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In Vitro Production of Estradiol by Bovine Granulosa Cells: Evaluation of Culture Condition, Stage of Follicular Development, and Location of Cells Within Follicles[1]

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ABSTRACT

In vitro estradiol (E_2) production by bovine granulosa cells was evaluated under several culture conditions, which included the presence or absence of fetal bovine serum (FBS; 2.5 and 10%), serum substitutes (1% Nutridoma [Boehringer-Mannheim, Indianapolis, IN], 2% UltroSer G [IBF Biotechnics, Villenue-la Garenne, France]), selenium (Se; 10 ng/ml), lipoprotein (0.25% Excyte/ml), O_2 concentration (5 and 20%), and two attachment factors (Pronectin F and PepTite-2000). Dulbecco's Modified Eagle's medium:Ham's F-12 medium (1:1 mixture) containing 1 μM androstenedione, 1 μg/ml insulin, and 0.1% BSA was the basal medium evaluated. The optimum conditions determined were the basal medium in 5% O_2. These conditions were then used to ascertain whether or not E_2 production by granulosa cells varied with respect to location of cells within a follicle. Follicular fluid was aspirated and centrifuged to obtain granulosa cells expected to be primarily luminal and cumulus cells. Follicles were then bisected, and remaining mural granulosa cells were removed by scraping the follicle wall with a fine plastic loop. Aspirated granulosa cells secreted more ($p < 0.01$) E_2 than scraped cells. Production of E_2 during Days 0 to 2 of culture by aspirated (0.15 ± 0.05 ng/μg DNA) and scraped (0.02 ± 0.01 ng/μg DNA) granulosa cells from small follicles (< 8 mm) was less than that by aspirated (6.30 ± 2.20 ng/μg DNA) and scraped cells (1.90 ± 1.00 ng/μg DNA) from large follicles (≥ 8 mm). During Days 2 to 4 of culture when compared to Days 0 to 2, E_2 production increased for aspirated (but not scraped) granulosa cells from small follicles (0.66 ± 0.23 ng/μg DNA)). In contrast, E_2 production decreased ($p < 0.05$) over time in culture for aspirated (2.10 ± 0.50 ng/μg DNA) and scraped (0.16 ± 0.07 ng/μg DNA) granulosa cells from large follicles. Thus, granulosa cells proximal to the basement membrane may be less differentiated with regard to E_2 production than cells distal to the basement membrane. In addition, aspirated granulosa cells from small follicles appear to continue to differentiate toward E_2-producing cells during culture, a characteristic difficult to obtain with bovine granulosa cells.

INTRODUCTION

Development of ovarian follicles is regulated by endocrine, paracrine, and autocrine actions and interactions of numerous protein and non-protein factors [1—5]. Endocrine actions of gonadotropins on follicular development are well characterized. However, the mechanisms involved in intraovarian regulation of follicular development via autocrine and paracrine pathways are not well defined. Limitations in methodology and the relatively high cost of many of the proposed autocrine/paracrine factors have made in vivo studies difficult if not impossible. In vitro culture of follicular cells is an alternative system by which the actions of proposed autocrine and/or paracrine factors can be evaluated. The responses of cultured granulosa cells to various regulatory factors differ between species. In addition, treatment of animals with hormones that alter follicular development also alters the in vitro response of granulosa cells to regulatory factors (see Hutz [6] for review). Differences in granulosa cell responses to various regulatory factors would be expected because of differences in follicular development associated with species variation in estrous cycle length and ovulation rates or because of alterations in follicular development induced by hormonal treatment of animals. However, findings from in vitro studies are meaningful only if gonadal cells maintain their in vivo biochemical characteristics during culture.

Aromatase activity, a key biochemical function of granulosa cells, is maintained during culture of granulosa cells from rats [7, 8], primates [9—11], and swine [12, 13] but not cows [14—19]. Therefore, development of an in vitro culture system that will sustain estradiol (E_2) production by bovine granulosa cells will facilitate studies evaluating possible paracrine and autocrine pathways involved in regulating follicular development of a monovulatory, long-estrous-cycle animal.

Serum and serum substitutes, oxygen tension and antioxidants, lipoprotein supplements, and attachment factors alter the capacity of granulosa cells to produce steroids in vitro. For example, addition of serum to culture medium enhances or suppresses granulosa cell function, depending on the duration of culture and the species [9, 15, 16, 20—22]. High O_2 concentration (i.e., ~20%) adversely affects cytochrome P450 enzymes involved in steroidogenesis [23—25], but the effects can be reduced or prevented by inclusion of antioxidants in culture medium [23, 24]. Supplementation with either low density (LDL) or high density (HDL) lipoprotein enhances steroidogenesis [22, 26, 27]. While research concerning the effects of attachment factors on granulosa cell function is limited, positive results have been obtained in cultures containing fibronectin [11, 18].

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[1]Mention of names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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The biochemical capacity of granulosa cells may differ among areas within a follicle. Mural granulosa cells from rodents contain greater levels of cytochrome P450 side-chain cleavage ($P450_{scc}$) [28], cytochrome P450 aromatase ($P450_{arom}$) [29], and hCG-binding capacity [30, 31] but express less insulin-like growth factor (IGF)-1 mRNA [32] than luminal or cumulus granulosa cells. Likewise, mural granulosa cells from porcine follicles secrete higher levels of E_2 during culture than luminal cells from the same follicles [13]. Expression of IGF binding proteins is also compartmentalized within different layers of human and rat granulosa cells [33, 34]. The capacity for DNA synthesis by granulosa cells also differs by location within a follicle [35]. Differences in steroidogenic capacity of bovine granulosa cells from different areas within a follicle have not been evaluated. However, E_2 production by bovine granulosa cells varies with developmental status of follicles. Follicular fluid concentrations of E_2 are positively correlated with follicle size [36], and in vitro secretion of E_2 is correlated with follicular fluid concentrations of E_2 [37].

