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ESTABLISHMENT OF A HUMAN GRANULOSA CELL CULTURE FOR EVALUATION OF THE BIOLOGICAL ACTIVITY OF HUMAN RECOMBINANT GONADOTROPHINS

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CONTENTS

INTRODUCTION

During the follicular phase of the human menstruation cycle, an orderly sequence of events take place which ensures that the appropriate number of follicles are ready for ovulation. The initial stimulus originates in the hypothalamus with the release of gonadotropic hormone-releasing hormone (GnRH), which stimulates the pituitary gland to secrete gonadotrophins. In a growing follicle, the differentiation of the granulosa cells is initiated by follicle-stimulating hormone (FSH), which induces receptor development for luteinizing hormone (LH) and steroid production. The granulosa cells of the developing follicle have the ability to synthesize all three classes of sex steroids. However, significantly more oestrogens than either androgen or progestin is produced. The production of oestrogens requires interaction between the granulosa and theca cells. The two-cell two-gonadotrophin theory originally described by Falck (1959) is a logical explanation of the events involved in ovarian oestrogen biosynthesis (Fig. 1). In the antral follicles, LH receptors are present only on the theca cells, and FSH receptors only on the granulosa cells. Theca cells are characterized by steroidogenic activity in response to LH, resulting specifically in androgen (androstenedione and testosterone) production. Androgens produced by theca cells are converted to oestrogens by the aromatase enzyme within granulosa cells under the stimulatory control of FSH (Shoham and Schachter 1996). Together, FSH and oestradiol promote an accumulation of FSH receptors, allowing the follicle to respond to relatively low concentrations of FSH. Furthermore, FSH combines with oestrogen to exert mitogenic action on granulosa cell proliferation and to increase the production of follicular fluid in which oestrogen becomes the dominant substance. Besides oestrogens, a wide variety of hormones, growth factors, enzymes and peptides are present in the follicular fluid. Some of them are synthesized locally by the granulosa cells in response to FSH (inhibin, activin and insulin-like growth factor-I [IGF-I]).

In the absence of FSH, androgens will predominate in the follicular fluid. The role of androgens in early follicular development is complex. Androgens at low concentrations are thought to stimulate FSH responsiveness and can enhance their own aromatization at intermediate stages of follicular development (Harlow et al. 1988). At higher concentrations,

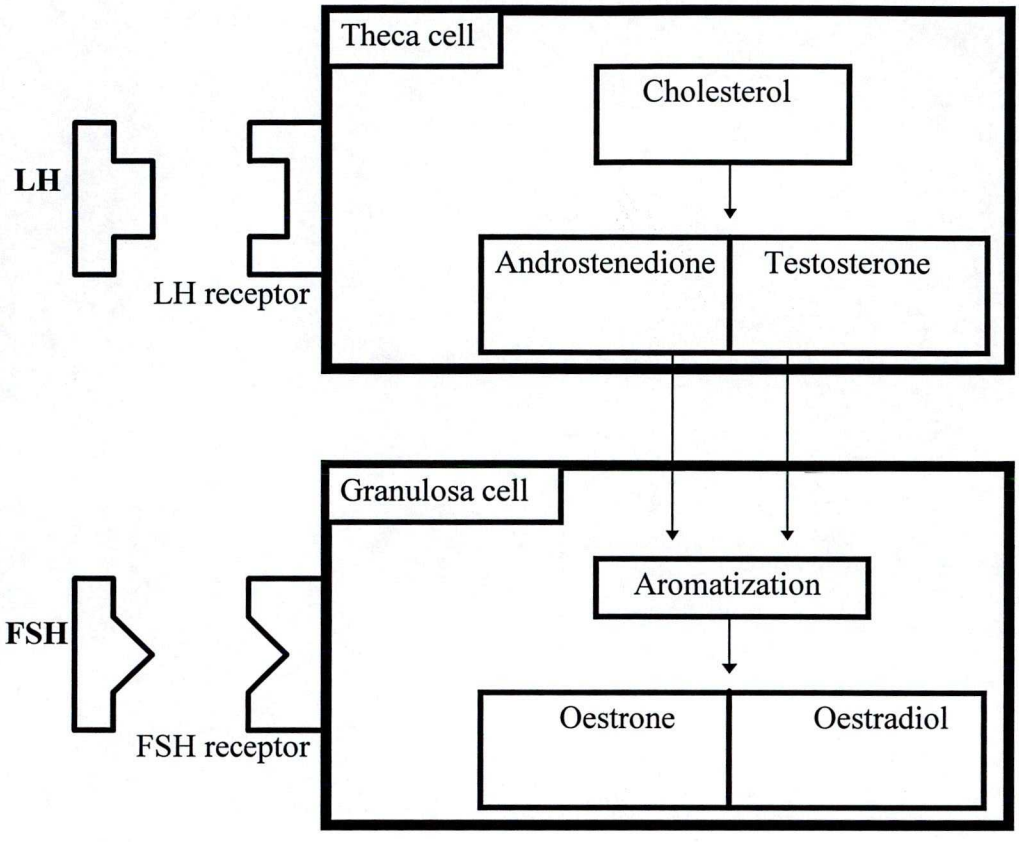

Figure 1. Schematic interpretation of the two-cell two-gonadotrophin theory for ovarian oestrogen production.

the limited capacity of aromatization is overwhelmed and the granulosa cells convert androstenedione to a more potent androgen, 5α-dihydrotestosterone, rather than oestradiol and the follicle becomes androgenic and ultimately atretic (McNatty et al. 1979). Additionally, the mode of androgenic modulation of FSH action on primate granulosa cells has been found to switch from stimulatory to inhibitory as follicular development advances (Harlow et al. 1988). Development-related changes in androgen receptor density appear to be involved in the selection of the dominant follicle since it was recently demonstrated that specific androgen receptor immunostaining is most abundant in the granulosa cells of early antral follicles, whereas it remains low or absent in preovulatory follicles, thereby protecting the granulosa cells against the potentially deleterious effects of androgens (Hillier et al. 1997). In summary, the success of a follicle depends upon its ability to convert an androgen environment to an oestrogen environment.

During the midfollicular phase, the granulosa cells begin to express LH receptors in response to FSH. As a result of FSH stimulation, a preovulatory follicle develops that is capable of ovulating in response to LH. During the late follicular phase, the preovulatory follicle produces increasing amounts of oestradiol, reaching a peak level approximately 24-36 h prior to ovulation. The LH surge initiates the resumption of meiosis in the oocyte, luteinization of the granulosa cells, the synthesis of prostaglandins and proteolytic enzymes essential for ovulation, and the physiological release of the oocyte and the cumulus mass of the granulosa cells. After the onset of the LH surge, the granulosa cells increase in size and undergo luteinization, transforming the follicle into corpus luteum. Under the stimulatory control of LH, the human corpus luteum secretes progesterone and oestradiol in order to render the endometrium hospitable to embryonic implantation. The progesterone levels produced by the corpus luteum rise sharply after ovulation, reaching a peak approximately 8 days after the LH surge. Thereafter, degeneration of the corpus luteum is inevitable unless pregnancy intervenes. If pregnancy occurs, survival of the corpus luteum is prolonged by a new stimulus called human chorionic gonadotrophin (hCG). This hormone is produced by the trophoblastic cells of the implanted embryo and serves to maintain the corpus luteum progesterone and oestrogen production until the placental steroidogenesis is established. If conception fails to occur, the corpus luteum deteriorates and the decreases in circulating levels of oestradiol and progesterone cause changes in the endometrium which lead to menstruation. As a consequence of a decline in luteal phase steroidogenesis, the FSH levels rise, initiating a new menstruation cycle.

The end-result of follicular development is usually one surviving mature follicle. In contrast with normal cycles, stimulation of multiple follicular development is required for assisted reproductive techniques (ART) such as *in vitro* fertilization (IVF) and embryo transfer in women suffering from infertility. For this purpose, follicular maturation is stimulated by FSH or human menopausal gonadotrophin in combination with clomiphene citrate (CC) or after desensitization of the pituitary gland by GnRH analogues. The induction of follicle development is monitored via the serum oestradiol concentrations and sequential ovarian ultrasonography. When the size of the leading follicle reaches about 17 mm or at least 3 follicles are greater than 13-15 mm in diameter and the serum oestradiol levels are higher than 600-800 pg/ml, ovulation is induced with an ovulatory dose of hCG (5,000-10,000 IU).

The follicles are aspirated either by laparoscopy or under vaginal ultrasound guidance 35-36 h after the hCG injection. The oocytes are identified by light microscopy and processed for fertilization.

The use of granulosa-lutein cells obtained as a by-product of IVF, is favoured as an *in vitro* model of ovarian function, because they can be easily harvested and cultured from follicle aspirates. These cells, however, are capable of responding with an increased steroid production to all three gonadotrophins. Therefore, the interpretation of the results obtained from *in vivo* or *in vitro* studies was compromised by the impurity of the hormone preparations used in these studies. The recent availability of recombinant FSH (rFSH; Keene et al. 1989), recombinant LH (rLH) and recombinant hCG (rhCG) provides an opportunity to investigate the fine regulation of the ovarian function *in vivo* or to examine their *in vitro* effects on granulosa cell steroidogenesis separately. Recombinant gonadotrophins are produced by recombinant DNA technology involving the use of a Chinese Hamster Ovary cell line, providing gonadotrophins with several advantages as compared with urine-derived preparations (Table I). These compounds have pharmacokinetic characteristics similar to those of pituitary and urine-derived gonadotrophins (Le Cotonnec et al. 1994, Le Cotonnec et al. 1998) and have been extensively studied in clinical trials. Recombinant FSH is the most advanced in terms of clinical assessment for the induction of multifollicular development in ART (Recombinant Human FSH Study Group 1995, Out et al. 1995, Mitchell et al. 1996).

Table I. The major advantages of producing the gonadotrophins *in vitro* by recombinant DNA technology

Fully controlled production process - no need for urine collection
High specific activity - greater than 10.000 IU/mg
High purity - complete absence of other gonadotrophin contamination
Low batch-to-batch variation
Excellent safety profiles - lack of co-administration of biologically active urinary proteins

The relative contributions of gonadotrophins to folliculogenesis have been a subject of interest for many years. During the past decade, the role of LH in follicle maturation has been questioned in primates (Chappel and Howles 1991). Recently, this concept was further developed by the introduction of recombinant gonadotrophins in the treatment of infertility. Recombinant FSH has been reported to induce normal multifollicular development with a concomitant increase in serum oestradiol concentrations in IVF patients, suggesting that, after pituitary desensitization, the residual levels of LH (0.3-1.0 mIU/ml) synergizing with locally produced IGF-I (Magoffin et al. 1990) and inhibin (Hillier et al. 1991) are sufficient to supply androgen substrates for oestradiol biosynthesis (Mitchell et al. 1996). Higher LH levels during the preovulatory period may impair gametogenic events due to an overstimulation of androgen production by the theca cells (Chappel and Howles 1991). On the other hand, the lack of LH activity in hypogonadotropic women results in very low oestradiol levels during follicular stimulation by rFSH (Schoot et al. 1992). Recombinant LH appears to be ideal adjunct therapy to rFSH in such patients in order to obtain adequate follicular steroidogenesis, as demonstrated by oestradiol secretion, endometrial growth and luteal phase progestereone secretion (Hull et al. 1994). In addition, rhCG was recently used successfully to trigger final follicular maturation prior to ART after rFSH-induced follicular development (Loumaye et al. 1996).The above results provide evidence that, in the course of ovarian hyperstimulation (where the aim is to obtain as many eggs as possible for fertilization *in vitro*), the role of LH during normal follicular development is focused on the production of the androgens necessary for oestrogen biosynthesis. By comparison, in normal ovulating patients,where the aim is to achieve monovulation and subsequent conception *in vivo*, the importance of LH in the selection of a dominant follicle and in the induction of ovulation is not questioned (Hillier 1994).

Besides clinical trials, the direct effects of recombinant gonadotrophins on human ovarian steroidogenesis are also extensively studied. The influence of rFSH on human granulosa cells from patients with spontaneous (Mason et al. 1993, Bergh et al. 1997) and hyperstimulated (Lambert et al. 1995) cycles in short-term cultures has already been demonstrated. Recombinant FSH induced dose-related increases in oestrogen production by the granulosa cells from normal preovulatory follicles (Mason et al. 1993, Bergh et al. 1997).

However, cells from preovulatory follicles after the LH surge or from gonadotrophin-stimulated cycles exhibited no stimulatory responses to rFSH (Mason et al. 1993, Lambert et al. 1995). Additionally, although FSH seems to be the principal regulator of oestrogen production in the follicular phase, elevated levels of oestrogens during the midluteal cycle are thought to arise from LH/hCG stimulation (Richardson and Masson 1981, Polan et al. 1986, Schipper et al. 1993). However, the direct effects of rhCG and rLH on granulosa cell oestrogen release have not yet been evaluated. Further, apart from the limited information concerning the stimulatory action of rLH on progesterone production (Bergh et al. 1997), no exact information is available on the direct effects of recombinant gonadotrophins on granulosa cell progesterone secretion.

The aims of the present study were

1. To establish a useful culture of human granulosa cells harvested from follicular aspirates of women undergoing *in vitro* fertilization.

2. To investigate the basal oestrogen and progesterone release from the granulosa cells *in vitro*.

3. To study the granulosa cell steroid production in the presence of appropriate substrates for oestradiol (testosterone and androstenedione) and progesterone (fetal calf serum as a cholesterol source) biosynthesis in order to provide optimum culture conditions for stimulation studies.

