered over 4 ml of 50% Percoll (Pharmacia, Amersham, UK). This was centrifuged at 300 g for 20 min to separate the luteinized granulosa cell buffy coat from red blood cells. The cells were removed using a pipette and washed with 8 ml of medium. After centrifugation at 300 g for 10 min, the pellet was resuspended and cell numbers assessed with the aid of a haemocytometer. Viable cell number was assessed by the trypan blue (Sigma) exclusion test, a record being made of the number of non-viable cells. Cells were then plated, in triplicate, at a concentration of 10 000 cells per well in 24-well plates and incubated at 37°C in a 95% air, 5% CO_2 humidified environment for up to 5 days. Medium was changed after 24, 72 and 120 h of culture. After medium collection, the wells were rinsed once with sterile phosphate-buffered saline (PBS) to remove unattached cells and debris before replacing with fresh medium. All media removed were stored at $-20°C$ for later hormone analysis.

Immunoassay of steroid hormones

The progesterone concentration of the follicular fluid and culture media was determined using an enzyme-linked immunosorbent assay (ELISA) (NETRIA, London UK). The intra-assay coefficient of variation of the assay was 5.9% and the inter-assay coefficients of variation were 5.6, 5.8 and 6% at 15, 25 and 60 nmol/l, respectively. The minimal detection concentration of progesterone was 1 nmol/l. The progesterone antibody is 100% specific for progesterone, showing cross-reactivity with: 5β-pregnane-3, 20-dione, 44%; 5α-pregnane-3,20-dione, 11%; 11-deoxycorticosterone, 2%; corticosterone, 0.3%; pregnenolone, 0.3%; testosterone, $<0.02\%$; cortisol, $<0.02\%$; estriol, $<0.03\%$; estradiol, $<0.03\%$; and oestrone, $<0.03\%$.

The estradiol concentration of the follicular fluid was determined using a commercially produced radioimmunoassay (Diasorin, Brussels, Belgium). The intra-assay coefficient of variation for the assay was $<10\%$ and the inter-assay coefficients of variation were 8.2, 3.6 and 6.6% at 35, 2600 and 6600 pmol/l, respectively. The estradiol antiserum is 100% specific for E_2, showing cross-reactivity with: estrone, 0.63%; estriol, 0.65%; ethinylestradiol, $<0.1\%$; progesterone, $<0.1\%$; testosterone, $<0.1\%$; and androstenediol, $<0.1\%$. The concentration of both progesterone and E_2 is known to be much higher in follicular fluid than in serum and, therefore, to reduce the concentration of steroids into the range measurable by the steroid assays, it was necessary to dilute the follicular fluid. For the estradiol assay, linearity of dilution had been tested by assaying two sera high in E_2 diluted with human serum as recommended. For the progesterone assays of both follicular fluid and media, all samples were diluted with culture media. Dilution in parallel was demonstrated using culture media.

Statistical analyses

All data were tested for non-normality using the Shapiro–Wilk test. Where data were found to be parametric, means were compared using the unpaired t-test, and where data were found to be non-parametric, medians were compared using the Mann–Whitney U-test. All means are expressed with the SD and all medians are expressed with the interquartile range. The χ^2 test or Fisher's exact test was used to compare frequencies, and a one-way analysis of variance to compare >2 variables. Differences seen were considered to be statistically significant if $P < 0.05$.

Results

A total of 224 follicles were aspirated from 41 women in the coasted group and 257 from 50 women in the control group. The range of diameter of the follicles aspirated was 10–39 mm and the range in volume was 0.1–26.5 ml. The correlation between the volume of fluid aspirated and the diameter of the follicle at the time of oocyte retrieval was statistically significant ($r = 0.91$; $P < 0.0001$).

