A simplified serum-free method for preparation and cultivation of human granulosa-luteal cells

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A simplified method for the preparation and long-term cultivation of granulosa-luteal cells in serum-free medium is described. The cells were harvested from women undergoing in-vitro fertilization, enriched by sedimentation and dissociated by enzymatic treatment. We demonstrated, by introducing a synthetic serum replacement (SSR2™), that these primary cell cultures cultivated in monolayers on an extracellular matrix may be used in experiments exceeding 7 days with low cell loss and cell death. No adverse effect on progesterone production was found. There was a high diversity in progesterone production between cells from individual patients. After several days in culture, the cells were challenged with human chorionic gonadotrophin which revived the rapidly decreasing progesterone production. We were unable to demonstrate an increase in cell number after 7 days of cultivation when the cells were grown in medium supplemented with either serum or SSR2™. The mitogens epidermal growth factor and basic fibroblast growth factor had no influence on proliferation. We also found that the present method prevents leukocyte contamination in the granulosa-luteal cell cultures. Compared with the common method based on the enrichment of granulosa-luteal cells on a density gradient (Ficoll™/Percoll™), this method saves time, labour and expense, in addition to augmenting purity.

Key words: cell preparation/granulosa cell culture/progesterone/proliferation/serum-free

Introduction

Human granulosa-luteal cells (luteal cells) are today usually harvested during in-vitro fertilization (IVF) from the pre-ovulatory follicles of hormonally stimulated women. These cells have been challenged with luteinizing hormone (LH) or human chorionic gonadotrophin (HCG), which may be regarded as a signal converting granulosa cells to luteal cells. This event has been described as the transformation of a protein-secreting cell to a steroid-secreting cell, which opposes proliferation and induces differentiation (Mestwerdt and Müller, 1982). In the growing follicle, the granulosa cells are separated from the theca interna by a basement membrane, interconnected by gap junctions in an avascular syncytium and organized in a multistratified pattern (Albertini and Anderson, 1974; Mestwerdt and Müller, 1982). Three different populations of these cells have been suggested, based on the content of enzymes and the secretion of proteins, steroids and acids (Inkster and Brodie, 1991; Whitman et al., 1991; Salustri et al., 1992). The mural population comprises the granulosa cells connected to the basement membrane, and the antral population comprises the cells closest to the antral cavity. The third population is suggested to constitute the ‘cumulus oophorus’. The heterogeneity indicates that the granulosa cells may be a family of cells rather than one distinct cell type. During oocyte retrieval, the cells are harvested by an invasive method which includes penetration of the vaginal wall, ovarian membranes and connective tissue. As these structures are vascularized, aspirates may be mixed with blood and thus contaminated with leukocytes.

A density gradient has been described to distinguish improperly granulosa cells from luteal cells (Chaffkin et al., 1992), though it is capable of selecting subpopulations of granulosa cells (Kasson et al., 1985). A high rate of leukocyte contamination has been described in primary cultures of luteal cells enriched by the density gradients Ficoll™ and Percoll™ (Beckmann et al., 1991; Best et al., 1994). These colloidal silica are not totally inert and have the potential of eliciting cellular activation in the leukocytes during preparation and cultivation (Pickering et al., 1989).

Under normal physiological conditions, only blood cells are surrounded by serum and the use of serum when cultivating other cell types may thus be questioned. Serum is an undefined substance containing multiple factors that might influence cell function in an unknown way. The content of polypeptides, hormones, growth factors and cytokines, or binding proteins and soluble receptors for these, is undefined. However, without serum, normal cell function may be endangered and experiments must be limited in time. Similarly, this represents an artificial condition that does not reflect the physiological interplay among a multitude of factors that exist in vivo.

