Culture of Chicken Granulosa Cells from Small Yellow Follicles: A Suitable Culture System

Alvaro G. Hernandez and Janice M. Bahr

Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

In the chicken ovary, one small yellow follicle (SYF) is selected daily to enter the hierarchy and ovulates a few days later. Cellular changes that occur in these follicles may provide an understanding of the mechanism of follicular selection. It was reported that granulosa cells isolated from SYF undergo apoptosis within 6 h in suspension culture. In this study, a culture method in which granulosa cells from SYF remain viable in vitro for 24 h is reported. Granulosa layers were isolated from SYF (Gr-SYF) and from the largest preovulatory follicle (Gr-F1) and dispersed in collagenase (1.5 mg/ml) for 15 min. Cells (1 × 10^6/500 μl for Gr-SYF and 2 × 10^7/500 μl for Gr-F1) were cultured either in 24 well plates in an incubator with 5% CO_2/95% O_2 at 39°C or in suspension in falcon tubes and incubated in water bath at 37°C, for 0, 6, 12 and 24 h. Theca cell explants (8 mm diameter) were cultured in 1 ml of the same medium for 6 and 12 h. Cell proliferation (n = 6) at 6, 12 and 24 h, DNA integrity and membrane permeability (n = 4) were determined by a colorimetric assay, gel electrophoresis and flow cytometry, respectively. Proliferation increased in Gr-SYF cultured in a plate at 12 and 24 h whereas it did not change in Gr-F1 cells at any time of culture. DNA fragmentation was observed in Gr-SYF cultured in suspension at 12 h and theca cells at 6 and 12 h but not in Gr-SYF cultured in the plate. Cell permeability and DNA stainability (indicative of apoptosis) were only increased in Gr-SYF cultured in suspension. In conclusion, we have identified culture conditions that will allow the culture of granulosa cells obtained from SYF, the stage prior to selection.

Key words: chicken, granulosa cells, culture system

Introduction

The chicken ovary contains thousands of cortical follicles less than 1 mm in diameter, hundreds of small and large white follicles (1–5 mm), an average of 7 small yellow follicles (SYF) (5–8 mm) and 4–7 preovulatory follicles (9–40 mm). Atresia is the most common fate of follicles less than 8 mm. Preovulatory follicles seldom become atretic and their normal fate is ovulation. It was estimated that only one of
every 20 follicles between 1–2 mm survives until ovulation (Gilbert et al., 1983). It is not known what causes this high rate of atresia among small follicles. Atresia could be a physiological process to ensure that only the healthier oocytes will ovulate. Follicular atresia occurs by apoptosis in all species studied to date, including the chicken (Yao et al., 1998; Guthrie et al., 1995; Hurwitz et al., 1996; Asselin et al., 2000).

A constant supply of preovulatory follicles is maintained by daily selection of one follicle from the pool of SYF. Regular selection of a SYF to the next level of maturation is required for normal reproduction and fertility. However, the mechanism by which SYF is selected is not presently known. A major problem concerning the SYF has been the lack of an appropriate culture system for the granulosa cells, delaying further studies in this area. Previous studies have reported that granulosa cells from SYF undergo apoptosis within 6 h in suspension cultures (Johnson et al., 1996, 1997; Johnson, 2000; Witty et al., 1996). Therefore, the objective of this research was to develop and validate a suitable culture system for granulosa cells from SYF. A comparison was made between two different culture systems, a) suspension cultures as previously published (Johnson et al., 1996) and b) plate cultures. The endpoints of our studies were: a) presence of DNA fragmentation, the hallmark of apoptosis, by agarose gel electrophoresis; b) cell proliferation, measured by a colorimetric assay and c) permeability of cell membrane and stainability of DNA, examined by flow cytometry.

Materials and Methods

Animals

Single-comb white Leghorn hens, 20 to 30 weeks of age with regular laying sequences of at least 6 eggs were used. Hens were maintained in individual cages, provided with feed and water ad libitum and exposed to a photoperiod of 17 h of light and 7 h of dark, with lights-on at 0500 h and lights-off at 2200 h. Oviposition was monitored daily at one hour intervals between 0800 and 1200.

