Estimation of viability of bovine granulosa cells

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Summary. Granulosa cells harvested from non-atretic, antral follicles of cow (and pig and sheep) ovaries were incubated over glass cover slips in medium containing 20% (v/v) donor calf serum. Cell attachment to the cover slips was rapid, being in most cases complete within 3 h at 37°C. There was little further change over the next 20 h. The number of bovine granulosa cells which attached to a cover slip was proportional to the volume of cell suspension added to the medium; and the amount of oestradiol secretion by attached cells in a testosterone-enriched medium increased in parallel with their number. Granulosa cells which did not attach within 3 h produced little oestradiol. There was no clear relationship between the number of nigrosin-impermeable cells in suspensions prepared from different follicles and plating efficiency. It is concluded that the 3-h attachment of granulosa cells in culture is a useful measure of the number of viable cells in a suspension and is to be preferred to less direct techniques based on dye exclusion.

Introduction

Estimation of the number of viable cells in a suspension is a common requirement when setting up in-vitro cell cultures. The usual technique is to expose a sample of the cells to a high molecular weight dye such as nigrosin (Paul, 1975), trypan blue (Campbell, 1979) or eosin (Hanks & Wallace, 1958) and to note the proportion of stained cells. Cells which exclude the dye are termed viable and those which admit dye, non-viable; it is implied that the viable cells will grow and develop normally in the culture medium, and that the non-viable cells will not. This distinction is not however invariable. There are reports of live cells which were permeable to nigrosin (Kaltenbach, Kaltenbach & Lyons, 1958), and of dead cells which excluded trypan blue (Tennant, 1964).

A method was required for estimating the viability of granulosa cells. Because of doubts about the appropriateness of dye exclusion techniques for this purpose, an alternative procedure has been developed based on the fact that mammalian cells grow optimally only when attached to a surface (Rappaport, Poole & Rappaport, 1960).

Materials and Methods

Cell culture

The solutions used for cell culture were obtained from Flow Laboratories (Irvine, U.K.). Cells were cultured in a solution of Medium 199 (modified with Hanks salt and 20 mM-Hepes buffer) containing donor calf serum (20% v/v), L-glutamine (2 mM), Fungizone (2.5 µg/ml),

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penicillin (50 i.u./ml) and streptomycin (50 µg/ml). Each culture consisted of supplemented Medium 199 (1.0 ml) to which was added cell suspension (25–150 µl). The cultures were incubated at 37°C, with air as the gas phase and without shaking, in a tissue culture box consisting of 25 compartments (19 × 19 mm: Flow Laboratories). A glass cover slip (18 × 18 mm) was placed in the base of each compartment. Before use, the cover slips were soaked in absolute ethanol, rinsed in deionized water, dried with paper tissue, heat sterilized, and cooled to room temperature. For the oestradiol secretion studies, the medium was removed after 3 h and replaced with an equal volume of medium to which testosterone had been added to make a final concentration of 1 mg/l.

Granulosa cells

Medium to large follicles (0.4–1.5 cm) were excised from the ovaries of recently killed cows, pigs or sheep, and the granulosa cells were removed by gently scraping into Medium 199 containing heparin (50 i.u./ml) to prevent clotting and aid cell dispersion. After centrifugation at 400–500 g and removal of the supernatant, the cell pellet was resuspended in Medium 199 alone. In this study only granulosa cells from non-atretic antral follicles were used. Such granulosa cells have been shown to have the capacity to secrete oestradiol (Moor, Hay, Dott & Cran, 1978). Follicles were considered to be non-atretic if the number of granulosa cells they contained met the criteria described by McNatty, Makris, De Grazia, Osathanondh & Ryan (1979a) and McNatty, Smith, Makris, Osathanondh & Ryan (1979b).

Cell counts

Dye exclusion was determined by diluting a sample of cell suspension 1:1 (v/v) with nigrosin (5 g/l in 0.154 M-NaCl) and counting the number of unstained cells after 5–10 min. Each estimate was based on a count of not less than 400 stained and unstained cells in a haemocytometer chamber. Cell attachment was determined by counting the number of cells adhering to a cover slip after removing it from the culture medium and rinsing it twice in Hanks' balanced salt solution. The cells on each cover slip were fixed in a solution of formaldehyde in 0.154 M-NaCl (10% v/v) for ≥30 min, dried in air at room temperature, and stained with haematoxylin (Harris, ortho modification) followed by eosin (Gurr, 0.1 g/l deionized water). An estimate of the total number of cells on a cover slip was made by counting the cells in 0.17 × 0.17 mm squares: 8 squares were observed at each of 8 or 12 evenly distributed sites giving 64–96 squares. Duplicate counts of the cells on 10 cover slips (16 000–32 000 cells per cover slip; mean, 23 595 cells) gave a coefficient of variation of 11.2% (Moroney, 1965).