Clinical observations

Patient characteristics were similar for both groups except that there were five cases of polycystic ovary syndrome (PCOS) in the coasted group (Table I). Patients were coasted between 2 and 7 days, with the median day coasting was commenced being day 11 of COS. The median day of HCG administration after COS was commenced was 1 day later in the coasted group ($P < 0.0001$). In the coasted group, the mean serum E_2 level on the day of HCG was 6690 pmol/l. Statistically significantly more follicles both ≤14 mm and ≥15 mm in diameter were seen in the coasted group. No differences were observed in the median number of oocytes retrieved although a lower fertilization rate was seen in the coasted group ($P < 0.01$). However, no significant differences were observed in the median number of embryos available for transfer. No patient, in either group, developed moderate or severe OHSS.

Oocyte retrieval and fertilization

The percentage of follicles from which an oocyte was retrieved appeared to be lower in the coasted group at most follicle sizes. No oocytes were retrieved from follicles >29 mm in diameter in the coasted group, although only small numbers of follicles of this diameter were aspirated. In the coasted group, fertilization was observed only in those oocytes retrieved from follicles of 12–28 mm in diameter, whereas in the control group fertilization was observed in oocytes retrieved from follicles of 11–35 mm in diameter.

Luteinized granulosa cell population in relation to follicle size, retrieval of an oocyte and fertilization

Both groups showed a similar trend towards increasing cell numbers as the follicle diameter increased, but neither showed a direct correlation between follicle size and cell number (coasted group $r = 0.07$, $P = 0.3$, and control group $r = 0.08$, $P = 0.2$). No significant differences were seen between the coasted and control groups in the number of granulosa cell derived from follicles from which an oocyte was retrieved or not (195 000 versus 170 000, $P = 0.4$, and 175 000 versus 150 000, $P = 0.3$) and whether the oocyte fertilized normally, abnormally or failed to fertilize.

Follicular fluid progesterone and E_2 in relation to follicle size, the retrieval of an oocyte and fertilization

No significant correlation was seen between the follicle size and progesterone concentration of the follicular fluid in both groups (Figure 1). Both groups showed a similar decrease in E_2 concentration as the follicle size increased. The correlation between follicle size and follicular fluid E_2 concentration was statistically significant in both groups (Figure 2). Both the coasted and control groups showed greater concentrations of follicular fluid progesterone and E_2 in follicles from which an oocyte was retrieved compared with if an oocyte was not retrieved [progesterone 198 798 (110 308–499–810] versus 155 873 (84 833–517 127), $P = 0.5$, and 194 643 (129 539–267 937) versus 141 950 (48 457–200 450), $P = 0.006$, respectively; E_2 1 333 435 (1 107 584–1 893 974) versus 1 240 798 (725 002–1 875 221), $P = 0.2$ and 1 160 432 (748 282–1 596 448) versus 1 093 728 (567–2 167 857], $P = 0.5$, respectively]. Both the coasted and control groups showed greater concentrations of follicular fluid progesterone and E_2 in follicles from which an oocyte was retrieved and normal fertilization occurred compared with failed fertilization [progesterone 204 240 (109 620–

568 700) versus 186 470 (89 500–499 810), $P = 0.7$, and 221 600 (129 020–270 540) versus 166 510 (132 660–217 260), $P = 0.9$, respectively; E_2 1 404 470 (1 109 220–1 829 100) versus 1 302 370 (1 035 630–2 192 130), $P = 0.9$, and 1 160 430 (666 600–1 877 740) versus 955 760 (682 260–1 575 160), $P = 0.9$, respectively]. The only differences that were statistically significantly different were higher levels of progesterone in follicular fluid derived from follicles where an oocyte was retrieved compared with where an oocyte was not retrieved in the control group.