Reagents

The following reagents, obtained from Sigma Chemical Company (St. Louis, MO), were used in the cell cultures: collagenase IV, Dulbecco’s Modified Eagle’s Medium (DMEM), Hepes, penicillin (100 U/ml), streptomycin (100 μg/ml), bovine serum albumin (BSA), insulin, transferrin, sodium selenite and Hank’s buffer salt solution (HBSS). Culture plates were tissue culture treated 24 well Costar plates (Corning Inc., Corning, NY). Fluorophore used for flow cytometry was Hoechst 33342, obtained from Molecular Probes, Inc. (Eugene, OR). Cell proliferation was determined with CellTiter® 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison).

Tissue Collection

Hens were killed by cervical dislocation 3.5 h after a midsequence oviposition. Small yellow follicles were removed and placed in sterile ice-cold Hank’s buffer salt solution (HBSS) immediately. Granulosa and theca layers were separated as previously described (Gilbert et al., 1977).
Cell Cultures

Plate Cultures. Granulosa layers were isolated from SYF (Gr-SYF) and from the largest follicle (Gr-F1) and dispersed by incubating in 1 ml of type IV collagenase (1.5 mg/ml) in DMEM for 15 minutes in 37°C water bath. The suspension was gently inverted every 5 minutes. After dispersion, cells were washed 3 times in 1 ml sterile HBSS supplemented with Heps (25 mM) and sodium bicarbonate (0.35 g/L) and resuspended in 1 ml of culture medium. Culture medium consisted of DMEM supplemented with Heps (25 mM), sodium bicarbonate (3.7 g/L), BSA (2 g/L) penicillin (100 U/ml), streptomycin (100 mg/ml), insulin (5 μg/ml), sodium selenite (5 ng/ml) and transferrin (5 μg/ml). Medium was filter sterilized and pH was adjusted to 7.4. Total cell counts and viable cell number were determined by the trypan blue exclusion method using a hemacytometer. Cell viability before culture was consistently higher than 90%. Culture medium was added to the dispersed cells to obtain a final concentration of 1 × 10⁶/500 μl for Gr-SYF and 2 × 10⁷/500 μl for Gr-F1 and cells were cultured in 24 well plates in a humidity controlled incubator with 5% CO₂/95% O₂ at 39°C. Cells were cultured for 0, 6, 12 and 24 h. After each culture time, the culture medium was aspirated and the adherent cells were removed from the wells by adding 500 μl of 0.25% trypsin-EDTA for 2 minutes at 37°C. After trypsinization, cells were washed 3 times in 0.5 ml HBSS, centrifuged at 200 g for 5 minutes, the supernatant was removed and the cells were either frozen at −70°C or processed for flow cytometry.

Circular theca cell explants 8 mm diameter were collected from F1 follicles, washed 3 times in HBSS and incubated for 0, 6 and 12 h in 1 ml of the same culture medium as used in the plate cultures.

Suspension Cultures. Granulosa cells from SYF were collected and dispersed as described above. Cells were incubated at a concentration of 1 × 10⁷/500 μl in 12 × 75 Falcon tubes in DMEM medium supplemented with Heps (25 mM), sodium bicarbonate (3.7 g/L), BSA (2 g/L) penicillin (100 U/ml) and streptomycin (100 mg/ml) and incubated in water bath 37°C for 0 and 12 h. Medium was filter sterilized and the pH was adjusted to 7.4. At the end of culture cells were centrifuged at 200 g for 5 min and either frozen at −70°C or processed for flow cytometry.

Determination of DNA Integrity

DNA was extracted from granulosa cells and theca cells as previously described (Yao et al., 1998). DNA was quantitated by absorbency at 260 nm and stored at −20°C. Fifteen μg of DNA were loaded onto a 2% agarose gel with ethidium bromide (0.5 μg/ml) and separated by electrophoresis for 3–3.5 h at 50 volts using single strength TAE (40 mM tris-acetate and 1 mM EDTA) as running buffer. Gels were visualized and photographed on a UV transilluminator.