Therefore, in vitro function of granulosa cells may vary due to culture conditions, developmental status of follicles, and location of cells within a follicle. The first objective of the present research was to evaluate different culture conditions for bovine granulosa cells collected from follicles at different stage of development in an attempt to identify optimum culture conditions for E_2 production. Specific effects evaluated were those of serum, serum substitutes, selenium (Se), O_2 concentration, lipoproteins, and attachment factors on E_2 production by bovine granulosa cells collected from small (~2–7 mm) and large (≥ 8 mm) follicles. The second objective of this research was to use the optimum conditions identified in the first objective to identify potential differences in steroid production by cells within follicles. Specifically, in vitro E_2 production was compared between cells obtained from aspirated follicular fluid (expected to be luminal and cumulus cells) and cells scraped from the follicle wall (expected to be mural cells).

MATERIALS AND METHODS

Tissue Collection and Cell Culture

Ovaries were obtained from cows at random stages of the estrous cycle within 10–20 min after slaughter at the Research Center's abattoir. Ovaries were placed in ice-cold Hanks' buffered saline solution and transported to the tissue culture lab. Follicles greater than 2 to 3 mm in diameter were dissected from the ovaries and grouped into small (< 8 mm) or large (≥ 8 mm) size categories. These size categories were selected on the basis of unpublished studies in which large increases in E_2 concentrations were observed in fluid from follicles ≥ 8 mm in size as compared to follicles < 8 mm in size. Follicular fluid was aspirated with a syringe and 22-gauge hypodermic needle. In some experiments, as indicated below, the follicular fluid was centrifuged to recover aspirated granulosa cells. Aspirated cells were resuspended in culture medium (1:1 mixture of Dulbecco's Modified Eagle's medium:Ham's F-12 medium [DMEM/F-12; Gibco BRL, Gaithersburg, MD] containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B [Sigma Chemical Co., St. Louis, MO]). Cells collected from aspirated follicular fluid were expected to be primarily luminal or cumulus granulosa cells. After aspiration, follicles were bisected while immersed in a petri dish containing Hanks' buffered saline solution. Granulosa cells attached to the follicle wall were removed by scraping with a fine plastic loop. These cells were pelleted by centrifugation and resuspended in culture medium. Cells scraped from the follicle wall were expected to be primarily mural granulosa cells.

Cells were seeded at 2.5 to 5.0 × 10^5 cells per well in 48-well culture plates containing 0.5 ml culture medium per well. Seeding rates were selected on the basis of preliminary studies demonstrating that in vitro E_2 production increased linearly between 1.0 × 10^5 and 5.0 × 10^5 cells per culture well. Unless specified otherwise, the culture medium was supplemented with 1 μM androstenedione, 1 μg/ml insulin, and 0.1% BSA (fraction V; Sigma). Cells were cultured at 39°C for 2 or 4 days under 95% air with 5% CO_2 unless otherwise stated. When cultures were maintained for 4 days, medium was collected and replaced on Day 2. Media from the first and second 2-day culture periods were frozen separately and subsequently analyzed directly by RIA for E_2 and progesterone (P_4) [38] using ^{125}I-labeled steroid [13]. Media from individual experiments were analyzed in single RIAs for E_2 and P_4, and the intraassay coefficients of variation ranged from 12 to 20% for E_2 and 10 to 15% for P_4. After collection of medium at the end of a culture, 100 μl of a 0.1% SDS, 1% cholate solution was added to each well to lyse cells; the culture plate then was frozen at −20°C until the quantity of DNA in individual wells was determined [39]. Unless otherwise indicated, cells from individual cows were cultured separately and each treatment was replicated in two to four culture wells for each cell population evaluated.

Evaluation of Serum, Serum Substitutes, and Se

Scraped granulosa cells from small and large follicles obtained from ovaries of 4 to 7 cows were used to conduct four experiments comparing serum-free culture medium to medium supplemented with Se (10 ng/ml), 10% fetal bovine serum (FBS; Hyclone Labs., Logan, UT), or serum substitutes, i.e., 1% Nutridoma (Boehringer-Mannheim, Indianapolis, IN) or 2% UltroSer G (IBF Biotechnics, Villenue-la Garenne, France). Supplements were added to culture medium before plating at levels suggested by the manufacturer. Cells were cultured for 2 days; then cultures were terminated and concentrations of E_2 and P_4 in media were determined and normalized for DNA content in the individual culture wells. Concentrations of E_2 and P_4 in the

FBS used in this and subsequent experiments were 9 and 190 pg/ml of undiluted FBS, respectively. Data were analyzed by a statistical analysis system (SAS) general linear analysis of variance procedure that included cow, follicle size, type of culture medium, and the interaction of follicle size and culture medium in the model. Analysis of E_2 concentrations was performed on $\log_{10}$-transformed data due to heterogeneous variances among cultures of granulosa cells from large and small follicles.

Evaluation of O_2 Concentration and Lipoprotein Supplement

The effects of O_2 concentration and lipoprotein supplement on in vitro steroid production were evaluated in a mixture of scraped and aspirated granulosa cells from small and large follicles. Cells were pooled from 2 to 4 cows on four different slaughter dates. Granulosa cells were cultured for 4 days under either 5% (90% nitrogen:5% CO_2) or 20% (95% air:5% CO_2) O_2 in the presence or absence of bovine lipoprotein supplement (Excyte, Pentex, Miles Inc., Kankakee, IL) that consists primarily of HDL. Lipoprotein treatment (0.25% [v/v]; equivalent to 26 μg cholesterol/ml medium) was added to the medium before plating, and culture medium was replaced on Day 2. An SAS general linear model procedure that included replicate (slaughter date), follicle size (large vs. small), culture duration (Days 0 to 2 vs. Days 2 to 4), lipoprotein (presence vs. absence), and O_2 (5% vs. 20%) was used to analyze DNA, E_2, and P_4. In the preliminary analyses, all possible interactions of follicle size, culture duration, lipoprotein, and O_2 were included in the model. Only the interaction of follicle size by culture duration was significant in the analyses of E_2 and P_4 data; therefore this was the only interaction included in the final analyses. An SAS least-squares means procedure was utilized for comparison of multiple means when significant ($p < 0.05$) differences were obtained in the general linear model.