Investigators have previously presented opposing results concerning luteal cell function, e.g. a mitogenic effect of epidermal growth factor (EGF; Tapanainen et al., 1987; Olsson et al., 1990). This may be explained to some extent by the heterogeneity of the cells originating from follicular aspirates. Especially when the role of cytokines in reproduction is to be investigated, both serum-free and leukocyte-free cultures are
an advantage. This study was undertaken to find a simplified and time-saving substitute for the density gradient procedure and to demonstrate a method for the long-term serum-free preparation and cultivation of purified luteal cell cultures by the introduction of a synthetic serum replacement, SSR2™ (Bertheussen, 1993).

Materials and methods

Luteal cells were obtained from women aged 25–37 years undergoing IVF after ovarian stimulation with human menopausal gonadotrophin (HMG; Humegon™, Organon®, Boxel, The Netherlands; 150 IU/day) from cycle day 3 onwards. In addition, on days 3 and 4 of the cycle, human follicle stimulating hormone (FSH; Fertinorm™, Serono®, Stockholm, Sweden; 150 IU/day) was administered. Serum oestradiol concentrations were measured daily, and follicular growth was monitored by serial vaginal ultrasonography. Ovulation was induced by administration of 9000 IU HCG (Pfyes™, Leo®, Ballerup, Sweden) when the largest follicle reached 18 mm in diameter and serum oestradiol concentrations corresponded to midcycle. When the largest follicle had reached a diameter of 18 mm, it had ovulated and the corresponding serum progesterone concentration exceeded the upper detectable limit.

Medium A

Medium A was sterile filtered physiological salt solution (0.9% NaCl) containing 2 mg/ml human serum albumin (Medi-Cult®, Copenhagen, Denmark) and 10 mmol/l HEPES buffer (cat. no. H-0887; Sigma).

Medium B

Ham’s F-12 medium (cat. no. N-6760; Sigma) and Dulbecco’s modified Eagle’s medium (DMEM; cat. no. 31600-091; Gibco®, Paisley, UK), 1:1, were supplemented with 100 000 IU/l penicillin, 0.1 g/l streptomycin and 1 mg/ml fetal bovine serum (cat. no. F-6131; Sigma). Synthetic serum replacement (SSR2™; cat. no. 2004; Medi-Cult) was supplemented at a concentration of 1 ml/l (0.1%).

Medium C

The same as medium B, but without fetal bovine serum.

Assay of progesterone

Progesterone of the luteal cells was assessed in two ways. Nine experiments were carried out ex vivo in 24-well plates with eight replicates and no medium exchange after 24 h. After 7 days of incubation, the cultures were trypsinized and cell numbers counted in a Bürker chamber. In addition, the incorporation of [3H]thymidine (cat. no. 2405901; ICN Biomedicals Inc., Irvine, CA, USA) was determined in cells cultivated in both 10% heat-inactivated fetal calf serum (FCS; Hyclone®, Lund, Sweden) and 0.1% SSR2™ (Medi-Cult). The specific activity of [3H]thymidine was 35 Ci/mmol, and each well was supplemented with thymidine that equalled 2 mCi/ml.

Cell number and viability in the luteal cell suspension (the pellet) were assessed in a Bürker chamber by Trypan blue exclusion and Trypan blue exclusion and the rate of [3H]thymidine incorporation with that of luteal cells was used at a final concentration of 2 mg/ml.

Cell number and viability in the luteal cell suspension (the pellet) were assessed in a Bürker chamber by Trypan blue exclusion and 1.0 × 10^5 disrupted single cells were seeded in 0.5 ml medium B (see below) on 24-well plates (cat. no. 3047; Falcon; 2.0 cm² surface area). When 96-well plates were used, the number of cells seeded was 2.0 × 10^5 in 0.1 ml medium. Before seeding, the wells were coated with fibronectin (cat. no. F-5764; Sigma). Incubation was carried out at 37°C in humidified air with 5% CO₂. After a 2 h incubation, the culture medium was removed gently and substituted with fresh medium B. After an additional 2 h incubation period, the plates were shaken roughly by hand to detach contaminating erythrocytes and subsequently washed twice with culture medium C (see below). The luteal cells were cultivated further in 0.5 ml or 0.1 ml 96-well plates medium C and fresh medium was added at each sampling time. When collagenase was employed, no fetal was used and the cells were seeded and cultivated further in medium C.