Progesterone secretion/10 000 cells in vitro in relation to follicle size, retrieval of an oocyte and fertilization

Culture was achieved for granulosa cells derived from follicles of all sizes with secretion and detection of progesterone. In both groups, progesterone secretion was observed to fall after 72 h, although in the coasted group progesterone secretion at 120 h was still greater than at 24 h (Figure 3). In both groups, progesterone secretion/10 000 cells was observed to be directly proportional to the follicle size. After 24 h, the coasted group and control group showed a statistically significant correlation between progesterone secretion/10 000 cells and follicle size ($r = 0.21$, $P = 0.04$ and $r = 0.22$, $P = 0.02$, respectively) (Figure 4a). After 72 and 120 h, whilst the control group continued to show a statistically significant correlation between progesterone secretion/10 000 cells and follicle size ($r = 0.31$, $P = 0.0008$ and $r = 0.22$, $P = 0.02$, respectively), the coasted group did not ($r = 0.16$, $P = 0.1$ and $r = 0.15$, $P = 0.1$, respectively, at 72 and 120 h) (Figure 4b and c).

At 72 and 120 h of culture, higher concentrations of progesterone were observed in the coasted group compared with the control group where an oocyte had been retrieved or not retrieved and where fertilization to 2PN or failed fertilization had occurred. These differences were not found to be
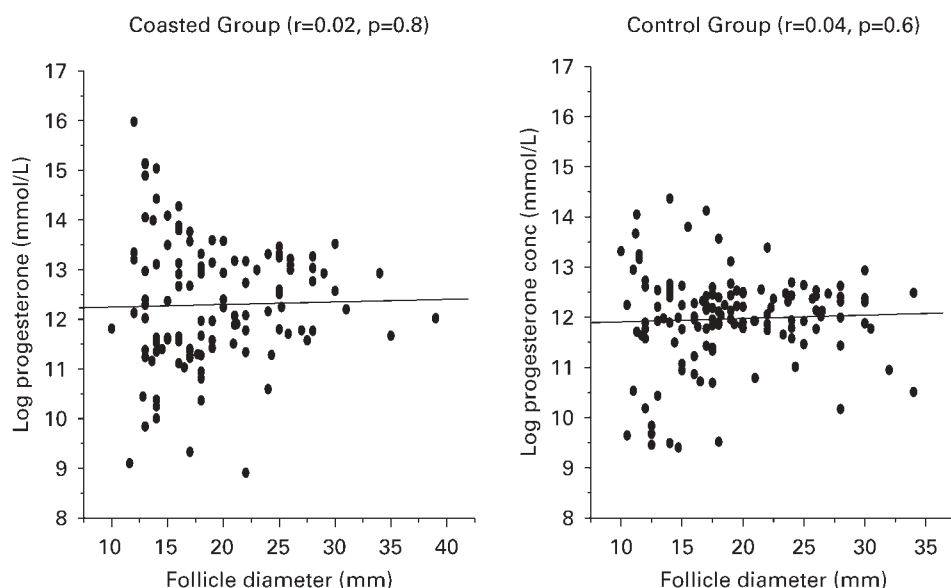

Figure 1. Follicular fluid progesterone concentration in relation to follicle size. Coasted group ($r = 0.02$, $P = 0.8$); control group ($r = 0.04$, $P = 0.6$)
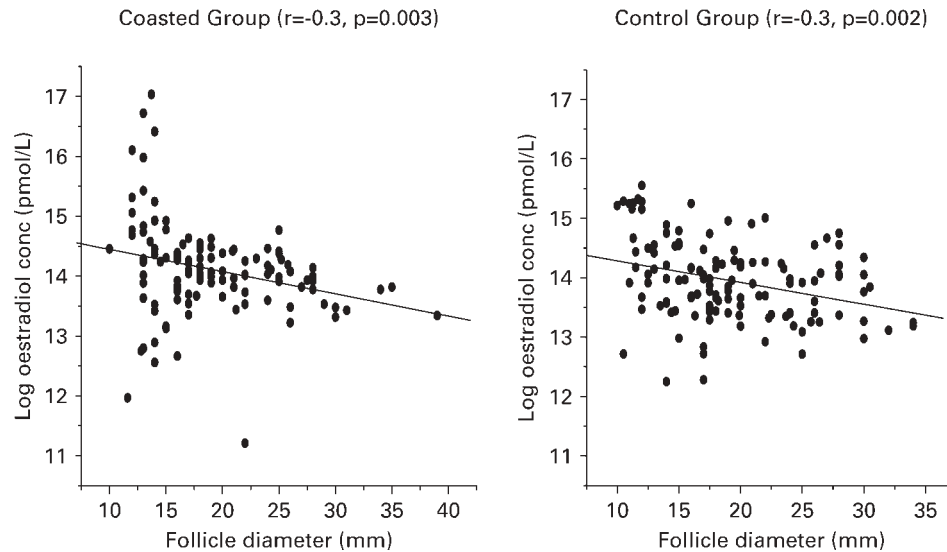
Coasted Group (r=-0.3, p=0.003) Control Group (r=-0.3, p=0.002)