Determination of Granulosa Cell Proliferation in Culture

Proliferation of Gr-SYF and Gr-F1 cells cultured in a plate as described above, was determined at 6, 12 and 24 h of culture by adding 100 μl of CellTiter® 96 for 1 h. Absorbance at 490 nm was then determined in 300 μl of culture medium. Background absorbance was determined in a well with culture medium and CellTiter® 96 but no
cells, and this value was subtracted from all other values. This reagent contains MTS
tetrazolium, which is bioreduced by cells into a colored formazen product soluble in the
culture medium. Absorbance measured at 490 nm is directly proportional to the
number of living cells. In preliminary experiments it was observed that determination
of proliferation with this method at 1 h of culture did not result in accurate readings, as
the cells are still recovering from the mechanical dispersion and adapting to a new
environment.

*Determinant of Membrane Permeability and DNA stainability by Flow Cytometry*

The DNA integrity and membrane permeability of fresh Gr-SYF cells and from
plate and suspension cultures at 24 and 12 h of culture, respectively, were analyzed by
flow cytometry. A 300 μl suspension of 500,000 cells/ml was prepared for flow
cytometry. Ten μl of a 1:10,000 dilution of Hoechst 33342 (from a stock solution of
10 mg/ml) were added to the suspension and incubated in a water bath at 37°C for 3
min. The cells were kept on ice for no longer than 15 min until analysis. Flow
cytometry was done at the Flow Cytometry Facility at the University of Illinois at
Urbana-Champaign using a Modified Coulter 753 (Beckman Coulter, Inc, CA, USA)
cell equipped with CICERO 8 parameter high speed sorting electronics.

*Statistical Analysis*

Cell proliferation was measured in Gr-SYF and Gr-F1 cultures at 6, 12 and 24 h of
incubation. Experiments were repeated 3 times and absorbance of each sample was read
twice. Data are expressed as fold increase over 6 h of incubation ± standard error of
the mean (SEM), with 6 h = 1. Data were analyzed by a one-way analysis of variance,
followed by Tukey’s Honestly Significant Differences test. P < 0.05 was considered
statistically significant.

*Results*

*Integrity of DNA During Culture*

The Gr-SYF and Gr-F1 cells cultured in plates had no DNA fragmentation (no
band present) at any of the times of culture (Fig. 1a and 1b). The Gr-SYF cells
cultured in suspension and the theca cell explants had evident DNA fragmentation at 12
h and at 6 and 12 h of incubation, respectively (Fig. 1c and 1d).

*Cell Proliferation*

Proliferation of Gr-SYF and Gr-F1 cells in plate cultures was determined at 6, 12
and 24 h (Fig. 2a and 2b, respectively). Proliferation, expressed as fold increase with
6 h = 1.0, was significantly higher (p < 0.0027) in Gr-SYF at 12 and 24 h (1.27 ± 0.07
and 1.53 ± 0.13, respectively) whereas it was not different in Gr-F1 cells at any time of
culture.

*Integrity of Plasma Membrane and DNA Stainability*

Flow cytometry was used to identify changes in DNA stainability and cell
permeability to the fluorescent dye Hoechst 33342 in Gr-SYF cells cultured in a plate
for 24 h and in suspension for 12 h, compared to fresh granulosa cells. Normal cells
(fresh granulosa cells) and Gr-SYF cells cultured in a plate for 24 h had lower Hoechst
33342 fluorescence than Gr-SYF cells cultured in suspension for 12 h (Fig. 3a, 3b and
Fig. 1. Integrity of DNA in (a) granulosa cells from the largest preovulatory follicle (F1) at 0 and 24 h of culture; (b) granulosa cells from small yellow follicles (Gr-SYF) cultured in plate for 0, 6, 12 and 24 h; (c) granulosa cells from 6 mm follicles incubated in suspension culture for 0 and 12 h and (d) theca cell explants cultured for 0, 6 and 12 h. DNA fragmentation was observed in suspension cultures at 12 h of incubation and theca cell explant cultures at 6 and 12 h of incubation. Experiments were repeated 3 times. Arrows indicate DNA fragments.