4. To investigate the effects of recently developed recombinant human gonadotrophins (rFSH, rLH and rhCG) on ganulosa cell steroidogenesis in long-term cultures. The effects of recombinant gonadotrophins were compared by using the same batch of granulosa cells in order to balance the possible intersubject variations in gonadotrophin sensitivity.

MATERIALS AND METHODS

Chemicals

Medium 199 Earle (M199), fetal calf serum (FCS), L-glutamine, gentamicin, Dulbecco's phosphate-buffered saline (PBS) and trypan blue stain were purchased from Seromed (Biochrom KG, Berlin, Germany). Medicol medium (Percoll 100%, isotonic) was from Medi-Cult (Copenhagen, Denmark). Hyaluronidase, testosterone, androstenedione, 5α-dihydrotestosterone (DHT), acetone, ethanol, methanol and hydrogen peroxide were from Sigma (Deisenhofen, Germany). Human recombinant gonadotrophins (rFSH, specific activity 15,000 IU/mg; rhCG specific activity 10,000 IU/mg; rLH specific activity 14,900 IU/mg) were generously supplied by Serono (Munich, Germany). Specific mouse IgG monoclonal antibody to human inhibin (32 kD α-subunit) was from Serotec (Camon Laboratory Services GmbH, Wiesbaden, Germany). Vectastain kit and diaminobenzidine tetrahydrochloride substrate (DAB) were from DAKO Diagnostica GmbH (Hamburg, Germany).

Protocol of Ovarian Hyperstimulation and Cell Isolation

Luteinized granulosa cells were obtained from women undergoing oocyte retrieval for IVF. Written consent was obtained and the experimental design was approved by the local ethic committee. Reasons for infertility were tubal occlusion, reduced male factor, polycystic ovarian disease (PCOD) or unexplained infertility. Patients with PCOD or at risk of ovarian hyperstimulation syndrome (OHSS) were excluded from the *in vitro* gonadotrophin-stimulation study. The mean age of these patients was 32.9 years (range: 23 to 45 years). Multiple follicular development was achieved with pure FSH (225 IU per day; Fertinorm HP®, Serono, Munich, Germany) until follicular maturity. Gonadotrophin therapy was preceded by complete desensitization of the pituitary gland with 0.1 mg per day of triptorelin (Decapeptyl®, Ferring, Kiel, Germany). The mean total FSH dose administered per patient was 2,300 IU (range: 1,500 to 3,150 IU). An average of 9 follicles larger than 13 mm per patient were induced (range: 3 to 17 follicles). For ovulation induction, 10,000 IU hCG (Pregnesin®, Serono, Munich, Germany) was injected 36 h prior to ultrasound-guided transvaginal follicle aspiration. Before ovulation induction, mean oestradiol concentrations

were determined in the range 870 to 3,000 ng/ml. Oocytes were immediately removed from the follicular fluid and processed for fertilization. Follicular fluids aspirated from the same patients were pooled and centrifuged for 3 min at 100 g. The resulting pellets were resuspended in PBS containing 1 % FCS and washed three times with PBS+1% FCS. Final pellets were incubated with 20 IU/ml hyaluronidase for 12 min at 37°C in a humidified atmosphere of 95% air + 5% CO_2. Cell clusters were separated by repeated gentle pipetting. Cell suspensions were washed and layered on 50% Percoll and centrifuged for 10 min at 2,000 g to separate the granulosa cells from the blood cells. The cells were aspirated from the interface and subsequently washed with culture medium (M199 supplemented with 100 μg/ml gentamicin, 2 mM L-glutamine and 10% FCS). Finally, the cells were resuspended in a small volume of culture medium and counted in a hemocytometer. Cell viability was determined by trypan blue exclusion and was found to be consistently higher than 80%. For the correlation study, aliquots were taken from individual granulosa cell preparations to determine the 24-h basal oestrogen production. When necessary, cells prepared from 2 or 3 patients on the same day were pooled to obtain a sufficient number of granulosa cells for the stimulation studies.

Identification of Granulosa Cells by Specific Inhibin Immunostaining

For morphometric analysis, granulosa-lutein cells (10,000 viable cells/well) were cultured in Lab-Tek chamber slides (Nunc Intermed, Naperville, IL, USA) for 24 h at 37°C in a humidified atmosphere of 95% air + 5% CO_2. After the incubation, the spent media were removed and the cells were gently rinsed with ice-cold PBS to remove unadhered blood cells and debris. The granulosa cells were fixed with methanol and acetone at -20°C for 5 and 3 min, respectively. To block endogenous peroxidase activity and non-specific binding, the cells were incubated with 1% hydrogen peroxide and normal horse serum, respectively. Thereafter, the slides were incubated with specific monoclonal antibody raised against the 32 kD α-subunit of human inhibin overnight at 4°C. In the negative controls, the primary antibody to inhibin was replaced with normal mouse serum. Specific binding was visualized by the avidin-biotin method, with the application of a Vectastain kit specific for mouse IgG and DAB as substrate for peroxidase. The cell nucleus and cytoplasm were counterstained with eosin-hematoxylin. Slides were examined by light microscopy and photos were taken at 40x magnification.

Cell Cultures

Granulosa cells were plated in multivell plastic dishes (Greiner GmbH, Frickenhausen, Germany) at a density of 100,000 viable cells/well and cultured at 37°C in a humidified atmosphere of 95% air + 5% CO_2. After a 24-h incubation, the cells were attached to the plates, forming monolayer cultures. For the correlation study, the spent media from the individual cell cultures were removed and stored for oestradiol analysis and the cultures were terminated. For other studies, the media were removed, the cells were washed with PBS containing 1% FCS, and fresh media were added to the wells. Incubation was continued uninterrupted for 48 h except for the study of the daily progesterone and oestradiol release from the unstimulated granulosa cells, where the cultures were maintained for up to 5 days with a daily medium change. After 3 days of preincubation, the media were replaced by fresh media containing 1% FCS for oestradiol stimulation studies and 2.5% FCS for progesterone stimulation studies. The cultures were maintained for an additional 6 days in the presence of near-physiological concentrations of rFSH (1-10-100 mIU/ml), rhCG (0.01-0.1-1-10 IU/ml) or rLH (0.001-0.01-0.1-1 IU/ml). The stimulation of oestradiol production was performed in the presence or absence of 10 ng/ml testosterone. For the evaluation of the influence of FCS on the basal and rhCG-stimulated progesterone production, spent media supplemented with 10% FCS were replaced after the initial 3-day preincubation by fresh media containing different concentrations (0-10%) of FCS, and the cultures were maintained for up to 9 days. Based upon preliminary dose-response curves, these experiments were performed in the presence or absence of 0.1 IU/ml rhCG. In all cases, the media were changed every 48 h and stored at -20°C for subsequent analysis. Androgens were dissolved as concentrated stock solutions in absolute ethanol and diluted in culture medium prior the addition to the cell cultures. The final ethanol concentration in the cultures was less than 0.01%.

Steroid Assays

Progesterone levels were determined by competitive enzymeimmunoassay (BioChem Immuno Systems GmbH, Freiburg, Germany). Oestradiol levels were measured by radioimmunoassay (BioChem Immuno Systems GmbH, Freiburg, Germany). The specific antibodies displayed no cross-reactivity with either testosterone, androstenedione or DHT. The average intra- and interassay coefficients of variation were 6.9 and 9.4, respectively, for progesterone and 5.1

and 7.5, respectively for oestradiol. The assay sensitivity was 2 ng/ml for progesterone and 10 pg/ml for oestradiol.

Statistical Analysis

The results were obtained from experiments performed in duplicate or triplicate. Single samples were taken from each well for oestradiol and/or progesterone determination. All experiments were repeated at least three times to ensure reproducibility. Data are presented as means ± SEM of replicate determinations. Linear regression analysis was performed to correlate the serum oestrogen levels (independent variable, n=21) with the oestrogen levels produced by the granulosa cells (dependent variable, n=21). The statistical significance of combined data was determined by Student's t-test for paired samples. All statistical parameters were calculated with Statistical Package for Social Sciences (SPSS) 6.0 software. Values of $P<0.05$ were considered to be significant.

RESULTS

Cell Morphology

Granulosa cells obtained from IVF cycles were seeded on plastic plates. The cells were cultured in culture medium supplemented with 10% FCS. The presence of serum allowed them to recover after follicular aspiration and improved their attachment and spreading to the culture plates. After a 24-h incubation, unadhered dead granulosa cells and contaminating blood cells were removed by washing with PBS and incubation was continued in the majority of cases for an additional 48 h without treatment. At the end of the preincubation period, the cells exhibited a flat appearance with smooth surfaces and multiple interconnections were noted between them (Fig. 1). Intracellular organelles, which appeared as small yellowish-brown granules, were spread throughout the cytoplasm.

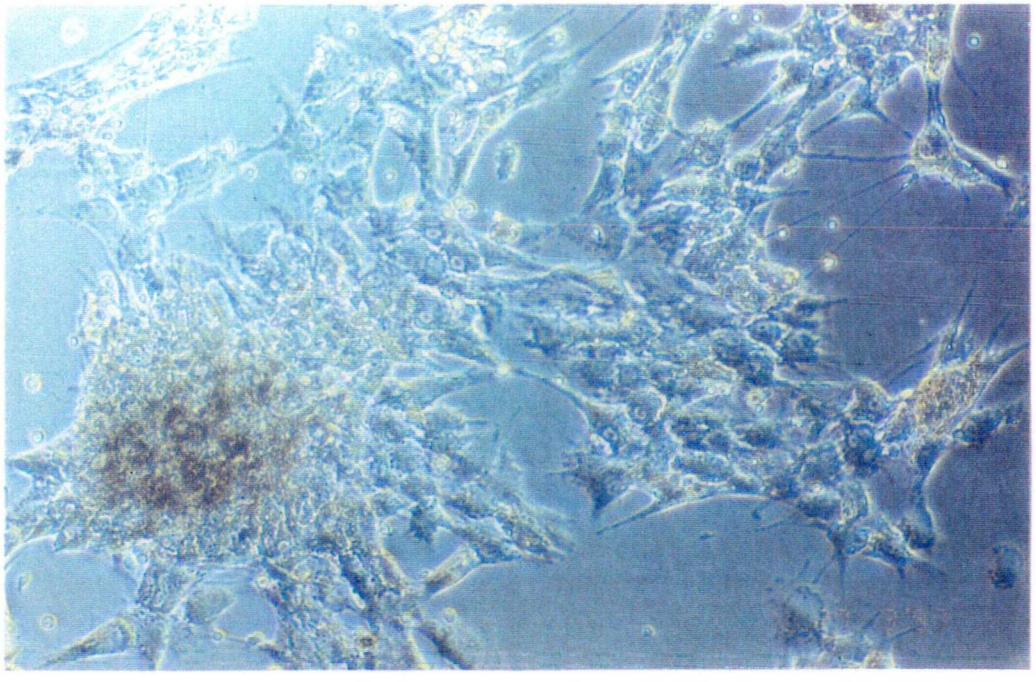

Figure 1. A picture of 3-day-old granulosa cells in culture. The cells were cultured in multiwell plates and were processed in the same manner as cell cultures for gonadotrophin stimulation studies. The photo was taken by phase contrast light microscopy at 40x magnification. Multiple cell-cell connections have already been established.

Identification of Granulosa Cells in Culture by Inhibin Immunohistochemistry

Inhibin is a glycoprotein which specifically inhibits FSH secretion at the pituitary level. It consists of two dissimilar peptides, known as the α and β subunits, linked by disulfide bonds. There are two forms of inhibin (A and B), each containing an identical α subunit and different but related β subunits. Granulosa cells were identified on the basis of the specific immunohistochemical detection of the inhibin (α subunit) exclusively present in these cells. The existence of reddish-brown staining in the cytoplasm indicates that the cells isolated and cultured from the follicular aspirates were granulosa cells (Fig. 2).

A **B**

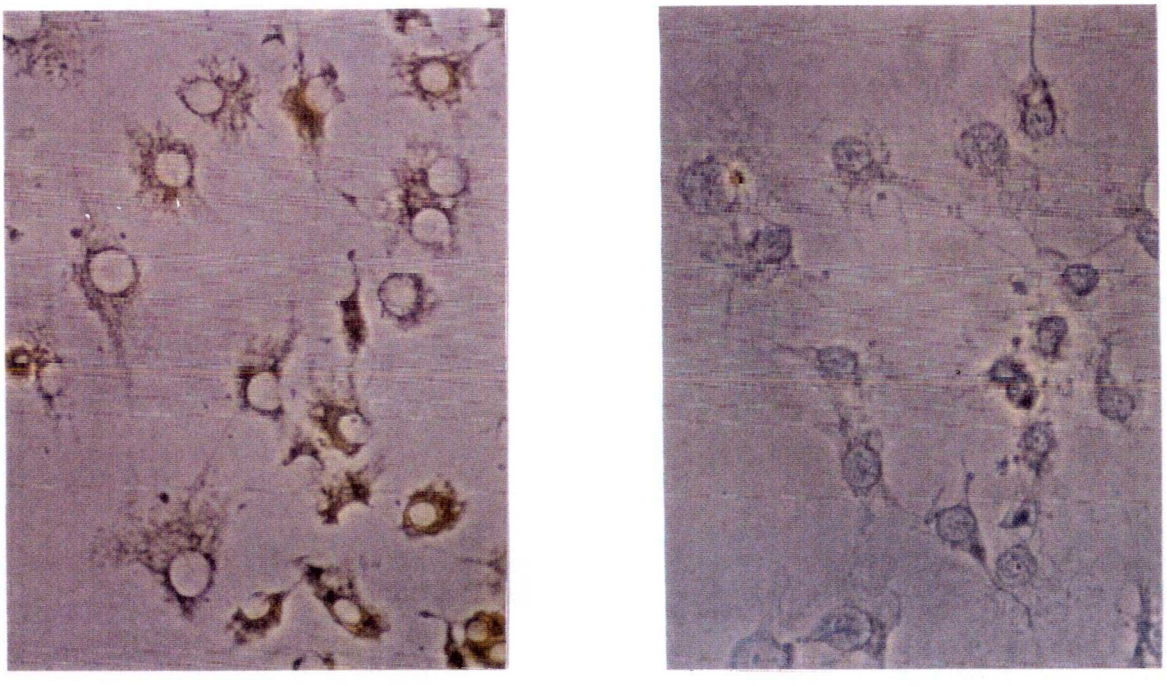

Figure 2. Immunohistochemical localization of the inhibin 32 kD α subunit in human granulosa cells. After a 24-h incubation in chamber slides, the cells were washed, fixed and incubated with primary antibody to the α subunit of inhibin (**A**, positive) or with normal mouse serum (**B**, negative). Reddish-brown staining in the cytoplasm denotes the presence of inhibin.