Figure 2. Follicular fluid estradiol concentration in relation to follicle size. Coasted group ($r = -0.3$, $P = 0.003$); control group ($r = -0.3$, $P = 0.002$)
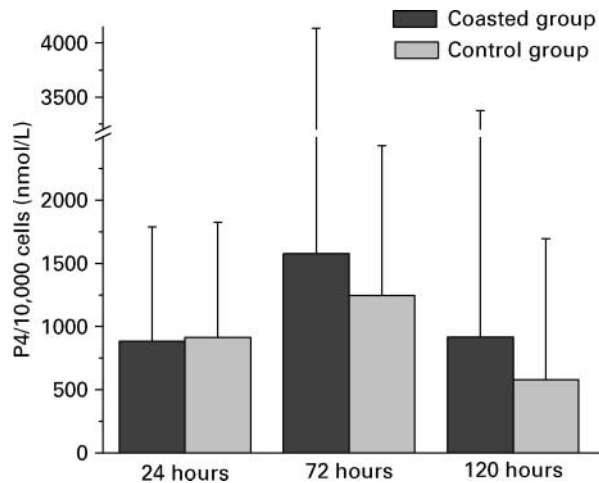

Figure 3. Progesterone secretion/10 000 granulosa cells *in vitro* over 120 h.

statistically significantly different (Tables III and IV). It should be noted that measures of progesterone and E_2 were extremely variable, as noted by the large interquartile ranges, both between patients and in the same patient. Thus, the possibility of significance in the results may be precluded because of the numbers of follicles studied.

Progesterone secretion/10 000 cells in vitro in relation to duration of coasting

Progesterone production in culture was significantly higher at 120 h in those women who were coasted for 2 days compared with coasting for 3–6 days [3299.2 (228.9–7726.7) versus 715.0 (201.8–1664.6) nmol/l, $P = 0.04$]. At 24 h, marginal significance was observed [2320.6 (421.9–3209.8) versus 715.6 (276.8–1370.0) nmol/l, $P = 0.05$] and at 72 h no significant differences were observed [3586.8 (804.3–9610.0) versus 1500.0 (283.0–3021.9), $P = 0.1$].

Discussion

This study, to our knowledge, is the first to evaluate the granulosa cell population in terms of cell number and steroidogenic activity, in individual follicles of women undergoing COS who have had gonadotrophin withheld for the prevention of OHSS. Whilst the ideal control group would have been women at risk of developing OHSS who had not been coasted, this was not ethically feasible in our clinical setting where coasting has been used successfully for many years. We therefore selected a group of women who had a so-called 'normal' response to COS in order to compare our findings with the coasted group. Clinically, as previously reported by others (Urman *et al.*, 1992; Sher *et al.*, 1995; Benadiva *et al.*, 1997; Dhont *et al.*, 1998; Tortoriello *et al.*, 1998), we have observed that coasting is an effective strategy to prevent moderate and severe OHSS when clinical protocols are adhered to. This is seen to be without compromise to the number of embryos available for transfer and pregnancy rates as compared with the control group.

