Modulation of intrafollicular oestradiol in explanted hamster follicles does not affect oocyte meiotic status

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Summary. Mature antral follicles were collected from PMSG-primed hamsters before the LH surge on Day 4 of the oestrous cycle and were incubated individually for 6 or 10 h under 95% O₂ and 5% CO₂. The relationship between follicle size and follicular fluid volume was established, and both the oestradiol output during incubation and the LH sensitivity of the follicle with respect to induction of meiotic resumption were determined. The data obtained from these initial studies were then applied to experiments in which the oestrogenic status of follicles was altered by pressure injection of solutions into the antral cavity to result in known concentrations in follicular fluid of oestradiol (0–500 µM), the anti-oestrogens, tamoxifen (0–200 µM) or enclomiphene citrate (0–500 µM), or an oestradiol-specific antiserum (1:200 dilution of serum). The proportion of immature oocytes (% GV) was determined cytogenetically after follicular incubation.

Significant correlations were established between follicle size and both follicular fluid volume ($r = 0.90$) and follicular oestradiol accumulation ($r = 0.96$). The minimum concentration of LH tested which induced maximal meiotic resumption (0% GV) was 0.5 µg/ml. The % GV was not significantly reduced by intrafollicular injection of either of the anti-oestrogens over the concentration ranges tested, or of the oestradiol-specific antiserum, which was injected in an amount adequate to bind all oestradiol accumulated during the incubation. Intrafollicular injection of oestradiol failed to increase the % GV after follicular incubation in the presence of 0.5 µg LH/ml. From these results we conclude that it is unlikely that oestradiol plays a primary role in the maintenance of meiotic arrest in follicle-enclosed hamster oocytes *in vitro*.

Keywords: hamster; follicles; oestradiol; meiosis

Introduction

The mechanisms which maintain the mammalian oocyte in meiotic arrest before the preovulatory surge of luteinizing hormone (LH) remain unknown (reviewed by Eppig & Downs, 1984; Thibault *et al.*, 1987). However, since meiosis resumes after removal of the oocyte from its follicular environment (Pincus & Enzmann, 1935), the general consensus is that arrest is due to a follicular arrester, the effect of which is somehow overridden upon exposure of the follicle to LH (reviewed by McGaughey, 1983). It has been proposed that either the intraoocyte level of arrester drops below the threshold required to maintain meiotic arrest (Dekel & Beers, 1978, 1980) or the action of arrester is bypassed by an alternative pathway which mediates the gonadotrophin-induced resumption of meiosis (Eppig & Downs, 1987). The nature of the arrester has not been established, although cyclic adenosine monophosphate (cAMP) has been shown to have an arresting function in oocytes of all species so far studied (reviewed by Racowsky, 1983). Nevertheless, results reported from several laboratories suggest that another follicular 'factor', in addition to cAMP, is also involved in the maintenance of meiotic arrest (reviewed by Racowsky, 1985a).

Recently, the possibility has been investigated that oestradiol may be the 'factor' (reviewed by Kaji *et al.*, 1987). A direct arresting action of oestradiol on the spontaneous maturation of cumulus-free oocytes of several species has been demonstrated (cow: Robertson & Baker, 1969; mouse: Nekola & Smith, 1974; Eppig & Koide, 1978; pig: Richter & McGaughey, 1979; Racowsky & McGaughey, 1982; hamster: Racowsky, 1985b), and oestradiol was found to induce a dose-dependent increase in the proportion of immature cumulus-enclosed hamster oocytes cultured in the presence of a sub-arresting level of the adenylate cyclase stimulator, forskolin (Racowsky, 1985b). Furthermore, testosterone has been shown to potentiate both dibutyryl cAMP-dependent arrest in mouse oocytes (Schultz *et al.*, 1983; Kaji *et al.*, 1987) and FSH-dependent arrest in pig oocytes (Racowsky, 1983). Since granulosa cells aromatize testosterone when stimulated by cAMP (Erickson & Ryan, 1975; Anderson *et al.*, 1979), it has been suggested that oestradiol might mediate these androgenic effects (Rice & McGaughey, 1981). In support of such an arresting function for oestradiol, the steroidogenic content of follicular fluid changes after the preovulatory surge of gonadotrophin such that the ratio of oestradiol:progesterone decreases to almost one-tenth in the pig (Gérard *et al.*, 1979; Ainsworth *et al.*, 1980), and by more than 95% in the hamster (Hubbard & Greenwald, 1982).