Oestrogen Secretion of Granulosa Cells under Basal Conditions

The basal *in vitro* oestrogen production of cultured human granulosa-lutein cells gradually decreased during the culture (Fig. 3). The oestrogen secretion on the first day of culture was high and displayed marked variability (421.3 ± 159.3 pg/ml, n = 13). A 50% reduction was observed during the next 2 days (211.3 ± 47.7 pg/ml) and the level continued to decline, to less than 40 and 20 % of the initial value after 5 and 7 days of culture, respectively. By day 9, it was close to the limit of detection of the oestradiol assay (16.7 ± 3.1 pg/ml).

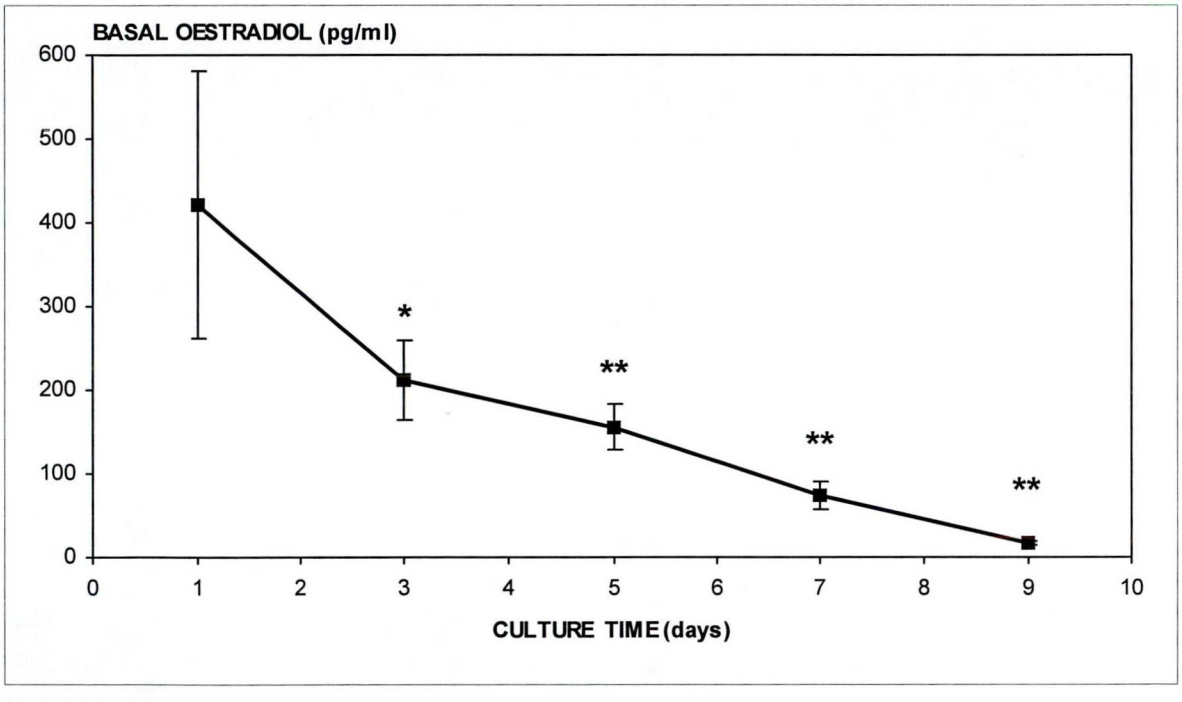

Figure 3. Baseline oestrogen production by human granulosa-lutein cells in culture. Granulosa cells were plated at a density of 100,000 viable cells/well and cultured for up to 9 days without any treatment. Media were changed on days 1, 3, 5, 7 and 9 of culture and stored for oestradiol analysis. Values are means ± SEM of the results of 13 experiments performed in duplicate. *, significantly ($P<0.05$) different from the initial value (day 1); **, significantly ($P<0.01$) different from the previous values.

The variability in oestrogen levels produced by the granulosa cells during 24 h in culture may originate from individual differences in the responsiveness of patients to *in vivo* gonadotrophin stimulation. To test this hypothesis, serum oestradiol levels measured on the day of hCG administration, which are valuable markers of *in vivo* aromatase activity, were

compared with the *in vitro* basal oestrogen production of the granulosa cells harvested from the same patients (Fig. 4). Both individual parameters exhibited wide variations in respect of oestrogen production (1,300-3,000 pg/ml for serum levels and 139-2,141 pg/ml for oestrogen levels produced by the granulosa cells during the first day). However, a close correlation was found between *in vivo* and *in vitro* oestrogen levels ($R=0.845$, $P<0.001$) providing direct evidence that the granulosa cells are capable of secreting oestrogen during culture at a rate that corresponds to that of *in vivo* oestrogen production.

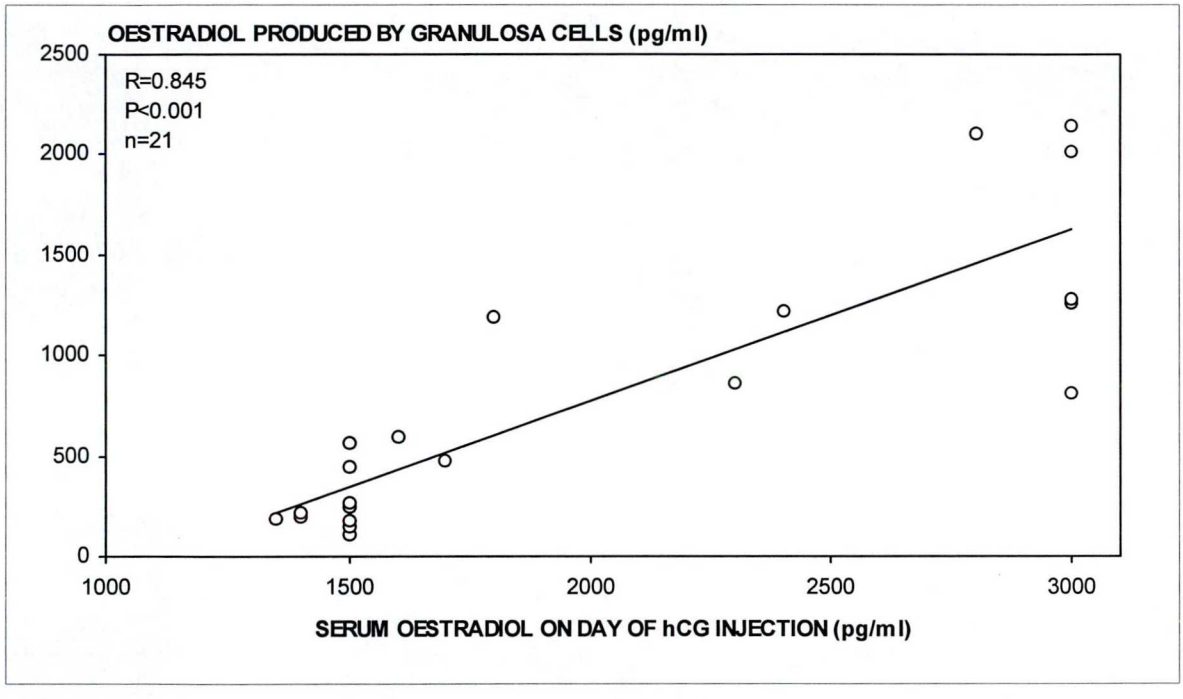

Figure 4. Correlation between the serum oestradiol levels measured on the day of hCG administration and the oestrogen production by granulosa cells from the same patients. The cells were harvested at the time of follicle aspiration after ovarian stimulation for IVF and cultured at a density of 100,000 viable cells/well for 24 h in medium containing 10% FCS.

Although the baseline oestrogen levels fell during culture (Fig. 3), the addition of testosterone or androstenedione in various concentrations (1-10-100 ng/ml) to the incubation medium markedly enhanced the oestrogen production in a dose-dependent manner (Fig. 5). There were no significant differences in oestradiol production between days 5, 7 and 9; the granulosa cells maintained their capacity to aromatize androgens for at least 9 days.

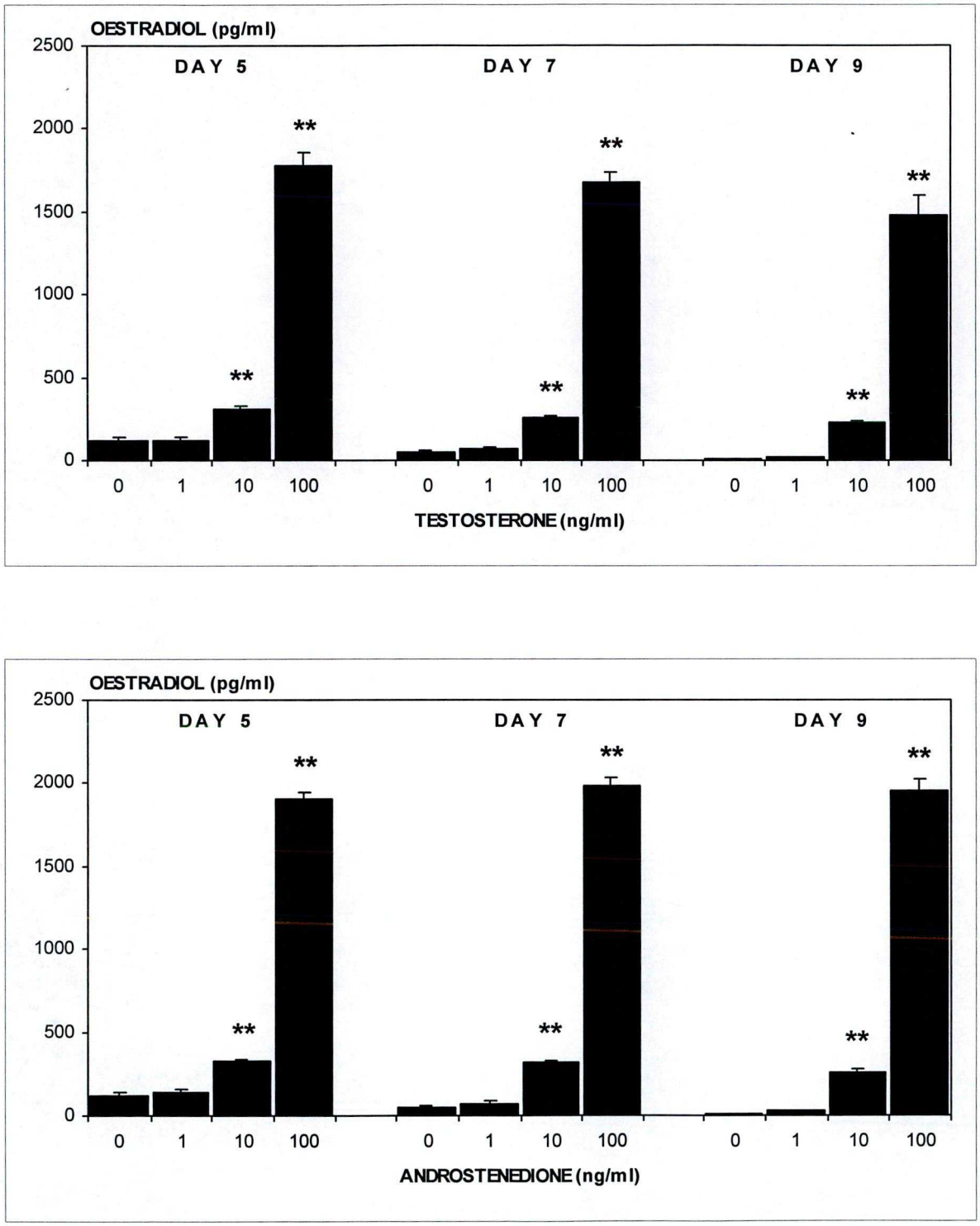

Figure 5. Time- and dose-dependent oestrogen production by granulosa-lutein cells in the presence of testosterone (A) and androstenedione (B). The cells (100,000 viable cells/well) were preincubated for 3 days in a medium containing 10% FCS and then cultured in a medium supplemented with 1% FCS and with 1-10-100 ng/ml androgen for 6 days. Data are means ± SEM of three independent experiments performed in duplicate. **, $P<0.01$ versus the control (0 ng/ml androgen).

Testosterone and androstenedione were equally well converted to oestradiol. In all subsequent experiments, 10 ng/ml testosterone was used as a substrate for aromatase when androgens were added to the media.