It is known that some oocytes undergo apoptosis during the coasting period and that whilst 'healthy' follicles can withstand brief reductions in gonadotrophin stimulation and E_2 secretion, follicles of marginal size or competence are not likely to tolerate coasting (Fluker *et al.*, 1999). Fluker also observed that smaller follicles (<14 mm) were more susceptible to gonadotrophin deprivation than larger follicles, finding little additional growth in the smaller follicles. The oocyte retrieval rate was certainly reduced by coasting, with a median of 13 oocytes collected from a median of 10 follicles ≤14 mm and 20 follicles ≥15 mm (Table II). In this study, although only 4–6 follicles were aspirated and analysed in each case, the oocyte retrieval rate per follicle size, as compared with the control group, appeared to be lower at most follicle sizes. No oocytes were retrieved from follicles >29 mm in diameter and fertilization was not seen in oocytes derived from follicles <12 mm and >28 mm in diameter.
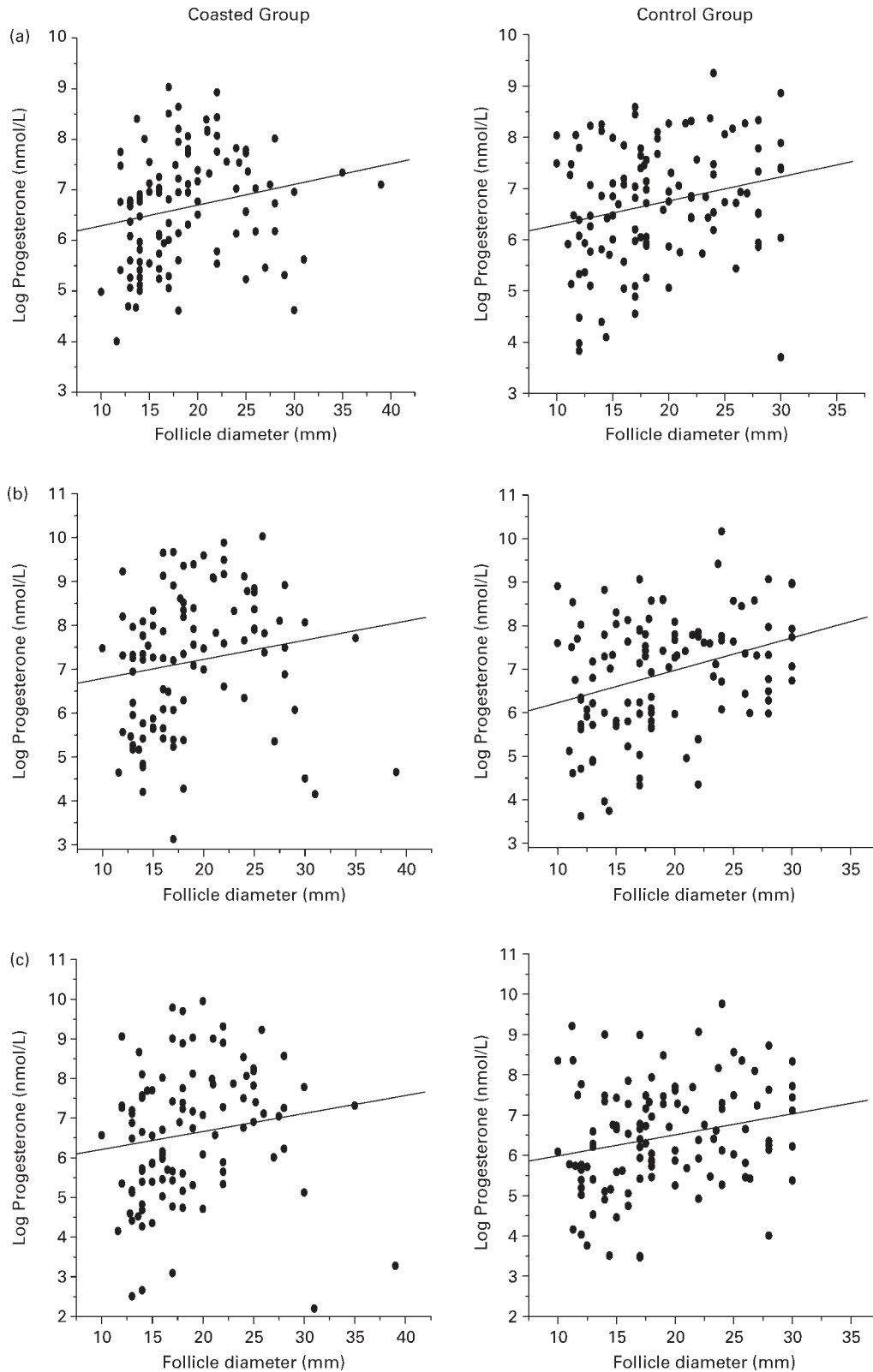
Figure 4. Progesterone/10 000 granulosa cells *in vitro* at (**a**) 24 h, (**b**) 72 h and (**c**) 120 h.