Taken together, these various results suggest that oestradiol may play a role in modulating cAMP-dependent meiotic arrest in mammalian oocytes. However, in all of the above studies, the oocyte was removed from its follicular environment and the effects of the various compounds on spontaneous maturation were determined. While such liberated oocytes undoubtedly provide useful models for investigating mammalian oocyte maturation, their application is somewhat limited by virtue of the oocyte being removed from the follicular environment. In addition, although the developmental ability of spontaneously matured mouse oocytes is normal (Schroeder & Eppig, 1984), there is evidence that sheep oocytes which mature in follicles exposed to LH *in vitro* differ physiologically from liberated oocytes which spontaneously mature in culture (Moor *et al.*, 1981; Moor & Osborn, 1983). In the present study, therefore, we have investigated the effect on meiotic status of modulating the oestrogenic content of follicular fluid in explanted hamster follicles. Two basic approaches were used for this study. In the first approach, follicles were incubated in the absence of LH and the effective concentration of follicular oestradiol was reduced either by the presence in follicular fluid of an oestradiol-specific antiserum or by an oestrogen antagonist. In the second approach, follicles were incubated in the presence of LH and follicular oestradiol was increased by the introduction of oestradiol into the follicles. A pressure-injection system has been used for these experiments in which a known volume of solution can be injected into the antrum.

Materials and Methods

Collection of follicles. Antral follicles were collected from mature golden Syrian hamsters (*Mesocricetus auratus*) that were maintained in a 14-h light:10-h dark (lights on at 05:00 h) cycle with food and water provided *ad libitum*. The day of oestrus (Day 1) was determined by the presence of the characteristic vaginal discharge. The pro-oestrous gonadotrophin surge occurred on Day 4. The animals were maintained for at least 2 consecutive cycles before use. Follicles were collected between 09:00 and 11:00 h on Day 4, before the gonadotrophin surge which begins at 14:00 h (Hubbard, 1983); all hamsters had been injected previously on Day 1 between 09:00 and 11:00 h with 30 i.u. PMSG (Sigma, St Louis, MO, USA) as described by Fleming & Yanagimachi (1980). The animals were asphyxiated with carbon dioxide, and the ovaries were removed immediately and placed in a sterile dish (Falcon Plastics; No. 1008) containing BMOC-3 medium (Brinster, 1971). Follicles were carefully dissected from the ovaries, cleaned free of connective tissue in BMOC-3 medium using two 1-ml tuberculin syringes with 26-gauge needles, and measured using an ocular micrometer at $\times 50$ magnification. When the follicle was not spherical, the length of the long axis was determined. Follicles within the size range 0.82–1.10 mm inclusive were used. Generally 2 hamsters were killed for each experiment and each experiment in any series was repeated at least 4 times. On average, 20 follicles were used from each hamster.

Incubation of follicles. The follicles were incubated separately in Starstedt tubes (No. 55.484; Starstedt, Princeton, NJ, USA) in 0.2 ml medium under an atmosphere of 95% O₂ and 5% CO₂ in a shaking water bath (Precision

Scientific Co.; 120 oscillations/min) at 37°C. Except as otherwise noted, follicles were incubated for 6 h, the start of incubation being from the time of an intrafollicular injection. The incubations were maintained in Medium 199 with Hank's salts and 25 mM-Hepes buffer (Gibco, NY, USA) supplemented with 4.5% BSA (Fraction V, Sigma), 1 mM-L-glutamine, 2.5 mM-lactate, 0.03 mM-sodium pyruvate, 12.5 mU insulin/ml and 50 µg gentamicin/ml. This medium was designated TCM-BSA. Stock solutions of insulin, sodium pyruvate and gentamicin were prepared in Medium 199, divided into appropriate aliquants and stored frozen at -20°C. A stock solution of LH (NIAMDD-oLH-24) in 10% BSA was prepared immediately before the first experiment of a series in which LH was required and was stored at 4°C for a maximum of 2 weeks. All media were sterilized through a 0.45 µm Nalgene filter (Nalgene Corp., Rochester, NY, USA) before use.