Progesterone Secretion of Granulosa Cells under Basal Conditions

In contrast with the oestradiol secretion, the basal progesterone production by the human granulosa-lutein cells was relatively low during the first day of culture (Fig. 6; 0.65 ± 0.11 μg/ml) and displayed far less intersubject variability. An increase in progesterone levels was observed during the next 2 days, reaching a maximum concentration on day 3 (4.47 ± 0.30 μg/ml). There was a 60% reduction between 3 and 5 days (1.70 ± 0.18 μg/ml) and the decrease continued to less than 5% of the peak value by the end of the culture (day 9: 0.21 ± 0.03 μg/ml).

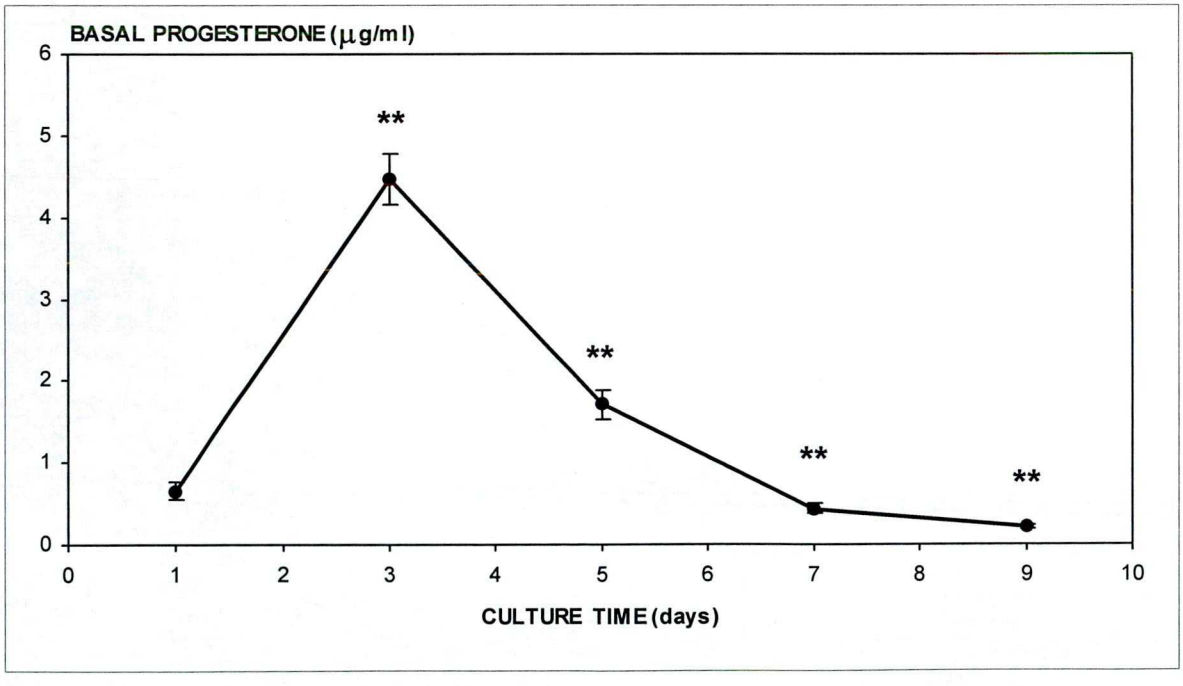

Figure 6. Basal progesterone production by luteinized granulosa cells during 9 days of culture. Cultures (100,000 viable cells/well) were maintained in a medium supplemented with 10% FCS for 3 days and subsequently incubated in a medium containing 2.5% FCS for additional 6 days. Samples were taken for progesterone analysis during the medium changes on days 1, 3, 5, 7 and 9 of culture. Values are means ± SEM (n=12). **, significantly different from previous values ($P<0.01$).

To evaluate the increment in baseline progesterone release during the first few days of culture, the daily progesterone secretion was determined in cultures maintained for up to 5 days. Besides progesterone, the basal oestradiol concentrations were measured (Fig. 7). The overall characteristics of the oestradiol and progesterone time-concentration curves revealed patterns similar to those demonstrated in Figs 3 and 6, respectively. However, the greatest decline in oestradiol level and the highest rise in progesterone concentration were noted between days 1 and 2 of culture.

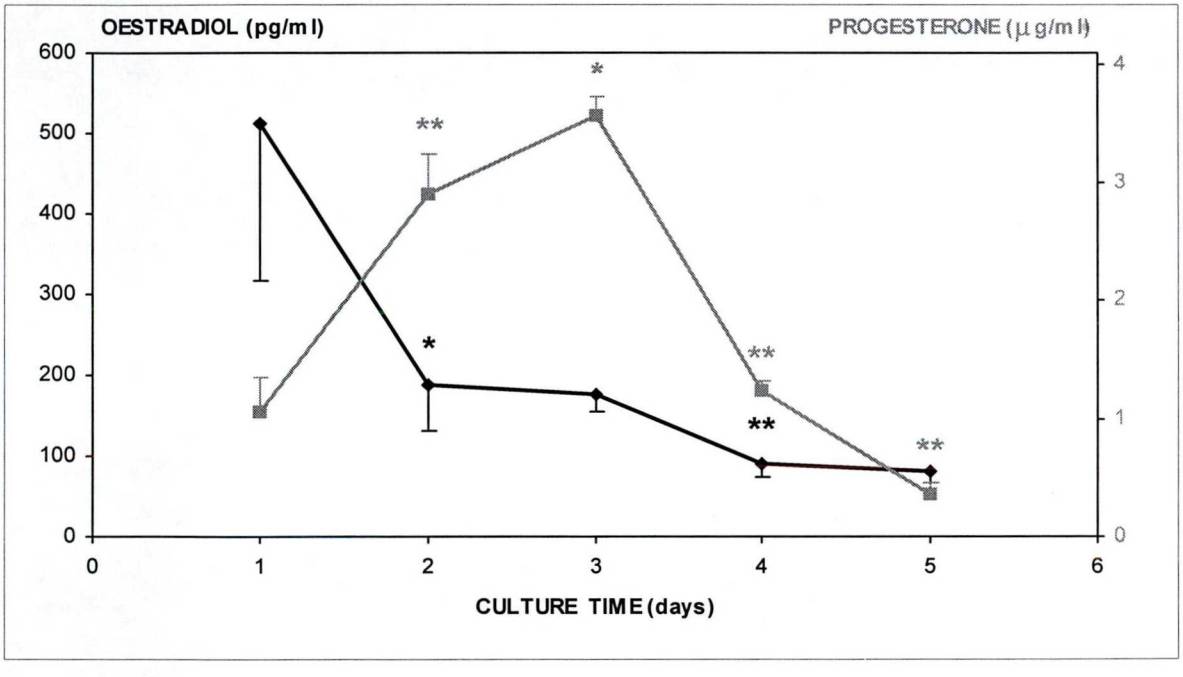

Figure 7. Daily baseline oestradiol and progesterone secretion of granulosa-lutein cells plated at a density of 100,000 viable cells/well. Cultures were maintained in a medium supplemented with 10% FCS for 3 days, followed by incubation in the presence of 2.5% FCS for 2 days. Media were changed daily and saved for steroid analysis. Data are means ± SEM (n=5). *, or **, significantly ($P<0.05$ and $P<0.01$, respectively) different from previous values.

The influence of serum supplementation on basal and rhCG-stimulated progesterone production is illustrated in Fig. 8. In the absence of serum, the granulosa cells produced relatively low levels of progesterone. Addition of serum to the culture medium (1.25-10% FCS, v/v %) caused significant dose-dependent increases in progesterone production as

compared with the serum-free medium. However, the time course of serum supplementation demonstrated declines in spontaneous progesterone production even in the presence of high amounts of FCS. During the early induction period (days 3-5), the progesterone secretion was significantly augmented by the presence of 0.1 IU/ml rhCG, even in the absence of serum.. However, during the next two stimulation intervals (days 5-7 and 7-9), the progesterone release from the granulosa cells in response to rhCG was elevated significantly above the basal level only in the presence of serum, reaching its maximum (approximately 7-8 fold higher than in the corresponding controls) on day 9 in the presence of 10% FCS.

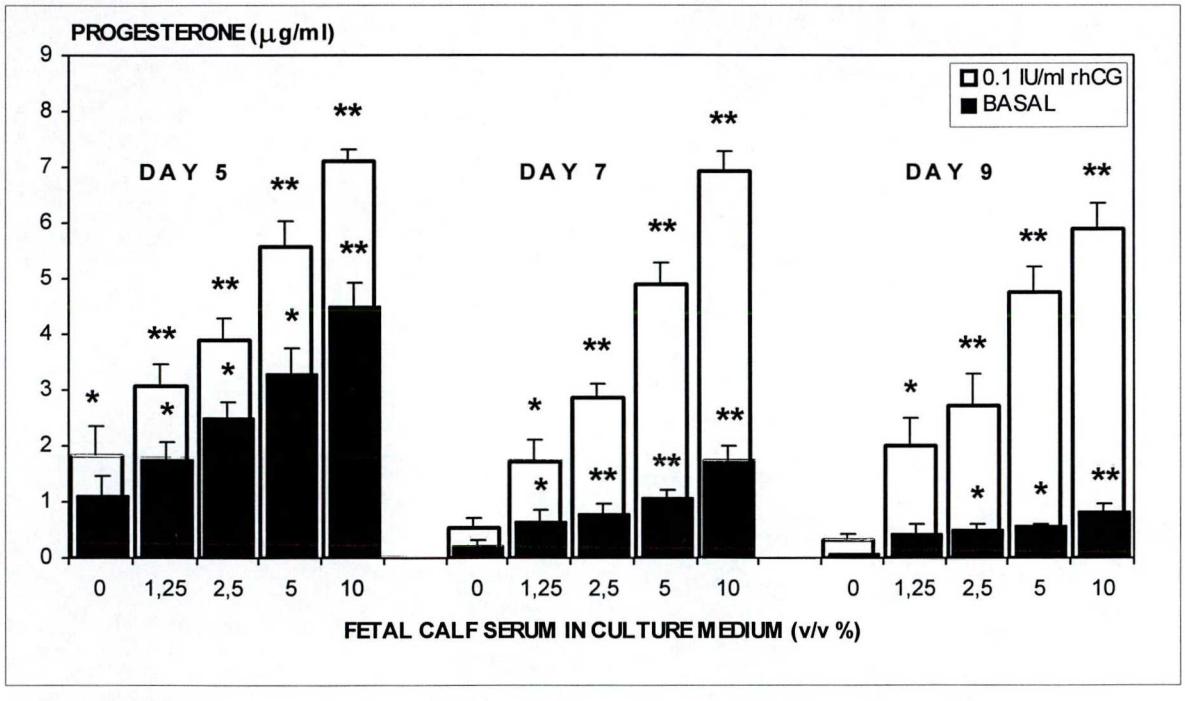

Figure 8. Basal and rhCG-stimulated progesterone production of human granulosa-lutein cells in the presence of different concentrations of FCS. The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium containing 10% FCS and then cultured for 6 days in a medium supplemented with 0-10% (v/v %) FCS in the presence or absence of 0.1 IU/ml rhCG. Data are means ± SEM (n=5). Significant differences were found between the basal (unstimulated) progesterone levels for serum-free (0% FCS) and serum-supplemented cultures, and for basal and rhCG-stimulated cultures (*, $P<0.05$, **, $P<0.01$).

Effects of Recombinant Gonadotrophins on Granulosa Cell Oestrogen Production

Oestradiol secretion by granulosa cells cultured for 9 days with different doses of rFSH (1-10-100 mIU/ml) in the presence or absence of exogenous testosterone are shown in Figs. 9 and 10, respectively. The inclusion of testosterone in the culture medium considerably augmented oestradiol production, which remained constantly high during the culture. However, in the presence of testosterone, rFSH failed to stimulate oestradiol production further (Fig. 9). Without added androgen, rFSH increased the oestradiol level in a dose-dependent manner (Fig. 10). Between days 3-5 and 5-7 of culture, rFSH at concentrations of 10 and 100 mIU/ml significantly stimulated the oestradiol accumulation but on day 9 only the highest dose was effective. It should be noted that in spite of the stimulatory effect of rFSH in the androgen-free environment, the basal and stimulated oestrogen levels decreased during the culture period, probably in consequence of the decreasing availability of aromatizable androgen substrates.