Media

Medium A

Medium A was sterile filtered physiological salt solution (0.9% NaCl) containing 2 mg/ml human serum albumin (Medi-Cult®, Copenhagen, Denmark) and 10 mmol/l HEPES buffer (cat. no. H-0887; Sigma).

Medium B

Ham’s F-12 medium (cat. no. N-6760; Sigma) and Dulbecco’s modified Eagle’s medium (DMEM; cat. no. 31600-091; Gibco®, Paisley, UK), 1:1, were supplemented with 100 000 IU/l penicillin, 0.1 g/l streptomycin and 1 mg/ml fetal bovine serum (cat. no. F-6131; Sigma). Synthetic serum replacement (SSR2™; cat. no. 2004; Medi-Cult) was supplemented at a concentration of 1 ml/l (0.1%).

Medium C

The same as medium B, but without fetal bovine serum.

Assay of proliferation

Provision of the luteal cells was assessed in two ways. Nine experiments were carried out ex vivo in 24-well plates with eight replicates and no medium exchange after 24 h. After 7 days of incubation, the cultures were trypsinized and cell numbers counted in a Bürker chamber. In addition, the incorporation of [3H]thymidine (cat. no. 2405901; ICN Biomedicals Inc., Irvine, CA, USA) was determined in cells cultivated in both 10% heat-inactivated fetal calf serum (FCS; Hyclone®, Lund, Sweden) and 0.1% SSR2™ (Medi-Cult). The specific activity of [3H]thymidine was 35 Ci/mmol, and each well was supplemented with thymidine that equalled 2 mCi/ml.

Assay of progesterone

Luteal cells from the luteal cell suspension were seeded as primary cell cultures in 24-well plates (1.0 × 10^5 cells/well, i.e. 5.0 × 10^5 cells/cm²) and cultivated in 0.5 ml medium C for 7 days. The medium was changed at 2 h. The supernatants were sampled and frozen at 24 h and every 48 h onwards. The supernatants were kept in a freezer at −20°C until analysis. After thawing, the progesterone concentration was assessed using an immunometric assay (Kodak Amerlite™, Amersham, UK). The coefficients of variation (CV) between and within assays were 7.4 and 7.3% respectively. Each sample was diluted initially 1:5 in medium C. When the progesterone concentration exceeded the upper detectable limit
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(160 nmol/l, i.e. 50.3 ng/ml), the dilution was increased. The maximum dilution needed was 1:50.

Immunocytochemistry

An immunocytochemical examination of contaminating leukocytes in the primary luteal cell cultures was performed at 24 h (after the second medium exchange). The primary antibody was monoclonal mouse immunoglobulin (Ig) G, a CD45 antibody (monoclonal mouse anti-human leukocyte common antigen; code no. M-0701; Dako®, Glostrup, Denmark). The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (cat. no. F-9006; Sigma). Human peripheral blood leukocytes, centrifuged on a density gradient (Lymphoprep™; Nygaard®, Oslo, Norway), were prepared from a healthy person and considered to be positive controls. Luteal cells were prepared with collagenase as the disrupting enzyme and grown on fibronectin-coated chamber slides (cat. no. 177445; Nunc®, Roskilde, Denmark). Three luteal cell suspensions were examined, with the luteal cells seeded in six replicates at a density of 2.0×10^5 cells/chamber. The leukocytes were seeded in two replicates with 1.0×10^5 cells/chamber. The wells, covered previously with phosphate-buffered saline (PBS; cat. no. D-8662; Sigma) supplemented with 1% bovine serum albumin (BSA, cat. no. A-8806; Sigma) for 10 min and subsequently washed twice in PBS, were labelled with the primary antibody for 1 h on ice after addition of the cells. This antibody (diluted 1:50) was added to both the luteal cells and the leukocytes, except for one well of each which served as the controls for non-specific binding. After the labelling, the wells were washed three times with PBS and the secondary FITC-conjugated antibody was added. Then the cells were allowed to rest on ice for 1 h in darkness and subsequently washed three times in PBS. The cultures were investigated using a fluorescence microscope (Diaplan™; Leitz®, Wetzlar, Germany) after fixation in 4% paraformaldehyde (cat. no. P-6148; Sigma).