Determination of follicular fluid volume with respect to follicle diameter. To be assured that injection of a known volume of stock solution resulted in the required intrafollicular concentration of solution, it was necessary to know the volume of follicular fluid in a given follicle. Therefore, a calibration curve for outside diameter of follicles against follicular fluid volume was generated. Twenty-five follicles collected from 3 hamsters between 09:00 and 10:30 h on Day 4 and >0.5 mm but <1.2 mm in size were cleaned and fixed in 2.5% (w/v) glutaraldehyde in 0.2 M-cacodylate buffer, pH 7.3, using the method for egg fixation as previously described by McGaughey (1978), and were subsequently dehydrated and embedded in epoxy resin. Several sections (2 µm) of each follicle were prepared in the region of maximum follicular outside diameter and were subsequently mounted on a microscope slide and stained with Wright's stain. The section in which the follicle was of maximum outside diameter was then mapped out on squared paper. The approximate volume occupied by the antral cavity was calculated by assigning the geometric shape which best fitted the perimeter of the cavity and by subtracting the average volume occupied by a hamster oocyte and the attached cumulus stalk (determined to be 0.006 µl).

Determination of oestradiol output by follicles. It was necessary to establish the oestradiol output by follicles into the follicular fluid and into the spent medium to estimate the dilution of antiserum required to bind all of the oestradiol secreted by the follicles during incubation. Therefore, after incubation, a measured volume (100 µl) of spent medium was removed, the follicles were rinsed 3 times in fresh medium, the outside follicular diameter was measured and the follicles were then carefully opened to release the follicular fluid. After the follicular shells had been rinsed, and the rinse combined with that fluid, the fluid was centrifuged at 2000 g in a 200-µl microcap (Biolab Inc., Lake Havasu City, AZ, USA) to remove any contaminating cells. Samples were stored at -20°C until assayed. The oestradiol content of samples was measured by radioimmunoassay after chromatographic separation of extracted steroids on celite microcolumns as described by Wingfield & Farner (1975). The oestradiol antibody was purchased from Endocrine Sciences (Tarzana, CA, USA) and the assay had a sensitivity down to 2.0 pg. The within- and between-assay coefficients of variation, derived from 5 assays were, respectively, 7.2% and 8.0%.

Titration assays to determine the dilution of oestradiol antiserum required to bind follicular oestradiol. Various dilutions of filtered (0.45 µm Millipore) oestradiol antiserum (No. 244-anti-E₂-6-BSA) were incubated for 6 h at 37°C with 1700 pg radiolabelled oestradiol, this being the mass equivalent to the greatest mass of oestradiol present in the spent medium and follicular fluid for any one follicle. Incubations were undertaken with 100 µl 0.1 M-phosphate buffer, pH 6.9, containing 0.1% gelatin. After incubation, unbound radioligand was absorbed by the addition of 200 µl dextran-coated charcoal, and 20 min later charcoal-bound radioligand was separated from antibody-bound radioligand by centrifugation. Radioactivity in the supernatant, indicative of antibody binding, was measured by liquid scintillation spectrometry (Beckman Model No. LS 7000).

Preparation of solutions for intrafollicular injections. Stock solutions of oestradiol (Sigma) and the anti-oestrogens, tamoxifen (ICI 46,474; Sigma) and enclomiphene citrate (MDL 16,289F; Merrell Dow Pharmaceuticals Inc., Cincinnati, OH, USA), were prepared on the morning of an experiment in ethanol-cleaned vials in 95% ethanol containing 0.1% methylene blue (Aldrich Chemical Co., Milwaukee, TN, USA). Oestradiol antiserum (No. 244 anti-E₂-6-BSA) was obtained from Dr G. D. Niswender and control normal rabbit serum was obtained from a sexually immature rabbit in our university animal care facility. The full-strength sera were filtered through 0.45 µm Millipore filters, stored frozen in 20 µl portions and thawed as needed on the morning of an experiment. Each serum was diluted 1:4 with an aqueous solution of methylene blue to result in a final concentration of 0.1% methylene blue in the injection solution. Except as noted otherwise, fresh methylene blue solutions were prepared for each experiment as the dye may degrade to toxic by-products upon storage (Conn, 1961). To reduce the likelihood of micropipettes clogging during injection, the dye solutions were filtered immediately before use with a glass-fibre filter (GFA, Whatman) or a 0.45 µm Millipore filter, for the ethanolic or aqueous solutions, respectively. The dye was used as a marker to confirm that solution had been successfully injected into the antrum.