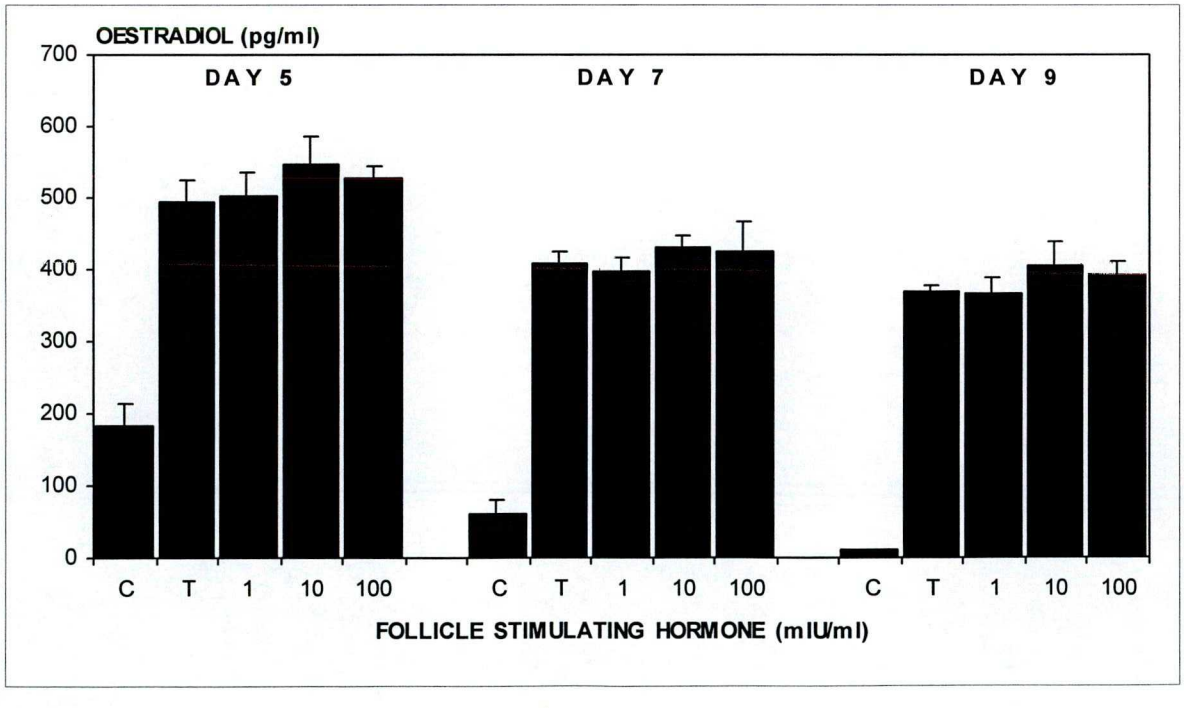

Figure 9. Oestradiol accumulation of human granulosa-lutein cells in response to rFSH stimulation in the presence of testosterone. The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10% FCS and then exposed to 1-10-100 mIU/ml rFSH in a medium containing 1% FCS and 10 ng/ml testosterone for 6 days. Data are means ± SEM (n=5). C, control without treatment; T, 10 ng/ml testosterone only.

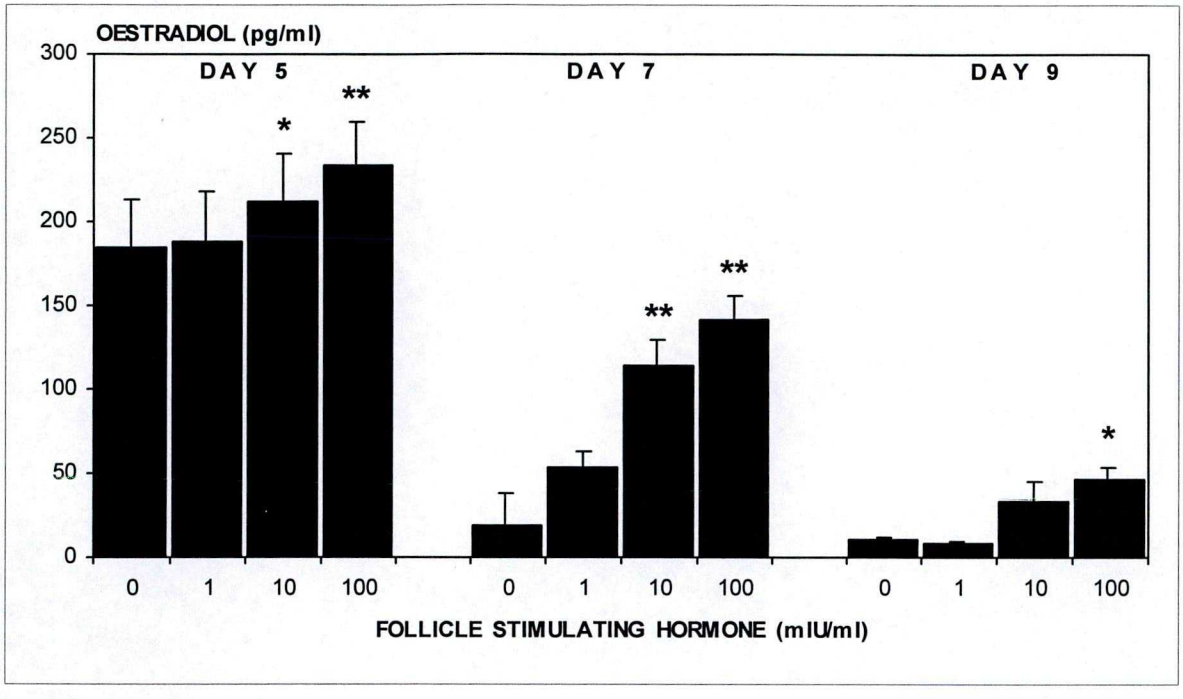

Figure 10. Oestradiol production by luteinized human granulosa cells cultured in an exogenous androgen-free environment in the presence of different doses of rFSH (1-10-100 mIU/ml). The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10% FCS and then incubated with rFSH in a medium containing 1% FCS for 6 days. Data are means ± SEM (n=5). *, $P<0.05$ and **, $P<0.01$ vs. control (0 mIU/ml rFSH).

To evaluate the possible influence of androgens in the stimulatory action of rFSH, the effects of an aromatizable (testosterone) and a non-aromatizable (DHT) androgen on the basal and rFSH-stimulated human granulosa-lutein cell oestrogen production were compared (Fig. 11). In contrast with testosterone, DHT in a concentration of 100 ng/ml was not converted to oestradiol by the granulosa cells since no differences were found between the control and DHT-treated cultures. Co-incubation of the cells with rFSH (100 mIU/ml) and DHT in the absence or presence of testosterone (10 ng/ml), resulted in slight, but non-significant increases in oestradiol production as compared with the rFSH-only and rFSH-testosterone cultures, respectively. Furthermore, treatment with combinations of rFSH-testosterone or rFSH-testosterone-DHT did not increase the oestrogen accumulation significantly over the control (testosterone-only) level. Again, only rFSH was able to augment the oestrogen production by the granulosa cells significantly. The rFSH-DHT treatment was also effective, but this could be accounted for by rFSH action.

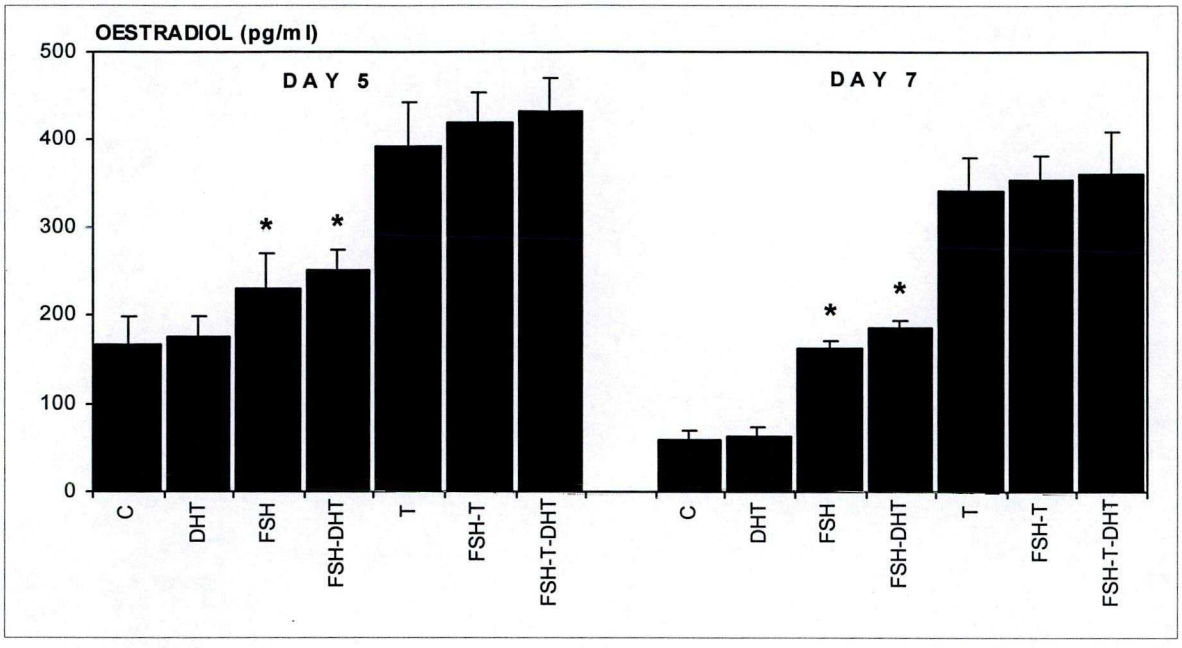

Figure 11. Influence of androgens on the basal and the rFSH-induced oestrogen production by granulosa-lutein cells. The cells (100,000 viable cells) were preincubated for 3 days in a culture medium supplemented with 10% FCS and exposed to combinations of 100 mIU/ml rFSH (FSH), 10 ng/ml testosterone (T) and 100 ng/ml 5α-dihydrotestosterone (DHT) in a medium containing 1% FCS for 4 days. Data are means ± SEM (n=4). *, Values are significantly ($P<0.05$) different from the control (C, without any treatment).

Oestradiol release from the granulosa-lutein cells in response to different concentrations of rhCG (0.01-0.1-1-10 IU/ml) in the presence or absence of exogenous testosterone are presented in Figs 12 and 13, respectively. Addition of the androgen to the culture medium markedly enhanced the oestradiol production in the both control and the rhCG-treated cultures (Fig. 12). However, in the presence of testosterone, the oestradiol production was significantly diminished by rhCG between 3 and 5 days of culture. No effect was noted during the next two days (days 5-7), whereas rhCG tended to stimulate the oestradiol accumulation during the final induction period (days 7-9), being significant at the lowest dose applied (0.1 IU/ml). In the absence of androgens, the differences in granulosa cell oestrogen responses to rhCG were more pronounced (Fig. 13). Between 3 and 5 days of culture, the oestradiol production was significantly inhibited by the presence of rhCG, whereas no effect was noted during the next two days. Between days 7 and 9 of culture, increases in oestrogen level were noted at doses higher than 0.01 IU/ml, but a significant elevation was found only at a dose of 0.1 IU/ml.

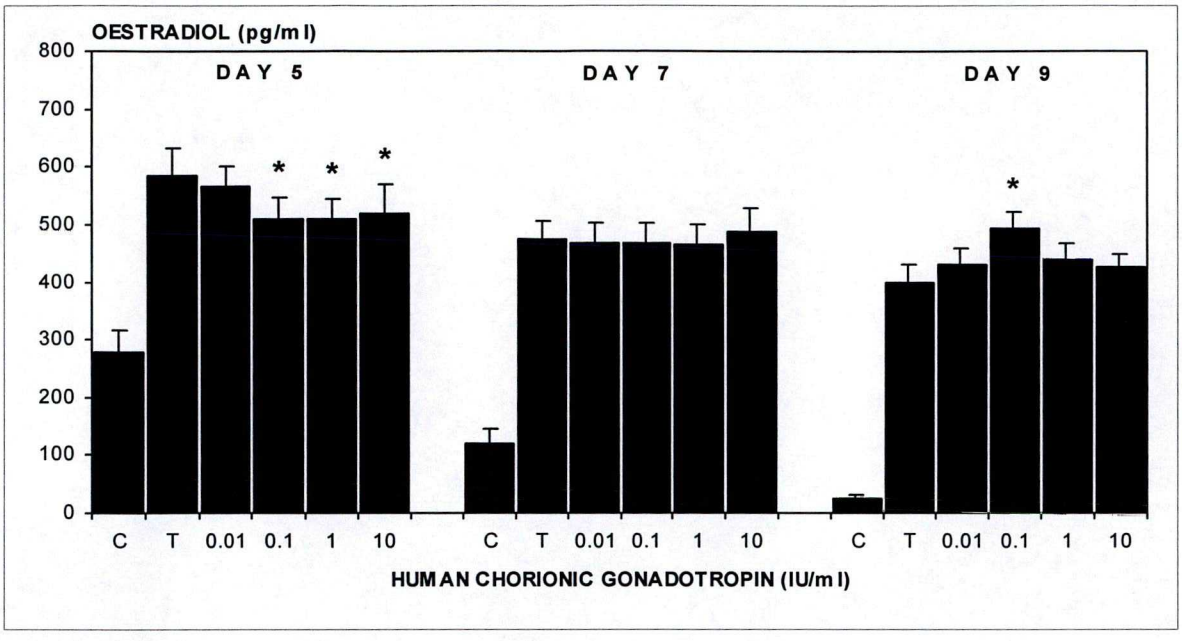

Figure 12. Oestradiol production of human granulosa-lutein cells in response to rhCG induction in the presence of testosterone. The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10% FCS and then exposed to 0.01-0.1-1-10 IU/ml rhCG in a medium containing 1% FCS and 10 ng/ml testosterone for 6 days. Data are means ± SEM (n=7). C, control cultures. *, $P<0.05$ vs. T (testosterone-only) cultures.