Evaluation of Cell Attachment Factors and 2.5% FBS

Scraped granulosa cells from small (≤ 5 mm), medium (> 5 to < 8 mm), and large (≥ 8 mm) follicles obtained from 3 cows were cultured in serum-free medium in the presence or absence of one of two cellular attachment factors or in medium containing 2.5% FBS. The attachment factors evaluated were Pronectin F (Protein Polymer Technologies, Inc., San Diego, CA) and PepTite-2000 (Gibco BRL, Gaithersburg, MD). Both of these products contain the RGD (Arg-Gly-Asp) adhesion sequence that promotes cellular attachment via integrin binding [40, 41]. Pronectin F was dissolved in the manufacturer's diluent to a concentration of 1 mg/ml as directed by the manufacturer. This solution was further diluted in PBS to a concentration of 20 μg/ml immediately before use. PepTite-2000 was dissolved in dimethylsulfoxide to a concentration of 1 mg/ml and was further diluted with PBS to a final concentration of 10 μg/ml before use. Culture plates were treated with 100 μl attachment factor per well for 2 h at room temperature. The attachment factor solution was then removed before addition of culture medium and plating with cells. Medium was replaced after 1 day, and cultures were terminated after a total of 3 days. Concentrations of the Pronectin F (2 μg/well) and PepTite-2000 (1 μg/well) used to treat plates were selected on the basis of dose-response studies performed with porcine granulosa cells (J.J. Ford, unpublished observations). Data were analyzed as described for the previous experiment using a model that included cow, follicle size (small, medium, and large), culture duration (Days 0 to 1 vs. Days 1 to 3), culture treatment (serum free, serum free + Pronectin, serum free + PepTite-2000, or 2.5% FBS), and the significant interactions of these main effects.

Evaluation of Intrafollicular Populations of Granulosa Cells

Granulosa cells from small (< 8 mm) and large (≥ 8 mm) follicles from individual cows (n = 4) or from pools derived from 2 to 4 cows (four different pools) were used to determine whether or not aspirated granulosa cells differed from cells scraped from the follicle wall. Cells were cultured for 4 days in serum-free culture medium under 5% O_2. Culture medium was collected and replaced after 2 days of culture. Data for concentrations of E_2, P_4, and DNA were analyzed by an SAS general linear model procedure that included replicate (n = 8), granulosa cell type (aspirated cells from large follicles, scraped cells from large follicles, aspirated cells from small follicles, or scraped cells from small follicles), culture duration (Days 0 to 2 vs. Days 2 to 4), and the interaction of granulosa cell type by culture duration. An SAS least-squares means procedure was utilized for comparison of multiple means when significant ($p < 0.05$) differences were obtained in the general linear model. E_2 and P_4 data were transformed ($\log_{10}$) before analysis due to heterogeneous variances among the different cell populations.

RESULTS

Evaluation of Serum, Serum Substitutes, and Se

Data from cells cultured in the absence or presence of 10% FBS revealed an interaction between follicle size and culture medium (Fig. 1). Including 10% FBS in the culture medium had no effect on E_2, P_4, or DNA when granulosa cells from small follicles were evaluated. In contrast, concentrations of DNA per culture well were lower ($p < 0.05$) and E_2 and P_4 were increased ($p < 0.05$) when expressed on a ng/μg DNA basis for granulosa cells from large follicles cultured in medium containing FBS. Because 10% FBS had opposite effects on DNA and steroid production, statistical analyses were also performed on E_2 and P_4 data ex-