Radioimmunoassay

Oestradiol was measured directly in culture samples after dilution (>10-fold) with a 0.1 M-phosphate buffer (pH 7) containing gelatine (1 g/l), sodium azide (0.015 M) and sodium chloride (0.154 M). An antiserum to oestradiol-6(O-carboxymethyl)-oxime-BSA (Gamma G, Beaconsfield, U.K.) was used at a 1:15 000 dilution. After incubation of radioactive oestradiol with sample and antiserum for 1 h at 37°C, unbound oestradiol was precipitated with dextran-coated charcoal and the supernatant counted. Interassay variability (coefficient of variation) for the assay was 13.9% for a sample containing 75 pg oestradiol.

Results

The number of bovine granulosa cells attached to the base of a culture dish after incubation for 20 h at 37°C was linearly related to cell density throughout the range 1×10^5 – 6×10^5 cells/ml

(correlation coefficient for 18 observations, 0.996). Attachment was complete within 2 h and changed little during the next 20 h (Text-fig. 1). The number of cells attached at this stage is termed the 'plateau cell count'. For ovine and porcine granulosa cells attachment took a little longer. The number of ovine cells which had attached after 2 h was $84.3 \pm 8.1\%$ (mean $\pm$ s.d.) of the plateau cell count; and the number of porcine cells in two similar experiments was $102.9 \pm 18.2\%$ and $65.4 \pm 13.4\%$. In all cases granulosa cell attachment was substantially complete within 3 h (95.1 ± 3.1 , 87.3 ± 5.7 and $86.9 \pm 11.0\%$ respectively). Testosterone had no significant effect on the plateau cell count of bovine granulosa cells (20 h attachment: with testosterone, $65\,501 \pm 12\,610$ cells (mean $\pm$ s.d., 6 observations); without testosterone, $70\,579 \pm 6130$ cells). In the experimental conditions which were used, $93.1 \pm 3.5\%$ (mean $\pm$ s.d., 4 observations) of the bovine granulosa cells attached to the inner surfaces of a culture dish after 3 h were on the cover slip lining the base.

Text-fig. 1. Rate of attachment of bovine granulosa cells when cultured at 37°C in medium containing 20% donor calf serum. Each point represents the mean $\pm$ s.d. of 3 observations. Different symbols indicate separate experiments. The numbers of observations (n) used to establish each value of the plateau cell count (c) were: O, $n = 23$, $c = 40\,484 \pm 3888$ (mean $\pm$ s.d.); $\triangle$, $n = 20$, $c = 146\,620 \pm 17\,868$; $\bullet$, $n = 11$, $c = 89\,494 \pm 9983$.

The amount of oestradiol secreted by bovine granulosa cells in the presence of testosterone increased in parallel with the plateau cell count throughout the range 2×10^4 – 12×10^4 cells (correlation coefficient for 18 observations, 0.964; mean oestradiol secretion, 229 pg/1000 attached cells/20 h). Comparable figures for similar experiments repeated on two other occasions were 0.873 and 0.973, and 649 and 250 pg/1000 attached cells. Testosterone was a necessary precursor for oestradiol synthesis. In three experiments, the secretion of oestradiol in the absence of testosterone was 0, 3.4 and 3.7% of that obtained in matching cultures to which testosterone had been added.

Unattached cells produced relatively little oestradiol. In experiments in which only $7.8 \pm 1.6\%$ (mean $\pm$ s.d., 18 observations) of a suspension of bovine granulosa cells had attached by 3 h, the attached and unattached cells were incubated separately for 20 h in medium containing testosterone. Oestradiol secretion (mean $\pm$ s.d., 18 observations) during this time was 264 ± 57 pg/1000 attached cells and 2.81 ± 2.28 pg/1000 unattached cells.

The number of cells attaching in the stated conditions was less than the number which were impermeable to dye. Suspensions of bovine granulosa cells were prepared on 9 separate occasions and the number of attached cells (A) was compared both with the number which

excluded nigrosin (E) and with the total number (T). The ratio $A/E \times 100$ varied between $23.3 \pm 4.8\%$ (mean $\pm$ s.d., 18 observations) and $68.4 \pm 7.8\%$ (12 observations), and had a median value of 45.5%. The correlation coefficient for the comparison of attached cells with nigrosin-impermeable cells was 0.824. Values of the ratio $A/T \times 100$ ranged from 7.8% to 53.4% (median, 16.6%). Comparable figures for the ratio $E/T \times 100$ were 24 to 78% (median, 37%).