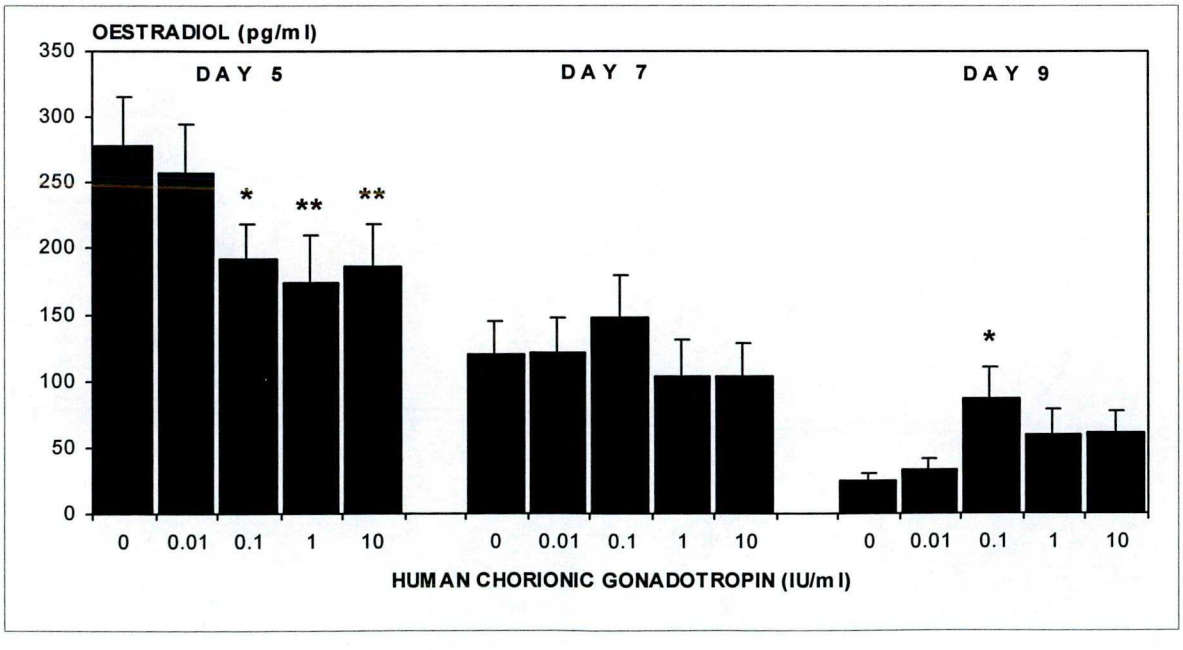

Figure 13. Oestradiol accumulation by granulosa-lutein cells cultured in an exogenous androgen-free environment in the presence of different doses of rhCG (0.01-0.1-1-10 IU/ml). The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10% FCS and stimulated with rhCG in a medium containing 1% FCS for 6 days. Data are means ± SEM. (n=7). *, $P<0.05$ and **, $P<0.01$ vs. control (0 IU/ml rhCG).

The granulosa-lutein cell responses to rLH with respect to oestrogen production in the presence of testosterone are demonstrated in Fig. 14. Recombinant LH used in concentrations of 0.001-0.01-0.1-1 IU/ml failed to alter the granulosa cell oestrogen production at any time during the treatment period. In the absence of the androgen, rLH was also ineffective in stimulating oestrogen production during the culture for 9 days (Fig. 15).

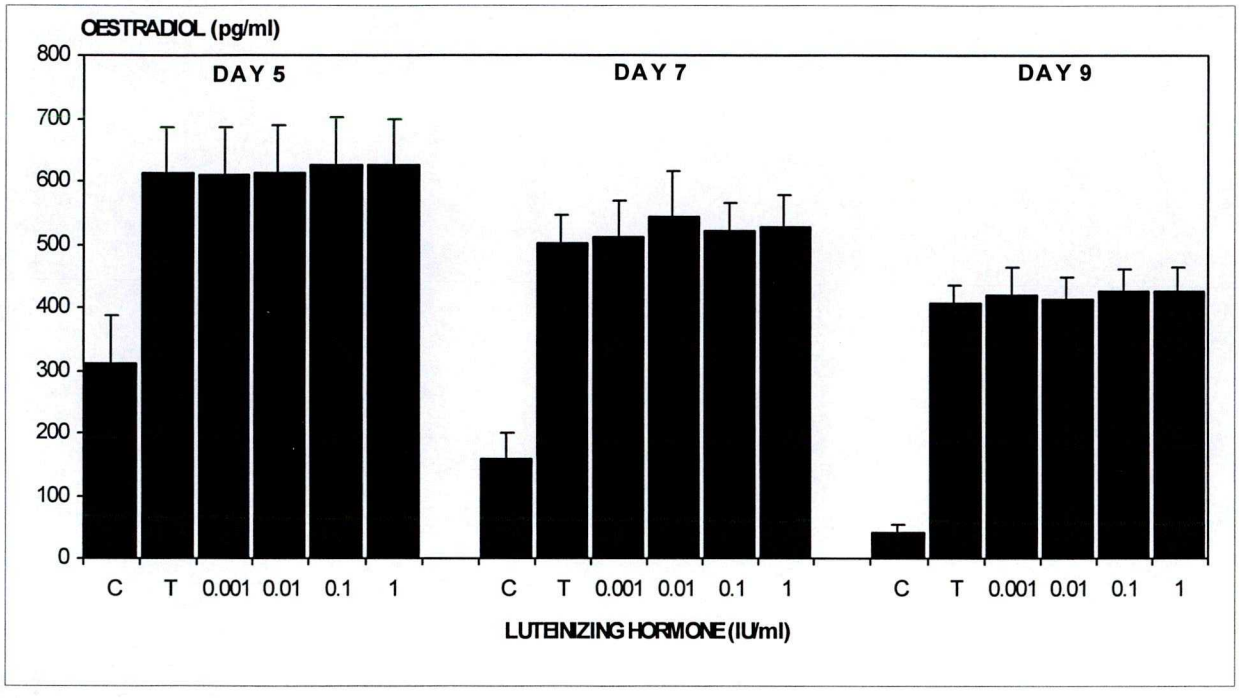

Figure 14. Oestradiol production of human granulosa-lutein cells in response to rLH induction in the presence of testosterone. The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10 % FCS and then exposed to 0.001-0.01-0.1-1 IU/ml rLH in a medium containing 1% FCS and 10 ng/ml testosterone for 6 days. Data are means ± SEM (n=7). C, control, untreated cultures. T, testosterone-only cultures.

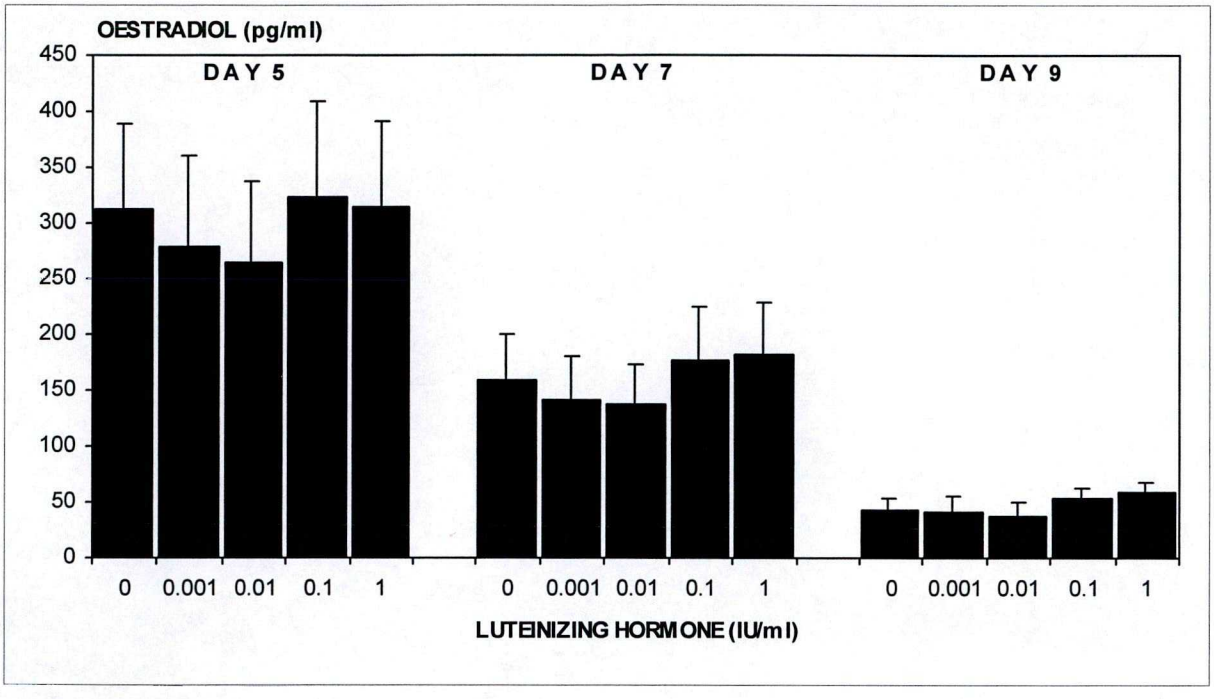

Figure 15. Oestradiol accumulation by granulosa-lutein cells cultured in an exogenous androgen-free environment in the presence of different doses of rLH (0.001-0.01-0.1-1 IU/ml). The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10% FCS and stimulated with rLH in medium containing 1% FCS for 6 days. Data are means ± SEM (n=7).

Effects of Recombinant Gonadotrophins on Granulosa Cell Progesterone Production

The progesterone responses of granulosa-lutein cells to recombinant gonadotrophin stimulation were assessed by using the same batch of cells cultured for 9 days including a 3-day preincubation. The culture medium was supplemented with 2.5% FCS providing cholesterol substrates for progesterone synthesis. All three recombinant gonadotrophins stimulated progesterone production. Recombinant FSH at doses of 1-10-100 mIU/ml and rLH at doses of 0.001-0.01-0.1-1 IU/ml caused clear dose-related increases in granulosa cell progesterone production (Figs 16 and 18, respectively). The granulosa cell progesterone secretion was also increased by the presence of rhCG (at doses of 0.1-1-10 IU/ml) in all time intervals (Fig. 17). However, rhCG at 0.1 U/ml caused maximum stimulation, with no further increases in progesterone production at higher doses. The overall effect of rhCG was similar to that of rLH with respect to the stimulation of progesterone production. Interestingly, only rFSH was able to augment progesterone production significantly at doses of < 0.01 IU/ml. In

all experiments, both the basal and the stimulated progesterone production declined with time. However, the reduction in progesterone level was more pronounced in the absence of recombinant gonadotrophins.

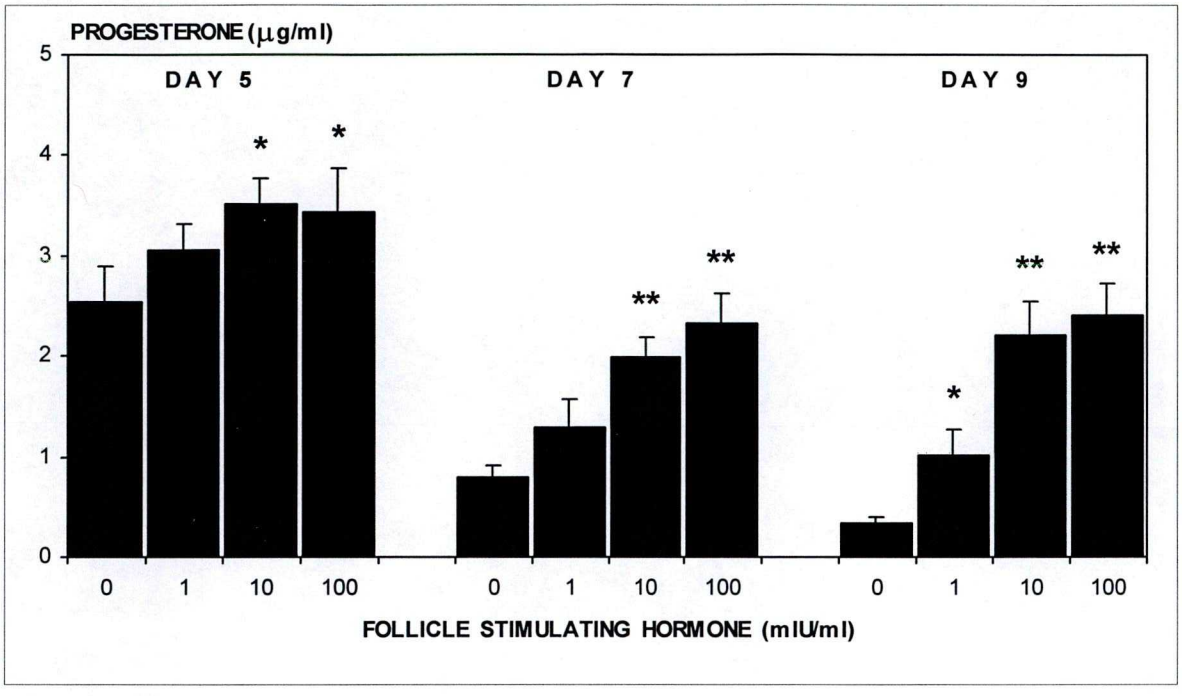

Figure 16. Progesterone production of luteinized human granulosa cells cultured in the presence of different doses of rFSH (1-10-100 mIU/ml). The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10% FCS and then incubated with rFSH in a medium containing 2.5% FCS for 6 days. Data are means ± SEM (n=6). *, $P<0.05$ and **, $P<0.01$ versus control (0 mIU/ml rFSH).