Pressure injection system and calibration of micropipettes. Intrafollicular injections were undertaken using a Picospritzer II valve delivery system (General Valve Corp., Fairfield, NJ, USA) and calibrated glass micropipettes under a stereomicroscope. The micropipettes were made from glass tubing of 1.0 mm o.d. with a filament (World Precision Instruments, Inc., New Haven, CT, USA) using a Narishige Type PN-3 glass microelectrode puller. Calibration of the micropipettes was accomplished by pressure-ejecting solutions (aqueous or 95% ethanolic) at a constant pressure (60 lbf/in²: 414 kPa) of N₂ and various pulse durations (10–320 msec). Each droplet of solution was ejected into a mineral oil drop on a coverslip square (11 mm²). The volumes of solution ejected from the tip were measured in one of two ways. In one, the amount of radioactivity present in an ejected drop was determined by liquid scintillation spectrometry as previously described (Sakai *et al.*, 1979). Stock solutions of [5,6-³H]uridine (aqueous

solution) and 1,2,6,7- $^3\text{H}(\text{N})$ progesterone (95% ethanolic solution) were used and, for each micropipette, duplicate samples were collected for each of the following pulse durations: 10, 20, 40, 80, 160 and 320 msec. In the other, the diameters of ejected droplets were measured using an ocular reticule after samples were collected for pulse durations identical to those indicated above. A linear relationship was established between the volume of solution ejected and the duration of pulse used (^3H)uridine solution: $y = 0.04x + 0.163$, $r = 0.99$; ^3H progesterone solution: $y = 0.027x + 0.883$, $r = 0.99$; 8 and 10 micropipettes used, respectively, where y = the drop volume and x = the pulse duration) and measured droplet volumes were highly correlated with droplet volumes calculated from radioactive determinations. All micropipettes were hand-trimmed and pre-calibrated before an experiment such that their ejection characteristics were consistent with those used to establish the initial calibration lines. After calibration, residual solution was ejected and the micropipettes were allowed to dry overnight.

Intrafollicular injection of solutions. Each solution was injected into the antrum to result in a 0.02 dilution of the follicular fluid. After a follicle was measured, its fluid volume was determined from the size vs volume calibration line (see Fig. 1). The required pulse duration to eject a volume of solution 0.02 that of the follicular fluid volume was then established from the micropipette calibration line. The solution was injected in the following manner. The follicle was held in position on a suction pipette such that the egg was above and to the left of the region of follicle wall to be penetrated with the micropipette. A filled micropipette was then placed in the micropipette holder and a drop of solution of the required volume was ejected under mineral oil onto the surface of a coverslip square to ensure patency of the micropipette. Immediately before follicular injection the follicle, attached to the suction pipette, was removed into a dry dish and any surplus culture medium was carefully dabbed off the follicle with a tissue. This step was essential to eliminate the possibility of culture medium rising into the micropipette by capillarity, thereby diluting the solution to be injected. Successful injection of solution into the antrum was assured by visualization of methylene blue dye in the antrum after injection of an additional drop of solution under oil on the coverslip square after follicular injection. If the pre- and post-injection drops of solution were not of comparable size, the intrafollicular injection was considered unsuccessful and the follicle was discarded. After successful injection, the follicle was immediately rinsed in TCM-BSA and placed in the appropriate tube for incubation.

Treatment of follicles after incubation. After incubation, each follicle was removed into a labelled watch glass containing 2% sodium citrate and was carefully opened to release the egg which was subsequently prepared for cytogenetic analysis (McGaughey & Polge, 1971).