The time elapsing between animal death and follicle excision appeared not to affect granulosa cell viability during the first 24 h. The percentage of nigrosin-impermeable cells in bovine follicles removed on the day of slaughter was $44.6 \pm 14.2\%$ (mean $\pm$ s.d., 10 observations). For granulosa cells harvested from ovaries which had been kept for 24 h at 4°C before follicle excision, the figure was $44.2 \pm 13.6\%$ (11 observations).

Discussion

Tests for viability must be relevant to the type of cell being cultured. For granulosa cells from large non-atretic follicles, one criterion of viability is their ability to secrete oestradiol. In this study cell attachment is shown to be proportional to the total number of granulosa cells added to a culture dish, and to distinguish between cells which can be stimulated to secrete oestradiol and unattached cells with little steroidogenic capacity. These observations have made possible the development of a simple technique for measuring the viability of granulosa cells which is applicable to oestrogen-secreting granulosa cells obtained from pig, cow and sheep ovaries. It remains to be seen whether the technique is applicable to granulosa cells in general.

Use of the technique need involve little delay. Granulosa cells attach rapidly in the recommended conditions (Text-fig. 1), and at worst the duration of an experiment will be extended by 3 h when viability is measured as a function of cell attachment. In practice, however, even this short delay may not be necessary. The concordance between cells which exclude dye and cells which attach (correlation coefficient, 0.824) means that cultures can be set up using dye exclusion to give a rough indication of the number of viable cells in a dish. For experiments which can be completed within 20 h a cell count at the end will usually suffice as a measure of the plateau cell count (Text-fig. 1). For longer experiments both 3 h and concluding cell counts will be necessary.

Doubts expressed in earlier reports (Kaltenbach *et al.*, 1958; Tennant, 1964) about the universal use of dye exclusion techniques for measuring cell viability, have been confirmed for bovine granulosa cells. Cells were harvested from a series of ovaries and their viability estimated both by cell-attachment and by dye exclusion. In every case the number of cells which attached was less than the number which excluded dye. Although there was a significant correlation between the two estimates, there were considerable discrepancies when the results of individual cell suspensions were compared. McNatty & Sawers (1975) studied the importance of the intrafollicular hormonal environment on the capacity of human granulosa cells to divide, and claimed that LH arrested mitosis. This conclusion was based on the ability of granulosa cells to multiply in culture. However, McNatty & Sawers (1975) did not report the amount of cell attachment in the initial stages of their cultures; only the number of cells excluding nigrosin or lissamine green was measured. It is possible, therefore, that LH inhibited cell division by inhibiting cell attachment, and the figures for mitotic indices are likely to be an underestimate of the true position.

Granulosa cells harvested on different occasions differed markedly in their content of viable cells, whether viability was measured as a function of cell attachment or by dye exclusion. This variation has been noted previously; McNatty & Sawers (1975) reported a lack of correspondence between the proportion of granulosa cells excluding vital dye and details such as the stage of the cycle, follicle size, and the hormone content of the follicle fluid. Their claim that

the time between the removal of an ovary and follicle excision might be a critical factor appears unlikely in view of Channing's (1969) failure to demonstrate such an effect in the horse, and there was no decrease in the proportion of viable granulosa cells when cow ovaries were kept for 24 h before follicle removal in the present study. The considerable variation in the proportion of viable cells in different preparations suggests that some aspect of the procedure customarily used to prepare granulosa cells for culture may be cytotoxic. Factors influencing the way in which granulosa cells attach to a surface may also be important. Cells are sensitive to the surface to which they attach (Rappaport *et al.*, 1960), and to the medium in which they are immersed (Channing, Tsai & Sachs, 1976). In this study plating efficiencies were low compared with the proportion of cells excluding dye, and the conditions used for the preparation of cover slips and media may not have been optimal for the attachment of granulosa cells. However, the technique can be used to measure the viability of granulosa cells, and to prepare samples of granulosa cells which are relatively homogeneous, are of known size, have an oestradiol-secreting capacity similar to that reported by McNatty *et al.* (1979a) for human granulosa cells, and which are not contaminated by unattached cells or by cell debris. The preparation has obvious advantages as a system for testing the effect of experimental variables on the culture of granulosa cells, and as a starting point for a quantitative investigation of their ability to divide and to secrete steroids.

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