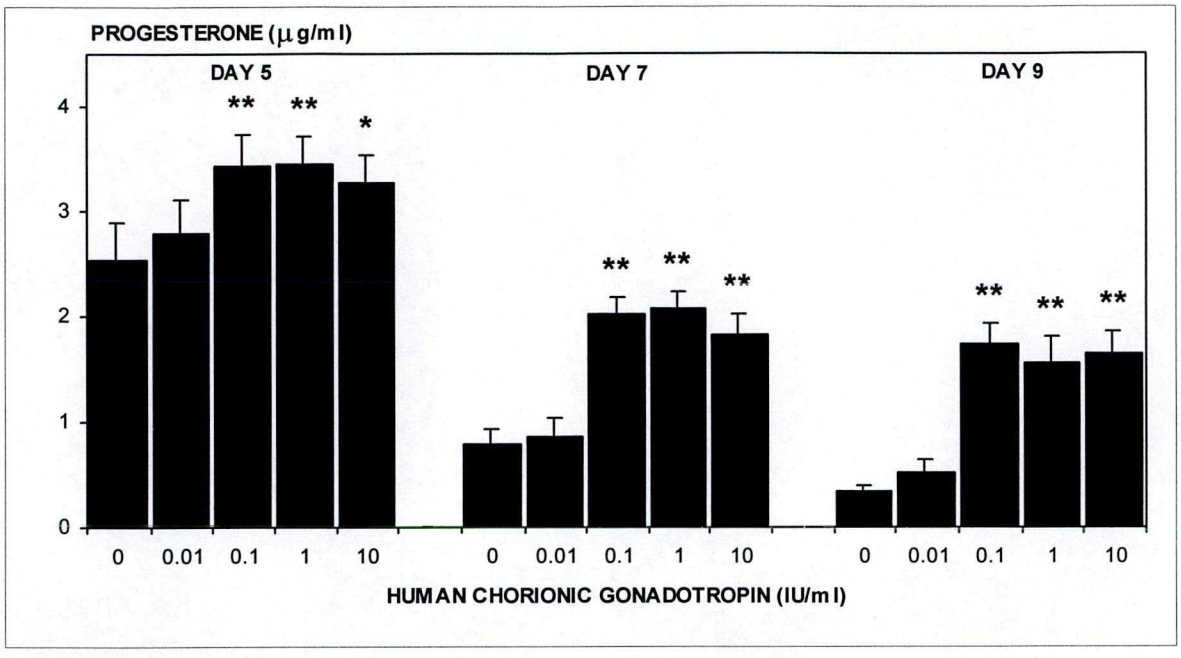

Figure 17. Progesterone responses of granulosa-lutein cells to rhCG induction. The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10% FCS and then exposed to rhCG (0.01-0.1-1-10 IU/ml) for 6 days in a medium containing 2.5% FCS. Values are means ± SEM (n=6). *, $P<0.05$ and **, $P<0.01$ vs. control (0 IU/ml rhCG).

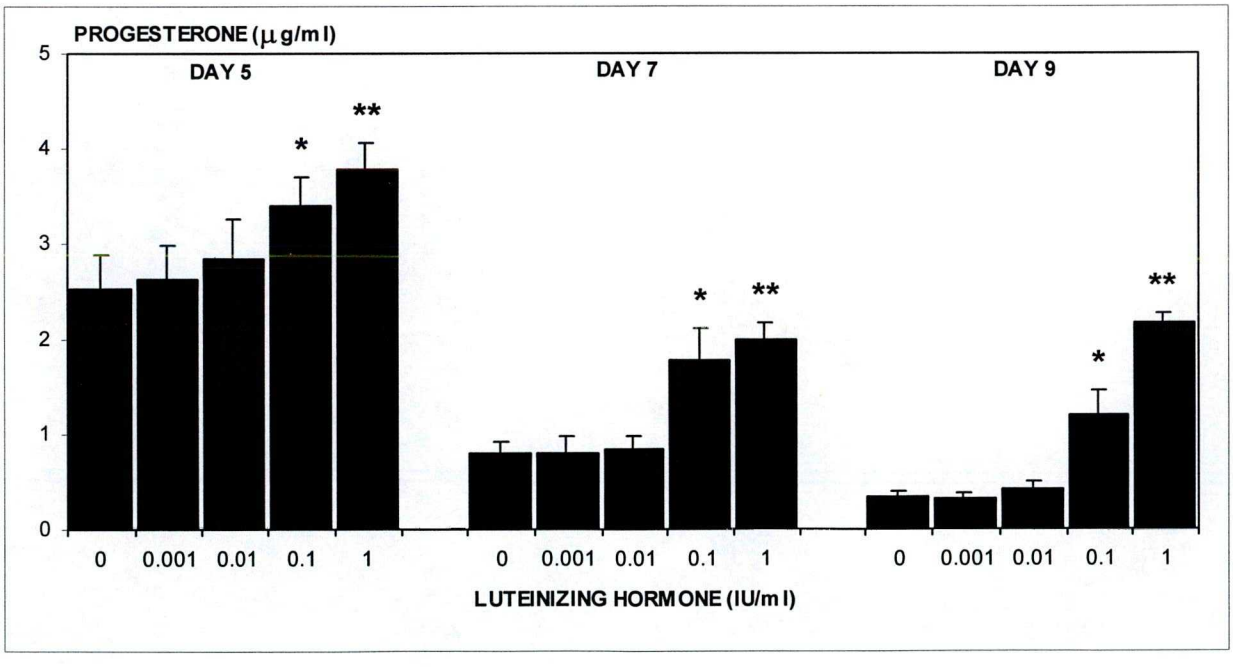

Figure 18. Progesterone accumulation of human granulosa-lutein cells in response to rLH. The cells (100,000 viable cells/well) were preincubated for 3 days in a culture medium supplemented with 10% FCS and then stimulated with rLH (0.001-0.01-0.1-1 IU/ml) for 6 days in a medium containing 2.5% FCS. Values are means ± SEM (n=6). *, $P<0.05$ and **, $P<0.01$ vs. control (0 mIU/ml rLH).

DISCUSSION

In this study, the *in vitro* biopotency of recombinant gonadotrophins (rFSH, rhCG and rLH) was assessed on the basis of their ability to stimulate the oestradiol and progesterone secretion of luteinized human granulosa cells in long-term cultures. The cells were harvested at the time of follicular aspiration after ovarian hyperstimulation for IVF. Several cell preparation methods have already been proved to reduce blood cell contamination in such cultures, thereby improving culture reliability (Beckmann et al. 1991, Best and Hill 1994, Figenschau et al. 1997). In our cultures, hyaluronidase digestion of the extracellular matrix, followed by enrichment of the granulosa-lutein cells by Percoll sedimentation resulted in a low rate of blood cell contamination and a reduced cell loss during the culture. Prior to an evaluation of the basal and the stimulated steroid production, granulosa cells in cultures were identified by specific inhibin immunostaining.

Basal Oestrogen Secretion

During the first day of incubation, the granulosa cells produced a high basal level of oestradiol and exhibited wide variations in oestrogen secretion, which is in agreement with other findings (Polan et al. 1984a, Wood et al. 1994). The individual differences found in basal granulosa cell oestrogen production were also reflected in the serum oestradiol concentrations measured on the day of hCG administration, since a close correlation was observed between the serum oestradiol concentrations and the *in vitro* basal oestrogen production of granulosa cells obtained from the same patient when assayed within the first day of culture. Thus, granulosa-lutein cells taken from patients with relatively low serum oestradiol levels (low responders to gonadotrophin stimulation) on the day of hCG injection produce less oestrogen *in vitro* as compared with cells from higher gonadotrophin responders. A weaker correlation was demonstrated between these parameters, when the serum level was correlated with the oestrogen production of the granulosa cells on day 3 (Hurst et al. 1992). On this day, however, our cells displayed far less variability in oestrogen production, suggesting that 1-day-old cultures could better reflect the *in vivo* sensitivity of patients to gonadotrophin stimulation. However, Polan et al. (1984a) reported that the individual

variations in granulosa cell aromatase activity were apparently not reflected in the peripheral oestradiol levels determined at the time of follicle aspiration. Since granulosa cells from woman at risk of OHHS (very high responders) were reported to secrete more oestradiol in culture than did cells from normal patients (Leya et al. 1992), our results support the concept that elevated serum oestrogen levels in women at risk of OHSS appear to be not only a result of the cumulative contribution from an increased number of preovulatory follicles: the individual follicles also possess an increased capacity for oestrogen production (Leya et al. 1992).

The basal oestrogen levels decreased to less than 50% of initial values after a 3-day preincubation and were less than 5% at the end of the culture. Thus, the granulosa cells were capable of secreting oestradiol spontaneously throughout the whole culture without androgen and gonadotrophin support. These findings are in contrast with those of Erickson et al. (1989), who reported that although the granulosa-lutein cells possess a very high aromatase activity in the first few days of culture, in the absence of exogenous androgens oestradiol production is negligible.

Although the basal oestrogen production gradually decreased during the culture, high aromatase activity was present in the granulosa cells as evidenced by the dose-dependent increases in oestrogen production in the presence of aromatizable androgens. The cells maintained their oestrogen-producing capacity for at least 9 days in the absence of added gonadotrophins. These results confirm the findings published by Bernhisel et al. (1987), who described granulosa cell culture maintained its steroidogenic enzyme activity for at least 12 days when an appropriate androgen substrate was present. However, Wood et al. (1994) reported that the basal oestrogen production decreased markedly during the culture, even in the presence of androstenedione.

Basal Progesterone Secretion

Besides the high aromatase activity present in the granulosa-lutein cells, these cells were able to secrete high amount of progesterone, in agreement with various other observations (Tapainen et al. 1987, Erickson et al. 1991, Schipper et al. 1993). The reason, why these cells produce both oestradiol and progesterone during the culture may be the heterogeneity of the cell population present in the follicular fluid at the time of ovum collection. Follicular

aspirates obtained from stimulated cycles contain cells at different stages of luteal differentiation such as granulosa cells, small luteal cells and large luteal cells (Whitman et al. 1989, Chaffkin et al. 1992). The granulosa cells secrete oestrogens in response to FSH stimulation, whereas the luteal cells are responsible for progesterone biosynthesis. Large luteal cells secrete more progesterone basally than do small luteal cells, and in contrast, small luteal cells produce more progesterone in response to LH/hCG (Ohara et al. 1987). In our experiments, the baseline progesterone production gradually increased during the first few days of culture, reaching its maximum on the day 3 of culture, followed by a subsequent decline, which could be partly explained by the lower FCS levels present in the culture medium. These results do not confirm previous findings (Schipper et al. 1993, Breckwoldt et al. 1996) where progressively decreasing levels of progesterone were noted during the culture, even in the presence of constantly high FCS levels. Richardson et al. (1992) demonstrated that, although the induction of ovulation by hCG injection in the course of IVF initiates the process of luteinization, the ability of the granulosa cells to produce progesterone is not fully developed at the time of follicle aspiration. The proportion of the luteal cells at the time of ovum collection appears to be about 20% (Whitman et al. 1989, Chaffkin et al. 1992). If we accept that progesterone release from the granulosa cells is a marker of luteinization (Holst et al. 1991), the increasing progesterone/oestradiol ratio during the preincubation period is direct evidence that the granulosa cells after follicle aspiration tend to luteinize in the culture, especially in the presence of serum. These results confirm and extend previous findings (Channing 1970, Richardson et al. 1992).

In human granulosa cells, cholesterol plays a key role as a precursor for steroid hormone biosynthesis (Carr et al. 1982). In most human steroidogenic tissues, it can be derived from two sources: *de novo* synthesis from acetyl coenzyme A, and the uptake of serum lipoproteins, especially low-density lipoproteins (Tureck and Strauss 1982, Richardson et al. 1992). The amount of cholesterol produced by *de novo* synthesis is inadequate to meet the needs of the granulosa-lutein cells for progesterone synthesis. Thus, these highly steroidogenic cells require cholesterol supplementation to synthesize progesterone both *in vivo* and *in vitro*. In our experiments, the granulosa cells produced significantly lower levels of progesterone in the absence of serum than in its presence. Furthermore, the stimulation of the granulosa cells by rhCG caused only moderate increases in progesterone production in the

absence of serum. These findings are in agreement with previous reports (Holst et al. 1991, Richardson et al. 1992). By comparison, the addition of serum to the culture medium resulted in dose-dependent increases in both baseline and rhCG-induced progesterone production. Our results clearly demonstrate that the granulosa-lutein cells require both gonadotrophin induction and cholesterol substrate for optimum progesterone synthesis.

In accordance with the results of other investigators (Tapanainen et al. 1987, Erickson et al. 1991, Schipper et al. 1993, Wood et al. 1994), we demonstrated that the granulosa-lutein cells harvested at the time of follicular aspiration spontaneously secrete high amounts of oestradiol and progesterone during the first few days of culture, suggesting the presence of a cell population with a maximally stimulated steroidogenic capacity due to *in vivo* stimulation with high doses of gonadotrophins. As a possible result of high baseline hormone levels and/or receptor down-regulation, the steroid production of freshly isolated cells could not be further stimulated by gonadotrophins (Tapanainen et al. 1987, Erickson et al. 1991, Schipper et al. 1993, Breckwoldt et al. 1996). To overcome this problem, granulosa-lutein cells were preincubated for 3 days in a culture medium containing 10% FCS prior to gonadotrophin induction, to allow them to recover after *in vivo* overstimulation by gonadotrophins. The induction of oestrogen and progesterone production by recombinant gonadotrophins was performed in the presence of 1% and 2.5% FCS, respectively, providing serum factors for optimum cell function and cholesterol substrates for progesterone biosynthesis. Further, no androgens were added to the cultures subjected to progesterone stimulation, since androgens have been reported to inhibit basal (Wood et al. 1993) and hCG-stimulated progesterone production (Polan et al. 1986).

Recombinant Gonadotrophin-Stimulated Oestrogen Production

In the absence of exogenous androgens, rFSH stimulated the oestrogen production of human granulosa cells in a clear, dose-dependent manner. The reduction in baseline oestrogen levels during the culture made the cells more sensitive to rFSH. These findings are in contrast with those of Lambert et al. (1995), where granulosa-lutein cells obtained from CC and gonadotrophin-treated cycles were investigated. However, Pellicer and Miró (1990) demonstrated that oestradiol production in response to FSH in the absence of androgens was increased only in GnRH-treated cells, no response being observed in CC-treated cells. They

measured higher oestradiol levels in cells treated with GnRH than in CC-treated cells, suggesting that the granulosa cells were probably less luteinized at the time of follicle aspiration in women treated with GnRH than in CC-treated patients (Pellicer and Miró 1990). Our results are in agreement with those published by Pellicer and Miró (1990). A similar phenomenon was found in relation to IGF-I-mediated granulosa cell oestrogen production, indicating that the stimulatory effect of IGF-I on granulosa cell oestradiol production was more pronounced in the absence of androstenedione than in its presence (Wood et al. 1994).