Statistics

Data were imported from a computer-based spreadsheet to a standard statistical package (Statgraphics® Plus for Microsoft® Windows™; Manugistics Inc.®, MD, USA). The samples were tested for normal distribution with Shapiro-Wilk’s W statistic and plotted in normal probability plots where outliers were traced. Non-parametric methods were applied to test for significance when sample distributions were unknown. The significance was tested at the level α = 5%, and P values <0.05 were accepted to be statistically significantly different. The types of test employed are denoted.

Results

Cell loss

A total of 16 luteal cell suspensions were prepared, divided into four groups (with four replicates in each group) and treated with/without trypsin and with/without fetuin (±T and ±F). The proportion of dead cells in the luteal cell suspensions was <0.1%, as assessed by Trypan blue exclusion. The cells were seeded in 24-well plates with 1.0×10^5 cells/well, and the numbers of living and dead cells lost when the medium was exchanged were determined in a Bürker chamber by Trypan blue exclusion. The counting schedule was in hours (days): 2 h (day 0), 24 h (day 1), 72 h (day 3), 120 h (day 5) and 168 h (day 7).

The addition of trypsin reduced total cell loss (detached/non-adherent living and dead cells). Statistical comparisons of the groups +T/+F with −T/+F, and +T−/−F with −T−/−F, both revealed a significant difference (P < 0.01; Mann–Whitney U test). This was probably the result of the loss of living cells at 2 h in the untrypsinated groups (Table I). As judged by microscopy, untrypsinated cells were improperly dissociated, formed multiple aggregates that did not adhere and were subsequently lost when medium was exchanged. No statistical difference in the loss of living and dead cells was found in either of the trypsinated groups when fetuin was added (Mann–Whitney U test). In the untrypsinated groups, the addition of fetuin decreased the loss of both living and dead cells (P < 0.01; Mann–Whitney U test). Cell loss was negligible (<1%) from 24 h onwards, and overall cell loss was least in group +T/+F, i.e. 2.1% of the number of cells seeded.

Progesterone production

The determination of progesterone production was carried out to estimate any adverse effects of the trypsin and fetuin treatments on this parameter of cell function. In all, 16 luteal

<table>
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<th>Time (h)</th>
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aNumber×10^3 out of 1.0×10^5 cells seeded expressed as a percentage (mean of 64 replicates).
bTreated with/without trypsin/fetuin (±T±F).
cell suspensions were each divided into four groups according to the set up described in Figure 1 (alternative 1). Four replicates in each group were divided further at day 3 to give tests and controls, containing two replicates at days 5 and 7. The tests were challenged with 1 IU/ml HCG on day 3 (Figure 2). No statistical differences in progesterone production were found between the groups at days 1, 3, 5 and 7 for either tests or controls (Mann–Whitney U test).

The general feature of the distribution of the progesterone concentration in the four groups (each containing four replicates from 16 patients) was a lack of normality caused by a positive skew of the samples (frequency distributions). Normal probability plots revealed some extremely high concentrations present in the samples. These concentrations were identified as coming from replicates originating from the same patient. In addition, extreme concentrations at the lower tail of the distribution were also traced. The mean progesterone concentrations in group +T/+F of the two most extreme patients (lowest/highest) were 15 and 5485 nmol/l respectively at day 1, which is a 366-fold difference (Figure 3).

The CV of replicates was calculated for the progesterone production. The CV of the replicates (cells from one patient) and the mean concentrations of the replicates) at each sampling point were calculated for group +T/+F. These were considered to be estimates of the variance in cell number between the wells containing cells originating from one patient, and of the variance in progesterone production between individual patients respectively. There were four replicates including day 3 in each of the 16 primary cultures, and two replicates from day 3 onwards, i.e. the previous four had been divided into tests and controls (Figure 4).