Discussion

Our novel finding is the identification of culture conditions in which granulosa cells from SYF remain viable in vitro for at least 24 h. In the plate culture, the Gr-SYF cells did not show any signs of DNA fragmentation up to 24 h of culture and cell proliferation increased with time of culture. Also, membrane permeability and DNA stainability were unchanged in Gr-SYF cultured in a plate compared to fresh cells.

Oligonucleosome formation, considered the hallmark of apoptosis, results in a distinct ladder-like pattern of DNA fragmentation. Furthermore, apoptotic cells exhibit increased plasma membrane permeability to certain fluorescent dyes (Darzynkiewics et al., 1992; Dive et al., 1992). Short term exposure of cells to low concentrations of Hoeschst 33342 results in more intense labeling of apoptotic cells. Theca layer explants were used as controls for DNA fragmentation as we had previous-
Fig. 2. Cell proliferation of granulosa cells at different times of culture expressed as fold increase of absorbance, with 6 h = 1.0 ± SEM. Proliferation of granulosa cells from small yellow follicles (a) and from the largest preovulatory follicle (F1) (b) cultured in plate for 6, 12 and 24 h. *P < 0.05.

Fig. 3. Membrane integrity and DNA stainability of (a) fresh granulosa cells from small yellow follicles and (b) granulosa cells from small yellow follicles cultured on a plate or (c) in suspension. Apoptotic cells exhibit increased plasma membrane permeability and DNA stainability to Hoechst 33342.

ly observed that they undergo apoptosis in short term culture. The fact that Gr-SYF cells in suspension cultures showed strong DNA fragmentation and increased stainability with Hoechst 33342 are unequivocal signs of apoptosis. However, when the same cells were cultured in a plate, cells proliferated and showed no signs of apoptosis for 24 h, suggesting that the conditions at which these cells are cultured
determine their fate in vitro. When cells from most solid tissues are dispersed and cultured, they grow as adherent monolayers, and attach to a substrate before they start proliferating. Dispersion of tissue with collagenase digests some of the extracellular matrix. Once in culture, cells must resynthesize the proteins of the matrix before they attach or cells can be provided with a matrix-coated substrate (Freshney, 1994). Suspension cultures are used for cells that can survive and proliferate without attachment. This ability is confined to hemopoietic cells, transformed cell lines and cells from certain malignant tumors (Freshney, 1994). Cells in the granulosa layer are surrounded by a matrix (glycoproteins, proteoglycans, collagen), which may explain why granulosa cells in in vitro culture must attach to a solid matrix and secrete a matrix for normal development and proliferation.

Suspension cultures in earlier publications and in our studies were performed in the absence of carbon dioxide (CO$_2$). Carbon dioxide is necessary to lower the pH, so the effect of elevated CO$_2$ tension is counterbalanced by increasing bicarbonate concentration. The inclusion of Hepes stabilizes the pH. However, CO$_2$ is still necessary, as the absence of CO$_2$ in the air eliminates dissolved CO$_2$ from the medium, which limits cell growth (Freshney, 1994). The addition of insulin (a growth factor that regulates glucose uptake and oxidation), transferrin (required as a carrier for iron and may have a mitogenic role) and sodium selenite (an antioxidant) have been proven to improve viability of cultured cells (Buck and Schomberg, 1987; Leist et al., 1996; Helmy et al., 2000).

In conclusion, conditions in which granulosa cells from SYF proliferate and remain viable (i.e. do not undergo apoptosis) in vitro for at least 24 h were identified. Suspension cultures in the absence of CO$_2$ and basal growth factor supplements proved to be inadequate to support growth of this cell type beyond 6 h of culture.

References


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