Statistical analyses. The cytogenetic data were analysed by Duncan's multiple range test using the SPSS computer program (Duncan, 1955; Nie *et al.*, 1975; McGaughey, 1978) after transformation to angles as previously described (McGaughey, 1978). Significant differences amongst experimental and control groups were approximated at $\alpha = 0.05$. Group means and their 95% confidence intervals were transformed back to percentages for tabulation.

The best-fit curve of the follicle size:follicular fluid volume data was established using least squares analysis and linear regression. This analysis was undertaken using the Curfit Program of Warne (1980) run on a Franklin 600 computer.

The steroid data were analysed by linear regression analysis or by Student's paired t test, with $P < 0.05$ considered statistically significant.

Results

Series 1: determination of follicular fluid volumes

A linear relationship was established between follicle diameter and follicular fluid volume (Fig. 1). This relationship was described by the equation $y = 0.126x - 0.197$, where y = the volume of follicular fluid and x = the follicle diameter; these parameters were highly correlated ($r = 0.90$).

Series 2: determination of oestradiol output by follicles

The oestradiol content of follicular fluid was very low (6.0 ± 2.1 pg/follicle) in follicles < 0.42 mm in diameter. While fluid from follicles 0.64 mm in diameter contained a significantly increased amount of oestradiol ($P < 0.001$, Student's paired t test), there was no further increase in fluid content of oestradiol as follicle diameter increased (Fig. 2). In contrast, there was a significant size-related increase in the amount of oestradiol accumulated into the incubation medium over the size of follicle examined ($y = 1603.8x - 723.4$; $r = 0.96$; where y = oestradiol content in spent medium, x = follicle diameter and r = correlation coefficient). During the 6-h incubation, the maximum amount of oestradiol accumulated by any follicle was 1700 pg.

Fig. 1. The relationship between follicle diameter and follicular fluid volume in hamster antral follicles. Follicles were collected on Day 4 of the oestrous cycle, were processed for electron microscopy, and fluid volumes were determined as described in the 'Materials and Methods'.

Fig. 2. Oestradiol output by incubated hamster follicles. Follicles were collected on Day 4 of the oestrous cycle, were incubated for 6 h in TCM-BSA and the oestradiol contents of the follicular fluid (□) and the spent media (▨) were determined for each follicle by RIA. The numbers in parentheses indicate the numbers of follicles used to obtain each histogram (mean $\pm$ s.e.m.).

Series 3: effects of intrafollicular injection of anti-oestrogens

Table 1 shows the effect of intrafollicular injection to tamoxifen to result in an antral fluid concentration of the drug in the range of 0–200 μM . Over the concentration range tested, tamoxifen failed to cause a significant dose-dependent reduction in the proportion of immature (GV-stage) oocytes. Furthermore, there was no significant difference between the proportions of GV-stage oocytes within follicles containing the highest concentration of tamoxifen tested (200 μM) and the control follicles which had not been injected.

Table 1. The effect of intrafollicular injection of tamoxifen on meiotic status of hamster oocytes in explanted follicles

Tamoxifen (μM)	No. of follicles	% of oocytes at GV stage*
0	40	78.2 $\pm$ 4.5 ^a (65.8–88.4)
25	37	78.8 $\pm$ 7.8 ^a (58.9–93.3)
50	36	74.7 $\pm$ 12.3 ^a (42.3–96.4)
100	35	81.7 $\pm$ 6.7 ^a (65.2–93.7)
200	38	81.2 $\pm$ 4.4 ^a (69.9–90.3)
Non-injected	36	89.6 $\pm$ 3.5 ^a (80.7–96.0)

*Mean $\pm$ s.e.m. (with 95% confidence limits) for 7 replicates.

Groups with the same superscript are not significantly different from each other, $\alpha = 0.05$.

Injection of follicles with enclomiphene citrate, another oestrogen antagonist, also failed to affect oocyte meiotic status during the 6-h incubation (Table 2).

Table 2. The effect of intrafollicular injection of enclomiphene citrate on meiotic status of hamster oocytes in explanted follicles

Enclomiphene citrate (μM)	No. of follicles	% of oocytes at GV stage*
0	24	87.1 $\pm$ 3.6 ^a (73.8–96.2)
0.5	23	86.4 $\pm$ 4.0 ^a (71.5–96.3)
5.0	24	87.1 $\pm$ 3.6 ^a (73.8–96.2)
50.0	24	90.5 $\pm$ 4.1 ^a (76.1–98.7)
500.0	24	79.5 $\pm$ 4.8 ^a (65.8–90.5)
Non-injected	24	90.5 $\pm$ 4.1 ^a (76.1–98.7)

*Mean $\pm$ s.e.m. (with 95% confidence limits) for 4 replicates.