**Cell density**

The effect of cell density on progesterone production was examined by cultivating different numbers of cells in wells of equal size using flat-bottomed tissue culture plates with a surface area of 2.0 cm². The cells were grown in 0.5 ml aliquots of medium, with four replicates at each density and six different cell densities in five experiments (five different luteal cell suspensions). Progesterone concentration was measured in supernatants collected every 48 h, except on day 1 which had a 22 h incubation interval (Figure 5A and B). By graphical estimation, both stimulated and unstimulated progesterone production was closely associated until the cell number was reduced to $2.5 \times 10^4$ cells/cm² ($5.0 \times 10^4$ cells/well). The apparent lack of decrease in progesterone production from days 1 to 3 in Figure 5A may be explained by the shorter incubation interval from days 0 to 1 (22 h), giving lower concentrations than expected if this interval had been identical to the others (48 h).

**Morphology**

The luteal cells were adherent to the fibronectin-coated wells after 2 h of incubation. In situations when aggregates were present, they tended to float at the surface of the medium, while single cells were attached. After the first incubation
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**Figure 2.** Mean progesterone production of granulosa-luteal cells from 16 luteal cell suspensions (patients) divided into four groups, treated with or without trypsin (±T) and fetuin (±F) on day 0 (0 h). There were four replicates with $1.0 \times 10^5$ cells/well in each group until day 3, when the cultures were divided into tests and controls with two replicates for each. Progesterone concentrations were measured at 24 h (day 1) and every 48 h onwards. The +F cells were cultivated in medium supplemented with fetuin for 24 h. The test groups (open symbols) were challenged with 1 IU/ml human chorionic gonadotrophin (HCG) on day 3.

**Figure 3.** The diversity of progesterone production between cells ($1.0 \times 10^5$ cells/well) from two different patients; four replicates on days 1 and 3; two replicates on days 5 and 7. Progesterone concentrations were measured after 48 h incubation intervals, except for concentrations on day 1 (24 h) which were measured after a 22 h interval. Closed symbols represent controls and open symbols represent tests (1 IU human chorionic gonadotrophin/ml on day 3). The error bars represent the SE.

**Figure 4.** Line A: coefficient of variation (CV) in progesterone production between the individual cell cultures (replicates) originating from one patient. The numbers shown are the mean of values obtained from 64 replicates (four replicates from 16 patients, group +T/+F) on days 1 and 3. On days 5 and 7 there were 32 replicates in each of two groups, a control and a test group. The latter group was given 1 IU/ml human chorionic gonadotrophin (HCG) on day 3. Line B: the mean of four replicates from each of the 16 patients was calculated and the CV between these patients is shown. From day 3 onwards there were two replicates in each of the control and the test (given 1 IU/ml HCG) groups.

period (2 h), the adherence was sufficient to avoid excessive cell loss (Table I). The general morphological feature was cells with several cellular processes (pseudopodia), in contrast to the spherical shape when seeded. The generation of pseudopodia increased during the subsequent 22 h (Figure 6). At 48 h, the cells appeared to be widespread (amoeboid), and when cell density reached $5.0 \times 10^4$ cells/cm$^2$, multiple interconnections between the cells were noted. The generation of pseudopodia seemed not to proceed after 72 h of incubation (Figure 7).

The erythrocytes in the cultures numbered $\sim 1.0 \times 10^2$–$1.0 \times 10^5$ estimated in a Bürker chamber, and this probably reflects the ratio of blood contamination in the luteal cell suspension. The gentle washing procedure at 2 h did not remove the erythrocytes completely, but they were totally displaced at 24 h as judged by microscopy.