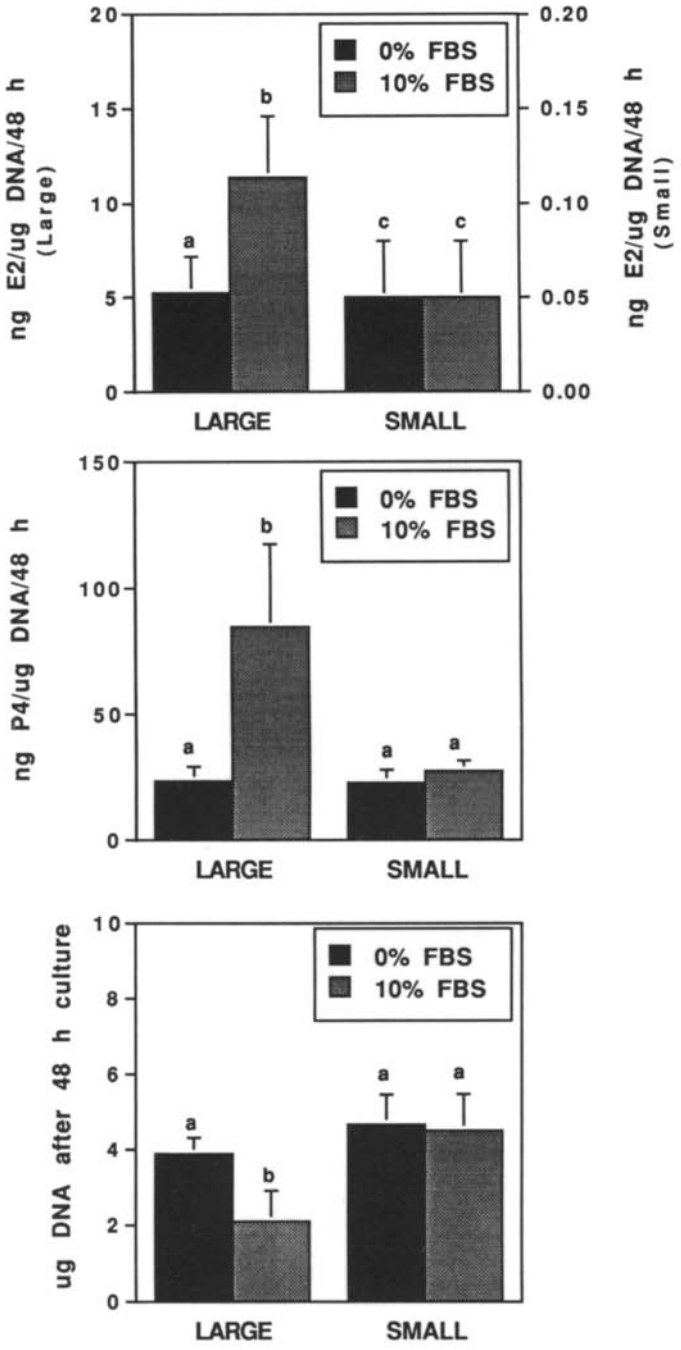

FIG. 1. Granulosa cell production of E_2 (top panel; note that two y-axes are used) and P_4 (middle panel) and cellular DNA (bottom panel) after 2 days of culture in DMEM/F12 in the absence (0%) and presence (10%) of FBS. Values represent mean ± SE for granulosa cells scraped from small (2 to < 8 mm) and large (≥ 8 mm) follicles obtained from four cows. Bars with different superscripts differ ($p < 0.05$) from each other.

pressed on the basis of ng/ml conditioned medium. The effects of FBS on E_2 and P_4 and the interaction between FBS and follicle size were not significant in the latter analyses.

Including the serum substitute Nutridoma in culture medium decreased ($p < 0.05$) DNA but had no effect ($p > 0.1$) on E_2 or P_4 production regardless of whether cells were from small or large follicles. Mean (± SE) concentrations of DNA pooled across follicle sizes ($p = 0.4$ for small vs. large) were 2.14 ± 0.15 and 2.81 ± 0.15 μg DNA/well for cells cultured with and without Nutridoma, respectively. The serum substitute UltroSer G did not affect ($p > 0.1$) DNA but increased ($p < 0.05$) E_2 and P_4. However, further investigations revealed that UltroSer G contained high concentrations of E_2 (~12 ng E_2/ml unconditioned medium containing 2% UltroSer G). Determination of the source of E_2 in these cultures was not further pursued. P_4 was not detected in unconditioned medium containing 2% UltroSer G. Mean (± SE) concentrations of P_4 pooled across follicle sizes ($p = 0.6$ for small vs. large) were 41.2 ± 6.5 and 17.0 ± 6.5 for cells cultured in the presence or absence of UltroSer G.

In a preliminary evaluation of the effects of antioxidants on granulosa cell function during in vitro culture, granulosa cells were cultured in the presence or absence of Se. Including Se at a concentration of 10 ng/ml medium had no effect ($p > 0.1$) on DNA, E_2, or P_4 (data not shown). It was concluded from these experiments that the culture supplements evaluated were not beneficial when used at the levels tested because 10% FBS and 1% Nutridoma were associated with decreased concentrations of DNA remaining in wells after 2 days of culture; UltroSer G contained high levels of E_2, thereby complicating evaluation of E_2 production by granulosa cells; and Se (10 ng/ml) did not enhance granulosa cell E_2 production.

As data for serum-free cultures in Figure 1 illustrate, size of follicle from which granulosa cells were derived affected E_2 production but had no effect on DNA or P_4. To further evaluate the effect of follicle size on E_2 secretion, E_2 data for the control cultures (i.e., nonsupplemented, serum-free medium) from the experiments with 10% FBS, serum supplements, and Se were combined and analyzed. Results from this analysis demonstrated that granulosa cells from large follicles produced more ($p < 0.01$) E_2 (6.04 ± 1.91) than granulosa cells from small follicles (0.08 ± 0.02 ng/μg DNA).

Evaluation of O_2 Concentration and Lipoprotein Supplement

Evaluation of the effects of O_2 concentration and lipoprotein supplement on granulosa cell function provided evidence that O_2 concentrations altered E_2 production (Fig. 2), but supplementing with lipoprotein was without effect (data not shown). Cells cultured in 5% O_2 secreted more E_2 ($p < 0.01$) than cells cultured in 20% O_2. As in previous experiments, granulosa cells from large follicles produced more E_2 ($p < 0.05$) than cells from small follicles. However, an interaction between follicle size and culture period existed. Concentrations of E_2 decreased ($p < 0.05$) with increased time in culture for cells from large follicles, whereas E_2 concentrations increased ($p < 0.05$) over time in culture for granulosa cells from small follicles.