Groups with the same superscript are not significantly different from each other, $\alpha = 0.05$.

An additional experimental series was conducted to examine the possibility that 6 h was an insufficient duration for these oestradiol-antagonists to manifest their effects. After 10-h incubation, however, neither of the drugs significantly reduced the proportion of immature oocytes below the control level (0 μM antagonist: 81.8 $\pm$ 10.7% GV; 200 μM -tamoxifen: 80.0 $\pm$ 11.7% GV; 500 μM -enclomiphene citrate: 77.3 $\pm$ 10.9% GV).

To determine whether tamoxifen or clomiphene exerted toxic effects upon the follicle which interfered with induction of meiotic resumption, injected follicles were incubated for 6 h and subsequently transferred to medium containing LH (0.5 $\mu\text{g/ml}$) for an additional 18 h. As shown in

Table 3. The lack of follicular toxicity induced by tamoxifen or enclomiphene citrate on LH-induced germinal vesicle breakdown in follicle-enclosed hamster oocytes

Treatment	No. of follicles	% of oocytes at GV stage*
None	19	25.0 $\pm$ 8.8 ^a (18.0-45.6)
200 μ M-tamoxifen	16	11.1 $\pm$ 7.8 ^a (12.0-34.7)
500 μ M-clomiphene	19	20.0 $\pm$ 8.6 ^a (14.9-41.3)

*Mean $\pm$ s.e.m. (with 95% confidence limits).

Groups with the same superscript are not significantly different from each other, $\alpha = 0.05$.

Table 3, compared with vehicle, neither oestrogen antagonist significantly reduced the proportion of oocytes which resumed meiosis in response to gonadotrophic stimulation.

Series 4: effect of intrafollicular injection of oestradiol antiserum

This series of experiments was undertaken to determine whether binding of oestradiol in follicular fluid with an oestradiol-specific antiserum might induce oocyte meiotic resumption in the absence of LH. A 1:4 dilution of antiserum was used for the injections since we had found previously that the resultant 1:200 dilution of antiserum in follicular fluid effectively bound the total maximum amount of oestradiol secreted by any follicle (1700 pg) during incubation. The injection micropipettes were successfully tip-filled with the diluted antiserum by capillary action. However, in the majority of experiments, a partitioning occurred in the micropipette tip such that there was a region of clear solution at the tip, followed by solution with a blue colouration typical of that observed when methylene blue was used. This partitioning was observed in both the antiserum and the control normal rabbit serum samples; for follicular injections, both solutions were used and follicles were grouped according to whether clear or blue solution was injected into the serum. The data show that there was no significant difference in the proportion of GV-stage oocytes among follicles that were injected either with control or oestradiol-specific antiserum or with the clear or blue solutions (Table 4). Furthermore, the proportion of immature oocytes

Table 4. The effect of intrafollicular injection of oestradiol antiserum on meiotic status of hamster oocytes in explanted follicles

Solution injected	No. of follicles	No. of replicates	% of oocytes at GV stage*
None	69	6	73.4 $\pm$ 4.4 ^a (58.7-85.9)
Oestradiol antiserum, blue	67	8	72.8 $\pm$ 9.1 ^a (50.9-90.0)
Oestradiol antiserum, clear	53	9	68.8 $\pm$ 9.2 ^a (50.3-84.7)
Control serum, blue	35	6	76.6 $\pm$ 6.6 ^a (53.6-93.3)
Control serum, clear	32	6	71.7 $\pm$ 7.1 ^a (52.1-87.8)

*Mean $\pm$ s.e.m. (with 95% confidence limits).