**Proliferation**

Five pilot experiments were carried out using $[^3]$H]thymidine incorporation to assess any difference in proliferation between the cells disrupted by either trypsin or collagenase. When trypsinated, fetuin was employed as antitrypsin. HeLa and HL60 cells were cultivated under similar conditions without the addition of enzymes. The results showed an equal rate of $[^3]$H]thymidine incorporation for both enzymatic treatments, and the basic incorporation rate was very low. The number of disintegrations per minute (dpm) after 6 h of incubation with $[^3]$H]thymidine ($2.0 \times 10^4$ cells/well) were $4.8 \times 10^2$, $2.2 \times 10^3$ and $1.6 \times 10^5$ for luteal, HeLa and HL60 cells respectively. After 96 h of cultivation without exchange of medium,
Figure 5. (A) Progesterone production with different numbers of granulosa-luteal cells per cm² (c/cm²). Closed symbols represent control groups, open symbols represent test groups (given 1 IU/ml human chorionic gonadotrophin on day 3). The concentrations on day 1 were measured after 22 h of incubation, and the remainder after 48 h. (B) The relationship between progesterone production and different densities of unstimulated granulosa-luteal cells during 7 days of incubation. The numbers are means of four replicates from five patients. From day 3 onwards there were two replicates.

Figure 6. A primary cell culture of granulosa-luteal cells dissociated with trypsin and seeded in fibronectin-coated wells at a density of $5.0 \times 10^3$ cells/cm². Appearance (magnification $\times 200$) after 24 h of incubation. Erythrocytes have been removed; the cells are attached and have started to produce pseudopodia.

Figure 7. A primary cell culture of granulosa-luteal cells dissociated with trypsin and seeded in fibronectin-coated wells at a density of $5.0 \times 10^3$ cells/cm². Appearance (magnification $\times 100$) after 72 h of incubation. Multiple cell–cell connections have been established.

6.0 $\times 10^3$ dpm were recorded in the luteal cell cultures. We included 17 luteal cell suspensions in a protocol with collagenase used as the disruption enzyme. Each of the 17 suspensions was divided in two and seeded in the same medium (DMEM/Ham’s F-12); one half was supplemented with 10% heat-inactivated FCS, and the other half with synthetic serum replacement (SSR2™). The influence on the proliferation of cells stimulated with EGF (10–10³ ng/ml), FGF (5–5×10³ pg/ ml), TNF-α (4×10¹–4×10³ U/ml), IFN-γ (1–1×10³ IU/ml), RU 486 (6.5–26.0 nM), progesterone (1×10²–1×10⁴ nM), HCG (1×10¹–1×10⁴ IU/ml) and FSH (1×10³–1×10¹ IU/ ml) was examined by incubation with [³H]thymidine for 96 h, where unstimulated luteal cells constituted the control cells. Each of the agents was tested in four to seven separate experiments with four to eight replicates. No mitogenic effect was found for any of these agents (t-test). There were no differences in [³H]thymidine incorporation between luteal cells grown in medium supplemented with either FCS or SSR2™ (t-test) for both stimulated and unstimulated cells (tests and controls).

Nine experiments in serum-free medium were carried out in 24-well plates with eight replicates of unstimulated luteal cells (1.0 $\times 10^5$ cells/well). After 7 days of incubation, no increase in cell number was found after estimation in a Bürker chamber.

Content of the leukocytes
Luteal cells were prepared as described in Figure 1 (alternative 1) and grown on fibronectin-coated chamber slides. Out of a
total of 12 chambers (2.0×10^5 cells/chamber), i.e. four test chambers from three different patients, we found two positively stained cells in the luteal cell cultures. These two cells were found in the same chamber. No non-specific binding was present, and positive controls ( peripheral blood leukocytes) were labelled.

**Discussion**

The main aim of this work was to simplify luteal cell preparation and to demonstrate monolayers of cells that may be used in long-term serum-free cultures. A major problem arises from the fact that luteal cells harvested from pre-ovulatory follicles usually occur as cell aggregates. Further processing is necessary for both enrichment and dissociation. The former is commonly performed by using density gradients (Percoll™/Ficoll™/Lymphoprep™), and the latter by mechanical disruption. In addition to being a time-consuming procedure, the density gradient has been described previously as being potentially harmful to cells, insufficient to separate luteal cells from leukocytes and capable of eliciting cellular activation (Memon et al., 1989; Pickering et al., 1989; Beckmann et al., 1991; Best et al., 1994). We consider enrichment by sedimentation as a method with low/no risk of cell damage or activation.