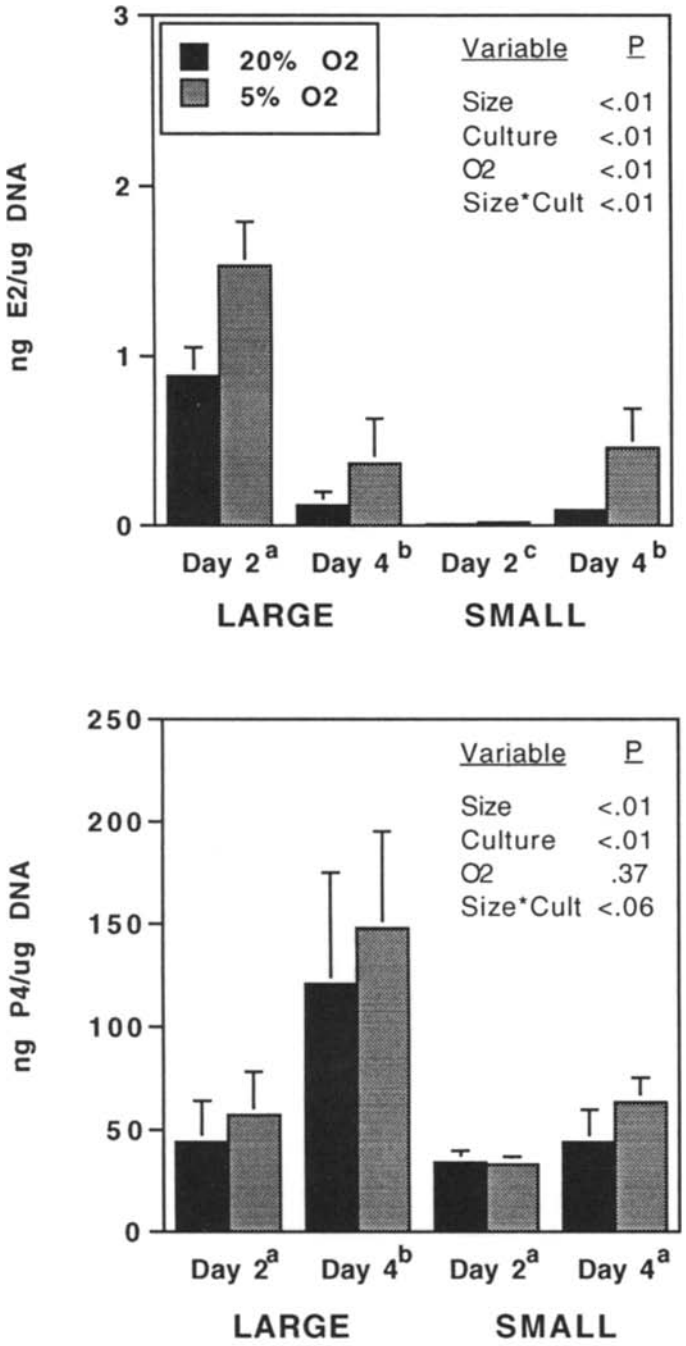

FIG. 2. Effect of O_2 concentration, follicle size, and culture duration on granulosa cell secretion of E_2 (top panel) and P_4 (bottom panel). Granulosa cells aspirated from small (~2 to < 8 mm) and large (≥ 8 mm) follicles were recombined with cells scraped from the same follicles and cultured for 2 or 4 days under 5 or 20% O_2. Data are expressed as the mean ± SE of four replications. Probabilities from the analysis of variance tables are shown within each panel. Size = follicle size; Culture = culture period; O_2 = oxygen concentration. [a-c]Denote differences observed in the interaction of follicle size by culture duration (Size * Cult).

O_2 concentration and lipoprotein supplement did not significantly alter DNA or P_4. Concentrations of DNA after the second 2-day culture period (mean ± SE = 3.1 ± 0.24 μg/culture well for cells from small and large follicles) were approximately 37% lower ($p < 0.01$) than after the first 2-day period (4.9 ± 0.28 μg DNA). In contrast to DNA, concentrations of P_4 were greater after the second 2-day culture period ($p < 0.01$) than after the first 2-day period. The magnitude of the increase in P_4 production was larger for cells from large follicles than for cells from small follicles ($p < 0.06$ for the interaction of follicle size by culture duration; see lower panel of Fig. 2).

Evaluation of Cell Attachment Factors and 2.5% FBS

Pretreatment of culture plates with the attachment factor Pronectin F or PepTite-2000 did not ($p > 0.2$) alter concentrations of E_2, P_4, or DNA (Fig. 3). Culture of cells in 2.5% FBS decreased ($p < 0.05$) concentrations of DNA measured at the end of the 3-day culture period compared to those in cells cultured in serum-free medium. This decrease in DNA resulted in a tendency ($p < 0.07$) for higher levels of E_2 to be observed in cultures containing 2.5% FBS when E_2 data were expressed on a ng/μg DNA basis (Fig. 3). However, no differences were observed when E_2 data were expressed on the basis of ng/ml medium. Concentrations of P_4 were not influenced by the addition of 2.5% FBS to culture medium, whether analyzed on a ng/μg DNA or a ng/ml medium basis.

As in the previous experiment, secretion of E_2 and P_4 was affected ($p < 0.05$) by size of the follicle from which cells were obtained (Fig. 3). In this experiment, small follicles were further subdivided into medium (> 5 to < 8 mm) and small (≤ 5 mm) categories. Granulosa cells from large follicles secreted an order of magnitude more ($p < 0.01$) E_2 than cells from medium follicles, which in turn secreted more ($p < 0.05$) E_2 than cells from small follicles. Granulosa cells from medium follicles secreted less ($p < 0.05$) P_4 than cells from large follicles. P_4 secretion by cells from small follicles was intermediate between, and did not differ from, secretion by cells from medium and large follicles.

Concentrations of E_2 declined ($p < 0.01$) with increased duration of culture while concentrations of P_4 increased ($p < 0.01$) over time (Fig. 3), whether data were expressed as ng steroid/ml medium or ng steroid/μg DNA. In addition, magnitude of the decline in E_2 production differed among granulosa cells from the different sizes of follicles ($p = 0.06$ for interaction of follicle size by duration of culture; see Fig. 3). This interaction differs from that observed in the previous experiment, where E_2 production by granulosa cells from large follicles decreased over time but production by granulosa cells from small follicles increased over time (see Fig. 2). In the previous experiment, granulosa cells scraped from the follicle wall were recombined with aspirated granulosa cells, whereas in this experiment, only cells scraped from follicle walls were used.