Groups with the same superscript are not significantly different from each other, $\alpha = 0.05$.

recovered from all injected follicles was not significantly different from that recovered from the non-injected group (75.3 ± 4.9 vs $73.4 \pm 4.4\%$ GV, respectively, $P > 0.05$, Student's paired t test). Incubation of injected follicles for an additional 4 h (total incubation time = 10 h) did not significantly reduce the proportion of GV-stage oocytes in either the control serum or antiserum-injected follicles (83.3 ± 9.8 and $65.4 \pm 12.3\%$ GV, respectively) over the respective value observed after 6-h incubation.

To determine whether the antiserum inferred toxicity on the follicle which interfered with induction of meiotic resumption, injected follicles were transferred after 6-h incubation to medium containing LH ($0.5 \mu\text{g/ml}$) for an additional 18 h. For these experiments, we were forced to use serum solutions prepared 48 h previously, due to a limited supply of oestradiol antiserum. Compared with the non-injected follicles, significantly more oocytes remained at the GV stage in those follicles injected with control serum or antiserum, although there was no significant difference in response between the two injected groups (Table 5).

Table 5. The effect of follicular injections of serum upon LH-induced germinal vesicle breakdown in follicle-enclosed hamster oocytes

Treatment	No. of follicles	% of oocytes at GV stage*
Non-injected	18	0 ^a (—)
Oestradiol antiserum	22	31.8 ± 8.0^b (21.9–49.0)
Control serum	21	27.3 ± 8.3^b (20.2–46.3)

*Mean $\pm$ s.e.m. (with 95% confidence limits) of 9 (non-injected) and 11 (each serum group) replicates. Groups with the same superscript are not significantly different from each other, $\alpha = 0.05$.

Series 5: effect of intrafollicular injection of oestradiol in the presence of LH

The first experiments in this series were undertaken to establish a concentration of LH which maximally induced meiotic resumption in cultured follicle-enclosed oocytes. Excised follicles were incubated for 6 h in TCM-BSA containing various concentrations of LH (0 – $0.5 \mu\text{g/ml}$). Figure 3 shows that LH at $0.5 \mu\text{g/ml}$ induced meiotic resumption in 100% of oocytes. The possibility was then tested that elevating the concentration of oestradiol in follicular fluid might overcome this action of LH to induce meiotic resumption. Oestradiol was injected to result in an antral fluid concentration in the range 0 – $500 \mu\text{M}$ and at no concentration did oestradiol significantly increase the proportion of oocytes which remained in meiotic arrest after incubation for 6 h with $0.5 \mu\text{g LH/ml}$ (Table 6).

Discussion

This study has examined the effect on oocyte meiotic status of modulating intrafollicular oestradiol in explanted hamster follicles. The results show that neither decreasing the effective follicular fluid concentration of oestradiol with an oestradiol-specific antiserum or an oestrogen-antagonist, nor increasing the follicular fluid concentration of oestradiol in the presence of LH, had any significant effect on oocyte meiotic status. These results therefore suggest that oestradiol is not of primary importance in the maintenance of meiotic arrest in the follicle-enclosed hamster oocyte.

To conduct this study, three aspects of follicular physiology had to be characterized: the relationship between follicle size and follicular fluid volume, the oestradiol output of the incubated

Fig. 3. The induction of meiotic resumption in follicle-enclosed hamster oocytes by LH. Follicles were collected on Day 4 of the oestrous cycle and were incubated for 6 h in TCM-BSA containing various concentrations of LH. Each histogram represents the mean $\pm$ s.e.m. of 6 replicates.

Table 6. The effect of intrafollicular injection of oestradiol on meiotic status of hamster oocytes in explanted follicles incubated in the presence of 0.5 μ g LH/ml

Oestradiol (μ M)	No. of follicles	% of oocytes at GV stage*
0	29	4.3 $\pm$ 0.2 ^a (3.9–4.8)
0.5	23	6.1 $\pm$ 1.7 ^a (2.8–10.3)
5.0	27	4.7 $\pm$ 0.4 ^a (3.7–5.9)
50.0	26	5.1 $\pm$ 0.9 ^a (3.2–7.3)
500.0	29	4.3 $\pm$ 0.2 ^a (3.9–4.8)
Non-injected	27	6.1 $\pm$ 0.5 ^a (3.7–5.9)

*Mean $\pm$ s.e.m. (with 95% confidence limits) for 5 replicates.