In the presence of testosterone, the cells produced a considerably higher baseline level of oestradiol, which remained constant up to the end of the culture. However, under these conditions the oestrogen accumulation was not significantly augmented by the presence of rFSH, suggesting that human granulosa-lutein cells in culture for 9 days are still able to convert androgens to oestrogens without the addition of rFSH. This finding is partly in contrast with what was reported by Erickson et al. (1989), who concluded that although FSH is unable to stimulate oestrogen output during the first 2 days in androgen-substituted granulosa cell cultures, the cells later increase their oestradiol production in response to FSH. There is agreement with the finding of Wood et al. (1994), who demonstrated that the incubation of granulosa cells with FSH caused merely a minor increase in oestradiol production in the presence of androgens. As regards the work with rFSH it was concluded that rFSH induced dose-dependent increases in both the oestradiol and the progesterone production of the granulosa cells from normal preovulatory follicles (Mason et al. 1993, Bergh et al. 1997), but surprisingly inhibited the accumulation of both hormones in the granulosa cells from a dominant follicle after the onset of the LH surge (Mason et al. 1993). The differences between these studies may be related to development-dependent changes in the granulosa cell responsiveness to FSH. Since follicular aspirates from stimulated cycles consist of a mixture of granulosa cells in different stages of differentiation (Whitman et al. 1989), the ratio of the cell types in the granulosa cell cultures may determine their sensitivity to FSH. Androgens are thought to augment the FSH-induced steroidogenesis in the growing follicles directly, but this ability is lost or could switch to an inhibitory effect during preovulatory development in primates (Harlow et al. 1988). Intensive androgen receptor immunostaining was observed in primate granulosa cells of preantral-early antral follicles, which declines during preovulatory development (Hillier et al. 1997). Thus, the lack of

modulation of either the basal or the rFSH-induced granulosa cell oestrogen production by the non-aromatizable androgen DHT could be accounted for by a diminished number of androgen receptors present in our granulosa cells. The role of androgens in oestrogen production therefore appears to be restricted to the substrates necessary for oestrogen biosynthesis at this stage of development.

In contrast with rFSH, marked changes were noted in the ability of the granulosa cells to respond to rhCG with respect to oestradiol production during the culture. Recombinant hCG inhibited oestrogen production within days 3 and 5 of culture, being significant at doses higher than 0.01 IU/ml, whereas no effect was found during the next stimulation interval (between days 5 and 7). These data confirm previous results with purified hCG (Christman et al. 1991, Breckwoldt et al. 1996), although no decrease in estrogen levels was noted during hCG treatment. However, hCG at high doses was reported to inhibit progesterone synthesis by human granulosa-lutein cells (de los Santos et al. 1993). During the final induction period (between days 7 and 9) rhCG produced an increase in oestradiol level, with a significant stimulation occurring at a dose of 0.1 IU/ml. The rhCG-dependent rise in oestradiol level was more pronounced in the absence of androgens, in accord with previous findings (Richardson and Masson 1981, Polan et al. 1986).

Recombinant LH was ineffective in stimulating the oestradiol production of the granulosa cells at any dose tested in either the presence or the absence of exogenous androgens. This is in contrast with the finding by Schipper et al. (1993), who demonstrated increases in oestrogen production after LH treatment, probably due to FSH contamination of their LH standard.

Recombinant Gonadotrophin-Stimulated Progesterone Production

The stimulatory action of rFSH on granulosa-lutein cell progesterone secretion confirms some previous findings with FSH (Pellicer and Miró 1990, Schipper et al. 1993), although these effects were thought to be caused by LH activity present in the FSH preparations (Pellicer and Miró 1990, Schipper et al. 1993). However, our data are in contrast with those where either FSH (Erickson et al. 1991, Wood et al. 1993) or rFSH (Mason et al. 1993, Lambert et al. 1995) was applied. Erickson et al. (1991) demonstrated marked differences in the FSH responses of the granulosa cells from stimulated or unstimulated follicles with respect to

progesterone production. In fact, clear, dose-related increases were obtained after rFSH stimulation in the granulosa cells from normal cycles (Mason et al. 1993, Bergh et al. 1997), whereas in the cells after the LH surge or in IVF granulosa-lutein cells, rFSH inhibited progesterone production (Mason et al. 1993, Lambert et al. 1995). These differences may be related to the difference in baseline progesterone production of the granulosa and the granulosa-lutein cells. The granulosa cells taken from normal preovulatory follicles produce small amounts of progesterone and can greatly increase their capacity to synthesize progesterone immediately after plating in response to gonadotrophins (Erickson et al. 1991, Mason et al. 1993, Bergh et al. 1997). Further, such cells are capable of secreting progesterone even under serum-free conditions (Erickson et al. 1991, Mason et al. 1993) or at low serum levels (Bergh et al. 1997). In contrast, substrate availability appears to be an important regulator of progesterone production in granulosa-lutein cells. Additionally, the granulosa cells harvested from stimulated cycles require a lag phase of a few days to regain their sensitivity to gonadotrophins. Thus, the lack of a response of granulosa-lutein cells to rFSH, described in other studies (Mason et al. 1993, Lambert et al. 1995), could be accounted for by the short-term, serum-free cultures applied in those studies.

In accordance with several previous observations involving the use of purified gonadotrophin preparations (Polan et al. 1984b, Hillensjö et al. 1985, Pellicer and Miró 1990, Erickson et al. 1991, Breckwoldt et al. 1996), rhCG enhanced the progesterone production by the granulosa cells. Maximum progesterone stimulation occurred at a dose of 0.1 IU/ml rhCG. The amount of rhCG required to elicit the maximum progesterone respons is the dose found effective in oestradiol stimulation and also the peak serum hCG level measured after 10,000 IU hCG administration for ovulation induction (Holst et al. 1991, de los Santos et al. 1993, Mannaerts et al. 1998).

Similarly to rFSH and rhCG, rLH augmented progesterone production in a clear, dose-dependent manner. These data confirm results obtained after purified LH stimulation (Veldhuis et al. 1983, Hillensjö et al. 1985, Schipper et al. 1993, Wood et al. 1993). In a recent paper, the effect of rLH on progesterone production was found to be biphasic, with lower responses at higher doses (Bergh et al. 1997). The stimulatory effect of rLH in these experiments provided evidence that the ineffectiveness of rLH in the stimulation of the

granulosa cell oestrogen biosynthesis could not be explained by the lack of response of the cells to rLH.

One important aspect that requires further evaluation is the difference in behaviour of recombinant gonadotrophins with respect to the stimulation of granulosa cell steroid production. As compared with rFSH, poor stimulatory responses to rLH and rhCG were noted with respect to granulosa cell oestrogen production. The induction of oestradiol production by rhCG required a longer lag phase as compared with rFSH. The lack of response to rLH and rhCG between days 3-5 and 5-7 of culture could be explained in part by the short preincubation period chosen for this study, since other investigators recommended a longer preincubation prior to gonadotrophin treatment (Christman et al. 1991, Schipper et al. 1993, Breckwoldt et al. 1996). This possibility is supported by the recovery of the granulosa cell responsiveness to rhCG during the closing stages of the culture (between days 7 and 9), although the oestrogen production was still unaffected by rLH at that time. By comparison, preincubation for 3 days was sufficient for the stimulation of granulosa cell progesterone production in the presence of rLH, rhCG or rFSH, suggesting that there could be slight differences between the gonadotrophin regulation of granulosa cell oestrogen and progesterone production at the receptor and/or second messenger level. It is assumed that the receptors for LH/hCG on the granulosa cells are occupied and probably down-regulated as a result of the high hCG doses employed for ovulation induction. However, the rLH and rhCG stimulation of progesterone production required a shorter preincubation period than that for oestradiol, suggesting the presence of some unoccupied LH/hCG receptors. The number of spare LH/hCG receptors appears to be sufficient for progesterone accumulation in response to either rLH and rhCG, whereas the stimulation of oestrogen production by these hormones may require more free LH/hCG binding sites and/or the presence of other factors synergizing with LH/hCG, such as IGF-I (Erickson et al. 1989, Christman et al. 1991). The differences found between rLH and rhCG with respect to oestrogen stimulation could therefore be related to the ability of rhCG to induce the formation of new LH/hCG receptors, as previously demonstrated (Polan et al. 1984b, Takao et al. 1997). Christman et al. (1991) additionally found that the second messenger mechanism for the induction of aromatase activity appears to be identical in short-term and long-term cultures and may be used by both FSH and hCG. Therefore, there could be differences in the FSH- and hCG-dependent cAMP production

necessary to induce oestrogen and progesterone synthesis. It was demonstrated that the low increase in cAMP level induced by FSH is sufficient to elicit maximum oestradiol responses, whereas higher levels of cAMP induced by hCG are required for the stimulation of granulosa cell oestrogen production (Breckwoldt et al. 1996). With respect to gonadotrophin-dependent progesterone production, there is evidence that, in spite of the desensitization of the gonadotrophin-sensitive adenylate cyclase as a result of *in vivo* exposure to large doses of hCG, the granulosa cells were still able to respond to hCG with an increased progesterone production (Jalkanen et al. 1986).

In conclusion, we demonstrated that rLH, rhCG and rFSH were able to stimulate granulosa cell progesterone synthesis in a dose-dependent manner. Before this study, the stimulatory effect of FSH on progesterone production was thought to be caused by LH activity present in the FSH preparations (Pellicer and Miró 1990, Schipper et al. 1993). However, our data provide direct evidence that rFSH completely devoid of LH activity is still able to stimulate progesterone production. In contrast, differences were found in the abilities of the cells to respond to rFSH, rLH or rhCG with respect to oestrogen production. Recombinant FSH enhanced oestradiol production in a dose-dependent manner. However, this effect was observed only in exogenous androgen-free cultures. By comparison, rLH did not affect the oestradiol production at any time during the culture at any dose tested, whereas rhCG tended to stimulate oestradiol production after an initial refractory stage. It should be born in mind that the results obtained from *in vitro* experiments should not extrapolate directly to *in vivo* mechanisms, our data suggest that in human luteinized granulosa cells, rFSH is capable of stimulating both oestradiol and progesterone production, whereas rLH and rhCG alone appear to be involved only in progesterone biosynthesis.

SUMMARY

For the evaluation of the *in vitro* biological activity of recently developed recombinant human gonadotrophins (rFSH, rLH and rhCG), a well-defined cell culture system was established, applying human granulosa cells harvested at the time of follicular aspiration for IVF. From an experimental viewpoint, the use of recombinant gonadotrophins completely free from other gonadotrophin activity allowed us for the first time to differentiate the effects of FSH from those of LH or hCG in the granulosa cells, which are capable of responding to all three gonadotrophins. The use of this culture system as a valuable model of the ovarian function also permits investigation of the influence of other hormones, growth factors, biologically active peptides, potential drugs and other compounds on ovarian steroid production.

The results led to the following main conclusions:

1. Human granulosa-lutein cells harvested from stimulated cycles were capable of secreting oestradiol spontaneously during the culture for 9 days.
2. The individual differences in basal granulosa cell oestrogen production during the first day of culture were reflected in the variations in serum oestrogen levels measured on the day of hCG administration. In other words, the granulosa cells are capable of secreting oestradiol *in vitro*, at a rate that corresponds to the *in vivo* sensitivity of patients to gonadotrophin stimulation.
3. The cells displayed a high aromatase activity and maintained their capacity to convert androgens to oestrogens for at least 9 days. In the presence of aromatizable androgens, the androgen/oestrogen conversion rate remained constantly high during the culture, even without gonadotrophin support.
4. In contrast with the oestrogen production, the granulosa-lutein cells require both serum supplementation and gonadotrophin stimulation for optimum progesterone biosynthesis.
5. After preincubation for 3 days, the granulosa-lutein cells responded to rFSH stimulation with regard to oestradiol production in long-term cultures but only in the absence of

exogenous androgens. The presence of androgens in the culture medium considerably enhanced the oestradiol levels in both control and rFSH-treated cultures, but rFSH failed to stimulate oestrogen accumulation further.

6. No modulatory effect of DHT was found on either the basal or the rFSH-induced granulosa cell oestrogen production. The role of androgens in oestrogen production at this stage of development appears to be restricted to substrates for aromatase.
7. As compared with rFSH, marked changes were noted in the ability of granulosa cells to respond to rhCG with respect to oestradiol production during the culture. In both cases, in the presence or absence of exogenous androgens, rhCG inhibited oestrogen secretion during the initial induction period. Later, it was either without effect or slightly stimulated oestradiol secretion. The stimulatory action of rhCG was more pronounced in the absence of testosterone.
8. Recombinant LH did not alter the oestradiol production by the same cell population during 9 days of culture at any dose tested. The lack of response to rLH was independent of the androgen supplementation of the culture media.
9. Recombinant rLH, hCG and rFSH were able to enhance granulosa cell progesterone synthesis. Recombinant LH and rFSH caused clear, dose-related increases in progesterone levels, whereas rhCG caused significant stimulation at the dose of 0.1 IU/ml, and no further increases were noted at higher doses.

The results relating to the use of pure recombinant gonadotrophins suggest that in human luteinized granulosa cells the progesterone biosynthesis is regulated by both LH, hCG and FSH, whereas the oestrogen production appears to be mostly FSH-dependent.

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