Evaluation of Intrafollicular Granulosa Cell Populations

A comparison of steroid production by granulosa cells obtained from follicular fluid (aspirated cells) and cells

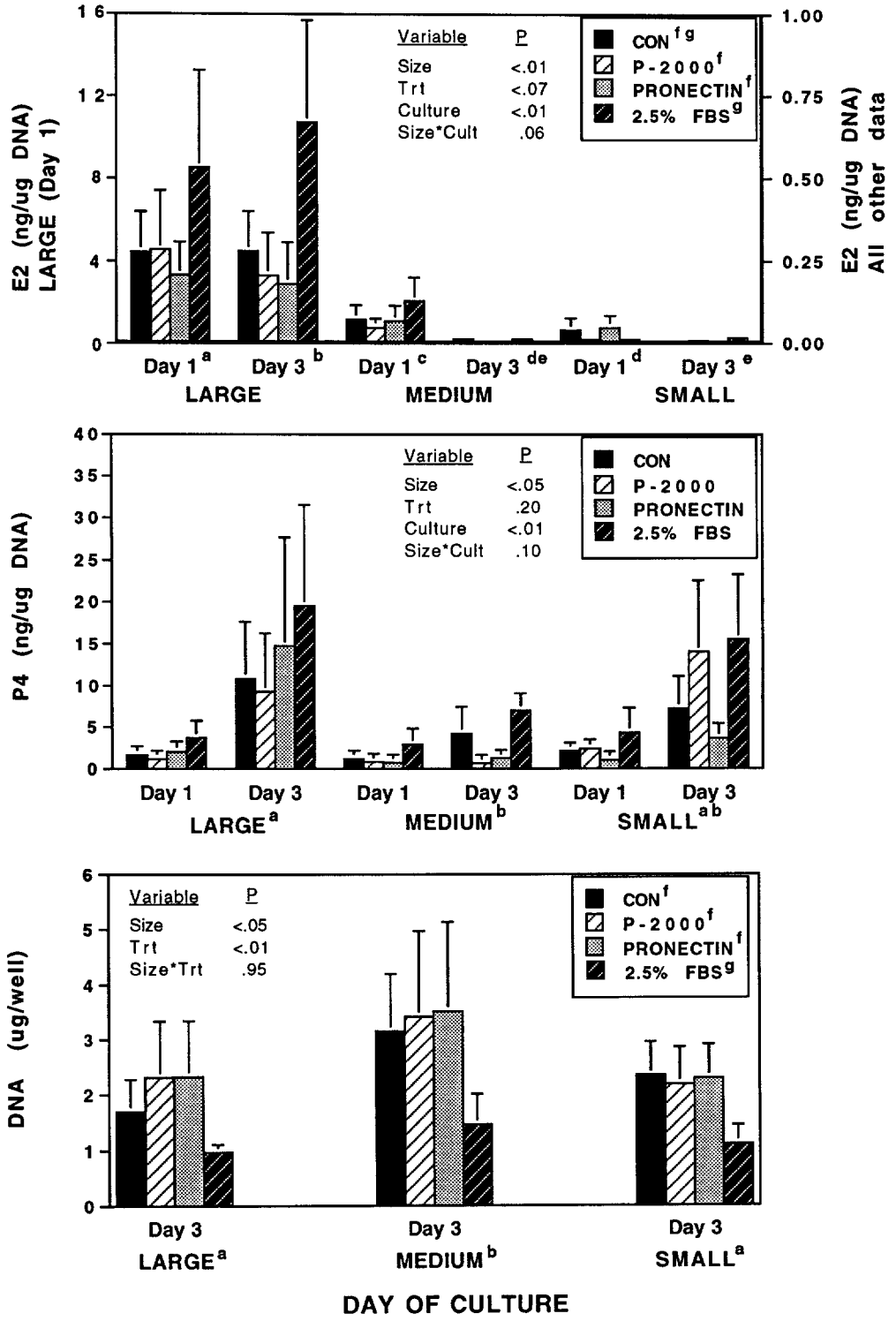

FIG. 3. Concentrations of E_2 (top panel; note that two y-axes are used), P_4 (middle panel), and DNA (bottom panel) in serum-free cultures (CON); serum-free cultures pretreated with one of two attachment factors, Peptite-2000 (P-2000) or Pronectin; or cultures containing 2.5% FBS. Granulosa cells were scraped from large (≥ 8 mm), medium (> 5 to < 8 mm), or small (2 to ≤ 5 mm) follicles and cultured for 3 days. Medium was collected after 1 (Day 1) and 3 (Day 3) days of culture. Probabilities from analysis of variance tables are shown in each panel. Size = follicle size; Trt = effect of medium and attachment factors; Culture = culture period. [a–e]Denote differences in the interaction of size by culture interaction (top panel) or differences between follicle sizes (middle and bottom panels). [f,g]Denote Trt differences ($p < 0.05$) for DNA and for E_2 data expressed on a ng/μg DNA basis. Differences were not observed when E_2 data were expressed on the basis of ng/ml medium. Values represent mean ± SE of cultures performed in triplicate for cells collected from three different cows.

scraped from follicle walls indicated that these populations differed (Fig. 4). Aspirated cells secreted more ($p < 0.01$) E_2 than scraped cells when compared within follicle size and culture period. In addition, an interaction between source of granulosa cell and culture period existed ($p < 0.01$). During the first 2 days of culture, granulosa cells from large follicles produced more E_2 ($p < 0.01$) than cells from small follicles. Secretion of E_2 by aspirated and scraped granulosa cells from large follicles decreased ($p < 0.01$) over time in culture (6.3 ± 2.2 vs. 2.1 ± 0.5 and 1.9 ± 1.0 vs. 0.16 ± 0.1 ng/μg DNA for aspirated and scraped cells on Day 2 vs. Day 4, respectively). In contrast, E_2 secretion by aspirated granulosa cells from small follicles increased ($p < 0.05$) over time in culture (0.15 ± 0.05 vs. 0.66 ± 0.23 ng/μg DNA for Day 2 vs. Day 4, respectively). Concentrations of E_2 in cultures of scraped granulosa cells from small follicles were lower ($p < 0.05$) than in all other cultures and did not change over time in culture (0.02 ± 0.01 vs. 0.01 ± 0.01 ng/μg DNA for Day 2 vs. Day 4, respectively).