Tissue dissociation involves disruption of the extracellular matrix and of individual cell–cell contacts. There are two common ways to achieve this: (i) mechanical dissociation, i.e. cutting, shearing, scratching and mincing, which may have the disadvantage of causing destruction of the cells and cell surfaces, e.g. destruction of receptors, membranes and cytoskeleton, as well as exposure of the cells to intracellular materials from destroyed cells; and (ii) enzymatic digestion of the extracellular matrix, e.g. using non-specific proteases such as trypsin, dispase, collagenase and pronase, or specific ones such as hyaluronidase.

Granulosa cells are kept together by gap junctions in vivo which are also reconstructed in vitro (Amsterdam et al., 1989), and the extracellular matrix inside the follicle has been reported to contain fibronectin, hyaluronic acid, proteoglycans, laminin and type IV collagen (Bortolussi et al., 1989; Salustri et al., 1992; Asem and Novero, 1993; Novero and Asem, 1993; Uhlin Hansen and Yanagishita, 1993). The complex composition of the extracellular matrix thus indicates no clear preference for which enzymes to use.

Trypsin is usually ineffective in tissue dissociation as purified substrate (Krieger et al., 1974; Sprang et al., 1987). The commercial tryptic preparations are commonly prepared from a bovine or porcine pancreas and are crude mixtures of proteases, polysaccharidases, nuclease and lipases, like the one used in our experiments. The addition of EDTA (a calcium chelator) to the trypsin solution, as applied here, is a necessity to prevent the enzyme from auto-digesting (Sipos and Merkel, 1970). Collagenase in pure solution, like trypsin, is ineffective in tissue dissociation (Harper and Kang, 1970) and has to be combined with proteases, lipases and polysaccharidases, like the crude source used here. Our observations of capability to dissolve the matrix revealed no difference between the two enzymatic treatments, but the combination of trypsin and fetuin seemed to give faster and stronger adherence. Fetuin is an antitrypsin that also promotes cell growth, adherence and differentiation (Nie, 1992; Yamamoto and Sinohara, 1993). This may explain the observation that adding fetuin decreased cell loss (Table I).

In the serum-free medium, cell loss was very high without coating the wells with fibronectin. The cultivation of granulosa cells on an extracellular matrix has been proved to promote cell adherence and the formation of cellular processes and gap junctions, i.e. resemblance of growth on a basement membrane (Furman et al., 1986; Amsterdam et al., 1989). The choice of the extracellular matrix and the surface it is layered onto is probably decisive for the limitation of cell loss (Aston et al., 1996a,b). Luteal cells have been suggested to be capable of degrading an extracellular matrix (Matrigel™), causing excessive loss of living cells (Aston et al., 1996a,b). This effect was prevented when HCG was present in the medium. By examination using an electron microscope, these authors found that cells cultivated for 14 days under serum-free conditions were highly damaged unless HCG was added to the medium. Estimated by a tetrazolium salt (MTT) reduction-activation (Memon, 1983), we found that luteal cells cultivated in a similar serum-free medium (DMEM/Ham’s F-12) with added synthetic serum replacement (SSR2™) and coated on purified fibronectin were still metabolically active after 1 month in cultures without exogenous HCG (results not shown). Regarding the low cell loss illustrated in Table I, this indicated that our culture conditions may be preferable.

A large body of evidence verifies the mitogenic effect of EGF on granulosa cells, although the reports are inconsistent for luteal cells. The lack of induced proliferation by EGF is in accordance with Olsson et al. (1990; density gradient not applied), but inconsistent with Tapanainen et al. (1987; density gradient applied) who also found EGF to be a luteal cell mitogen.