Thus there appeared to be a susceptibility to gonadotrophin withdrawal at all follicle sizes, but particularly in both the very small and very large follicles.

Granulosa cells are suggested to be more susceptible to coasting and becoming atretic than are the developing oocytes (Fluker *et al.*, 1999), and it is the size of the granulosa cell population available for luteinization following HCG that is said to determine both the incidence and severity of OHSS (Asch *et al.*, 1993; Sher *et al.*, 1995). We may have expected to see differences in the pattern of granulosa cell

Table III. Granulosa cell number and follicular fluid levels of progesterone and E_2 in relation to the retrieval of an oocyte

	Coasted group		P	Control group		P
	Oocyte retrieved	No oocyte retrieved		Oocyte retrieved	No oocyte retrieved	
Progesterone secretion/10 000 cells in 24 h (nmol/l)	878 (395–1730) (n = 52)	870 (266–1732) (n = 48)	0.7	846 376–1752 n = 84)	1025 (378–2540) (n = 29)	0.6
Progesterone secretion/10 000 cells in 72 h (nmol/l)	1542 (355–4280) (n = 52)	1600 (261–3251) (n = 48)	0.6	1149 (334–2444) (n = 84	1468 (417–2247) (n = 29)	0.9
Progesterone secretion/10 000 cells in 120 h (nmol/')	1147 (224–2409) (n = 52)	817 (208–2392) (n = 48)	0.6	583 (236–1504) (n = 84)	518 (310–2000) (n = 29)	0.5

Table IV. Granulosa cell number and follicular fluid levels of progesterone and E_2 in relation to fertilization of an oocyte

	Coasted group			P	Control group			P
	Fertilization to 2PN	Abnormal fertilization to 3PN	Failed fertilization		Fertilization to 2PN	Abnormal fertilization to 3PN	Failed fertilization	
Progesterone secretion/10 000 cells in 24 h (nmol/l)	1012 (449–2358) (n = 28)	272 (204–1045) (n = 3)	569 (247–1176) (n = 20)	0.06	805 (405–1764) (n = 47)	1073 (823–2154) (n = 11)	669 (239–1718) (n = 22)	0.3
Progesterone secretion/10 000 cells in 72 h (nmol/l)	1431 (306–7419) (n = 28)	434 (218–4208) (n = 3)	1754 (385–2740) (n = 20)	0.6	1245 (387–3012) (n = 47)	1500 (370–2406) (n = 11)	713 (303–2375) (n = 22)	0.3
Progesterone secretion/10 000 cells in 120 h (nmol/l)	1184 (250–3506) (n = 28)	267 (175–2325) (n = 3)	715 (111–1415) (n = 20)	0.1	602 (234–1540) (n = 47)	607 (353–2019) (n = 11)	437 (188–1535) (n = 22)	0.4