Production of P_4 varied by granulosa cell type when evaluated over both culture periods (Fig. 4). Aspirated cells from small (mean ± SE over both culture periods = 46 ± 13 ng/μg DNA) and large (69 ± 28) follicles secreted greater ($p < 0.05$) amounts of P_4 than granulosa cells scraped from small (12 ± 3) and large (36 ± 22) follicles. Concentrations of DNA per culture well decreased ($p < 0.05$) by approximately 34% between Days 2 and 4 of culture for all the cell populations evaluated. Mean (± SE) concentrations of DNA for aspirated cells from small (4.11 ± 0.99) and large (2.03 ± 0.49) follicles and for scraped cells from small (3.15 ± 0.63) and large (2.70 ± 0.69) follicles did not differ ($p > 0.2$) after 4 days of culture.

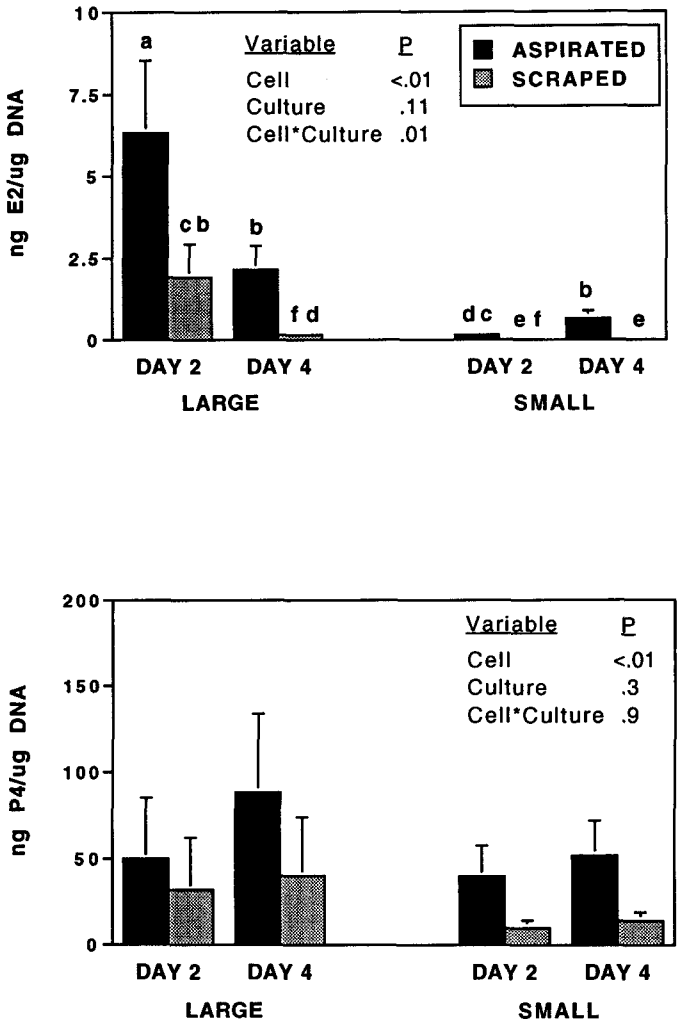

FIG. 4. Concentrations of E_2 (top panel) and P_4 (bottom panel) in serum-free medium collected on Day 2 or Day 4 of culture in 5% O_2. Granulosa cells from small (~2 to < 8 mm) and large (≥ 8 mm) follicles were obtained from aspirated follicular fluid (ASPIRATED) or were scraped from the follicle wall (SCRAPED). Probabilities from the analysis of variance procedures are shown in each panel. Cell = effect of source of cells; Culture = culture period. Data are expressed as the mean ± SE of cultures performed in triplicate from eight different replications. $^{a-f}$Bars with different superscripts were different from each other when data were transformed (log_{10}). P_4 production over the 4-day culture period by aspirated cells from large and small follicles did not differ but was greater than production by cells scraped from large and small follicles.

DISCUSSION

These experiments provide evidence that in vitro production of E_2 by bovine granulosa cells is significantly influenced by location of cells within the follicle, size of follicle from which cells originated, duration of culture, and incubator concentration of O_2. Results in Figure 4 illustrate that aspirated granulosa cells produce from twofold to several orders of magnitude more E_2 than scraped granulosa cells. This difference occurred in the absence of any differences in DNA concentrations, indicating that differences in E_2 production are not likely to have been due to differences in viability. Thus, luminal and/or cumulus granulosa cells appear to be more differentiated with respect to steroidogenic capacity than mural granulosa cells. This observation is the opposite of results in rats [28—31] and pigs [13] where mural granulosa cells were more differentiated with respect to steroidogenesis than luminal granulosa cells.

In all of the present experiments, granulosa cells from large follicles (≥ 8 mm) produced more E_2 than cells from small (2–7 mm) follicles. It is likely that apparent differences in E_2 production between cells from small and large follicles would have been even greater in magnitude if only cells from large estrogen-active follicles had been evaluated (i.e., in some instances, more than one large follicle was collected per cow). Subclassification of small-sized follicles into medium (> 5 to < 8 mm) and small (≤ 5 mm) categories also resulted in a positive association between follicle size and in vitro E_2 production. These findings support previous observations that in vitro granulosa cell steroidogenesis changes with developmental status of the follicle [19, 37].

An exception to the observation that granulosa cells from large follicles secreted more E_2 than cells from small fol-

licles occurred during Days 2 to 4 of culture in granulosa cells either partly (mixed; Fig. 2) or entirely (Fig. 4) derived from aspirated follicular fluid. In this exception, E_2 secretion was not different between cells from small and large follicles; rather, secretion of E_2 by cells from large follicles decreased with duration of culture, whereas E_2 secretion by mixed or aspirated cells from small follicles increased with duration of culture. Thus, granulosa cells aspirated from small bovine follicles continued to acquire an increased capacity to secrete E_2 during culture in a fashion similar to that observed during in vivo follicular development. In contrast, other populations of granulosa cells studied (i.e., scraped and aspirated cells from large follicles) appear to lose the capacity for E_2 production while the capacity for P_4 production is either maintained or increased. Data shown in Figure 3 and results from previous studies [14—17] demonstrate that the decline in E_2 production occurs primarily during the first day of culture. Thus, bovine granulosa cells aspirated from small follicles appear to be unique among the populations of granulosa cells studied in that they do not undergo changes in cellular differentiation similar to those that occur during luteinization (i.e., loss of aromatase activity and enhanced P_4 production) when cultured for 3 to 4 days [14—17]. This unique property of these cells makes them attractive candidates for in vitro experiments designed to evaluate the mechanisms involved in regulating bovine granulosa cell estrogen biosynthesis.