Benyo and Pate (1992) reported that IFN-γ reduced the cell number in 7 day old serum-free bovine mid-cycle granulosa cell cultures, but did not influence the proliferation of immature porcine granulosa cells (Yasuda et al., 1992). TNF-α (10 ng/ml) has been reported to double the cell numbers of human luteal cells (Percoll-enriched) during 10 days of culturing (Yan et al., 1993). We observed no mitogenic effect of this cytokine. Chaffkin et al. (1992, 1993) reported that progesterone at a concentration of 640 nmol/l inhibited luteal cell proliferation significantly, an effect that was antagonized by 64 mmol/l RU 486. These authors also found that ~20% of the cells in the cultures (Ficoll-enriched) were granulosa cells not luteal cells. Such heterogeneity is in accordance with investigations described by others (Ohara et al., 1987; Rao et al., 1991). An explanation of these opposing findings might be methodological differences such as harvesting, cultivation, selection and purity of the cell cultures. Percoll-enriched luteal cell cultures have been demonstrated to contain 40–52% lymphocytes and 6–14% macrophages (Best et al., 1994). Our findings by immunocytochemical labelling of the leukocytes in the luteal cell cultures showed a negligible rate of contamination.

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In our experiments the traditional human/bovine serum source was substituted with the synthetic equivalent SSR2™ (Bertheussen, 1993), but hormone production was triggered to significant rates after several days in culture. A more lengthy investigation of hormone production was limited by practical reasons in this study, but there were still adequate differences between tests and controls identifiable in the 7 day experimental protocol based on progesterone estimation. The serum-free medium employed here is devoid of steroid precursors. Progesterone synthesis is thus maintained by intracellular sources. This is in accordance with previous investigations performed on luteal cells cultured in serum-free medium, which demonstrated that thyroxine augmented gonadotrophin-induced steroid secretion and somatostatin enhanced LH-stimulated progesterone release, despite the lack of steroid precursors in the medium (Holst et al., 1995; Wakim et al., 1996). Although luteinization is greatly increased in medium supplied with serum (Holst et al., 1991), our results suggest that the nutrients in our medium are sufficient to maintain granulosa cell function in long-term experiments. Previously, time limitation has been a major disadvantage using serum-free medium (Epstein-Almog and Orly, 1985). Present results show that these problems may have been solved by the introduction of SSR2™.

We demonstrated linearity between cell number and progesterone production at densities $\geq 2.5 \times 10^4$ cells/cm². At lower densities a deviation occurred in this association (graphical estimation of Figure 5B) whereby a linear relationship was not evident. We traced some unusually high progesterone concentrations in the distributions, and these were assumed to be from wells containing cell aggregates. By exclusion of these values (outliers) and correction of the progesterone concentrations for the reduced cell numbers, we revealed no statistical differences in progesterone production at the lower densities (Mann–Whitney U test). A cell aggregate may contribute more to error occurring among small cell numbers than high. To prevent this problem, a filtration of the luteal cell suspension is recommended. Thus in our experiments, progesterone production is apparently not influenced by cell density.

We revealed a considerable variation between the individual patients in both basal and stimulated progesterone production, which is in accordance with previous reports (Yie et al., 1995; Wakim et al., 1996). The latter authors reported a correlation between granulosa cell steroid output and the concentration of melatonin in the follicular fluid. Wakim et al. (1996) suggested that the observed variability in their study could be attributed to fluctuating melatonin concentrations caused by seasonal variation. Our experiments covered several seasons and the variance present (Figure 4) may thus have a similar explanation.

In summary, we have presented a simplified method for the preparation and serum-free cultivation of luteal cells. The enrichment and disruption of the cells are both time saving, and treatment with trypsin and futilin does not influence progesterone production. The cells need not be exposed to foreign materials such as silica, and contamination with leukocytes is negligible. We were unable to detect an increase in cell number during 7 days of incubation, and proliferation was not induced when the cells were stimulated by mitogens. Our results suggest that this method provides reliable conditions for luteal cell function in long-term serum-free cultures and, regarding the present interest of elucidating the roles of cytokines in reproduction, this method also has safer experimental conditions.

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