number relative to the follicle size, oocyte retrieval and fertilization between the two groups. However, we observed similar numbers of cells isolated per follicle size in both groups and, whilst both groups showed a trend towards a greater number of granulosa cells if an oocyte was retrieved which subsequently fertilized, these differences were not statistically significantly different in either group. Coasting may exert its therapeutic action by reducing the functioning capacity of the cells and not just their number. This study supports this hypothesis as the coasted patients' granulosa cells appear to have a greater innate capacity to produce progesterone than the control patients. Follicular fluid levels of progesterone and E_2 showed similar concentrations of the steroids in both groups with respect to follicle size. The coasting process appears to have brought the functioning capacity of the granulosa cells in line with the 'normal' responders. It must be remembered, however, that follicular fluid represents a composite of granulosa cell secretion and, because of this, follicular fluid steroids may not entirely be representative of the secretory pattern of the granulosa cells at the time of oocyte aspiration.

The culture of granulosa cells *in vitro* over 5 days clearly demonstrated a difference in granulosa cell function between the two groups. In both groups, progesterone secretion declined after 72 h of culture, although in the coasted group progesterone secretion at 120 h was still greater than at 24 h, unlike in the control group, reflecting a more sustained ability of the cells to secrete progesterone. Whilst these differences were not statistically significant, they did provide evidence of a potential trend. The duration of coasting also appeared to have an impact on the sustainability of the cells

to secrete progesterone in culture, with a significantly greater production of progesterone in women coasted for 2 days compared with 3–6 days. In the control group, after 24, 72 and 120 h we observed a significant correlation between progesterone secretion and follicle size, with greater progesterone secretion/10 000 cells in relation to follicle diameter. This may be expected and explained by the fact that as the follicle size increases, the follicle becomes more mature, as do the granulosa cells which acquire a greater complement of LH receptors available for luteinization and hence progesterone production. There appeared to be an increase in progesterone secretion in most follicle sizes at 72 and 120 h in the coasted group, but, in the coasted group, progesterone secretion did not directly correlate with follicle size after 72 and 120 h. Furthermore, we may be seeing much lower rates of progesterone secretion than we would have observed had these patients not been coasted and that gonadotrophin withdrawal has the effect of reducing the functional capacity of all granulosa cells, but that certain follicles are more susceptible than others probably related to the maturity of the follicle at the time of coasting. Whilst follicle maturity has been related to follicle size (Wittmaack *et al.*, 1994), the two are not totally dependent (Salha *et al.*, 1998) and, whilst coasting probably exerts most effect in the very small and very large follicles, arrest of maturation is likely to be seen in follicles of all sizes. Eight percent of follicles studied in the coasted group came from five women with PCOS. Whilst there may be differences in granulosa cell function between different aetiological causes of subfertility, we do not feel that the results have been significantly skewed by including these women. In conclusion, no cases of moderate or severe OHSS

were observed in the coasted group. Differences are observed in the function of granulosa cells derived from individual follicles in the coasted group of women compared with the control group. These differences may be secondary to withholding gonadotrophins or may be representative of women at risk of developing OHSS. In the coasted group, progesterone secretion over 120 h in culture appears to be sustained for a greater period of time. This probably reflects the greater functioning capacity of the granulosa cells in women at risk of OHSS which is likely to be diminished by the coasting process. Indeed, if coasting lasted >2 days, a less sustained secretion of progesterone by the granulosa cells was observed in culture. It is our belief that in women at risk of OHSS who are not coasted, it is likely that the functioning capacity per granulosa cell is much higher, which, together with the increased number of follicles, leads to a much greater total functioning granulosa cell mass that not only has the capacity to produce chemical mediators at higher concentrations per cell but is also likely to be able to produce them for a more prolonged period of time.

Acknowledgements

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