Of the numerous culture conditions evaluated in the present experiments, the only factor that appeared to have a consistent positive influence on granulosa cell production of E_2 was O_2 concentration. Culture of granulosa cells in 5% O_2 resulted in an increase in E_2 concentrations after 2 or 4 days of culture compared to concentrations in cells cultured in 20% O_2 (95% air:5% CO_2). This observation is novel, as in all studies referenced in the present paper, bovine granulosa cells were cultured in ~20% O_2 (i.e., 95% air:5% CO_2). Porcine granulosa cells cultured in 5% O_2 produced more E_2 and P_4 than cells cultured in 20% O_2 [42]. These results for porcine cells differ from the current findings in that P_4 was not altered when bovine granulosa cells were cultured in 5% O_2. While the mechanism by which reduced O_2 increased E_2 concentrations was not determined in the present experiments, previous research has demonstrated that cytochrome $P450_{17\alpha}$ hydroxylase/lyase in mouse Leydig cells [24] and cytochrome $P450_{11\beta}$ hydroxylase in bovine adrenocortical cells [23] are adversely affected by high (19%) O_2 concentrations. Thus, elevated O_2 tension may also be detrimental to $P450_{arom}$. While Se, which is known to act as an antioxidant, was found to be without effect during a 2-day culture period, additional studies evaluating various concentrations of this and other antioxidants for longer-term cultures may be warranted.

The decreases in DNA concentrations observed for granulosa cells cultured in FBS provide evidence that FBS may decrease cellular attachment or viability. The decreased DNA concentrations in the presence of FBS resulted in an apparent increase in E_2 production by granulosa cells cultured in FBS when data were normalized to DNA concentrations at the end of culture. However, E_2 concentrations expressed on the basis of ng/ml culture medium were not different between serum-free conditioned medium and conditioned medium containing FBS. An explanation of these results may be that less cells remained attached to the culture wells after removal of culture media. Alternatively, cell viability might have been reduced by FBS while E_2 secretion by the remaining viable cells was increased. In previous studies, 10% FBS increased E_2 production by bovine granulosa cells during Days 2 to 7 of culture and concentrations of DNA were higher at the end of cultures [16]. Skinner and Osteen [15] found that aromatase activity (expressed on a μg DNA basis) in 24-h cultures of granulosa cells was also increased by 10% FBS when compared to the activity in serum-free cultures. However, aromatase activity in cells cultured in serum-free medium containing FSH (100 ng/ml) and insulin (5 μg/ml) was similar to that of cells cultured in 10% FBS. While the reasons for the inconsistencies between studies are unclear, some of the discrepancy may be explained by supplemental factors such as insulin, FSH, and other substances that are present or absent in the serum-free media used by different researchers. In addition, variation in the composition of different sources and lots of FBS may also contribute to differences among studies. It is the ambiguous nature of FBS that has prompted researchers to develop serum-free systems. As with FBS, the inclusion of commercially available serum substitutes (Nutridoma and UltroSer G) in culture medium did not result in beneficial effects on bovine granulosa cell E_2 production.

Addition of a commercially available preparation of bovine lipoprotein (Excyte) did not enhance granulosa cell steroid production or viability in the present study. This same lipoprotein enhanced proliferation of bovine granulosa cells in response to heparin-binding growth factor [22], and both HDL and LDL enhance P_4 production in subcultured bovine granulosa cells [26]. Recently, Bao and coworkers [43] demonstrated that HDL but not LDL stimulated P_4 production by primary cultures of bovine granulosa cells from large follicles, whereas both HDL and LDL increased cell viability. The concentration of HDL used in the study of Bao and coworkers [43] was about twofold greater than that used in the present study. As bovine follicular fluid contains HDL [26] and previous studies demonstrated either beneficial effects on P_4 production and cell viability or, as in the present study, no effect, further evaluation of lipoprotein effects in serum-free culture condition may be warranted.

Pretreatment of culture wells with commercial preparations containing the RGD cellular attachment sequences did not result in any beneficial effects (Fig. 3). Saumande [18] reported that precoating culture wells with fibronectin, which also contains RGD integrin-binding sequences [40],

increased cell viability and E_2 production while P_4 production was decreased. Whether the differences between studies are due to the source or level of attachment factor or other factors remains to be determined. As granulosa cells secrete fibronectin [44], endogenous production of fibronectin may also influence the results of studies in which the effects of supplemental attachment factors are evaluated.

It was concluded from the present experiments that several factors influence steroid production by bovine granulosa cells during in vitro culture. Most important are the observations that E_2 production is dependent on location of the cells within the follicle and the developmental status of the follicle. In addition, the results indicate that reduced O_2 concentrations may also favor E_2 production. It is also important to consider the implications of the interactions between medium supplements and origin of granulosa cells and the interaction between granulosa cell origin and duration of culture when a bovine granulosa cell culture system is evaluated. Thus, while FBS, serum substitutes, attachment factors, Se, and lipoprotein were not beneficial in the present experiments, interpretation of these results requires consideration of the origin of granulosa cells (i.e., location within follicle and stage of follicular development), duration of culture, O_2 concentration, the presence or absence of additional factors in the particular serum-free medium used for the experiments, and the concentration of the culture additive evaluated. Results from these experiments are currently being used to continue to evaluate factors of potential importance for maintaining and stimulating E_2 production by bovine granulosa cells obtained from follicles at different stages of follicular development from individual animals.

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