Groups with the same superscript are not significantly different from each other, $\alpha = 0.05$.

hamster follicles in the absence of gonadotrophin, and the LH sensitivity of the follicles with respect to induction of meiotic resumption.

The size range of the antral follicles used in these experiments (0.65–1.05 mm) is consistent with that of large healthy Graafian follicles collected on Day 4 of the hamster oestrous cycle as observed by Greenwald (1962) and Roy & Greenwald (1985). While the calculated follicular fluid volumes may be slightly underestimated due to tissue shrinkage during fixation, nevertheless a linear relationship was established between follicle diameter and follicular fluid volume (Fig. 1). This observation indicates that follicular growth in preovulatory follicles is accompanied both by an increase in the number of layers of granulosa cells (Roy & Greenwald, 1985) and by a proportionate increase in fluid accumulation.

Analysis of the follicular oestradiol output during the 6-h incubation period revealed a size related increase in oestradiol accumulation into the spent medium over the range of follicles examined (Fig. 2). This observation is consistent with previous findings that (1) more oestradiol was detected in media in which granulosa cells from large hamster Graafian follicles were incubated than in spent medium of granulosa cells from medium-sized follicles (Makris & Ryan, 1975); and (2) the oestradiol content of media in which excised hamster follicles were incubated increased commensurate with the day of the oestrous cycle upon which follicles were collected (Hubbard &

Greenwald, 1982). The amount of oestradiol output into spent media observed in this study is similar to that observed previously for short-term incubations of hamster follicles (Wang & Greenwald, 1984).

Consistent with the well-documented stimulatory action of LH upon meiotic resumption of follicle-enclosed oocytes of small laboratory mammals (reviewed by Tsafri, 1979), the proportion of maturing hamster oocytes in this study was dependent upon the concentration of LH in the incubation medium. As shown in Fig. 3, 100% of oocytes exhibited GVBD when the follicles were exposed to 0.5 µg LH/ml. However, in the absence of LH in the incubation medium, about 20% of follicle-enclosed oocytes had, nevertheless, undergone GVBD after incubation for 6 h (Fig. 3). The basis for this observation is unknown but it seems unlikely that it was due to perturbation of cellular metabolism by the high O₂ tension used since the structural integrity of such incubated follicles looked entirely normal as judged by both thin-section and freeze-fracture electron microscopy (unpublished observation). The possibility exists that prostaglandins (PGs) may have been involved since PG synthesis was probably stimulated during follicular collection as sensitized tissues secrete PGs (reviewed by Salmon & Flower, 1979), and PGE-2 has been found to stimulate meiotic resumption in follicle-enclosed rat oocytes (Lieberman *et al.*, 1976). A similar LH sensitivity of follicle-enclosed hamster oocytes, with a comparable basal level of meiotic resumption in the control group that was incubated in the absence of LH, was observed by Hubbard (1986).

Intrafollicular injections of either of the anti-oestrogens, tamoxifen or enclomiphene citrate, failed to stimulate meiotic resumption in the follicle-enclosed hamster oocytes during incubation for 6 h (Tables 1 and 2, respectively). In light of the previous observation that GVBD in explanted hamster follicles was induced by 1 µg FSH/ml only after 6-h incubation (Hubbard, 1986), the possibility existed that the kinetics of maturation in response to the anti-oestrogens might be attenuated. However, incubation of injected follicles for an additional 4 h failed to reveal an enhancement of meiotic resumption over that seen at 6 h. Lack of oocyte response was not due to a toxic effect of either drug upon GVBD since the proportion of immature oocytes after follicular exposure to LH for 18 h was similar to the anti-oestrogen and control groups (Table 3).

From the above results, we conclude that oestradiol antagonists are unable to modulate the meiotic status of the follicle-enclosed oocyte, and it is therefore unlikely that oestradiol plays a primary role in the maintenance of meiotic arrest. This conclusion is further supported by the finding that intrafollicular injection of oestradiol-specific antiserum failed to affect significantly the proportion of maturing oocytes compared with that observed in the corresponding control group, although both sera reduced the maturational response compared with the non-injected group (Table 4). The most likely explanation for the latter observation is a toxic influence of methylene blue by-products produced upon storage of the injection solutions.

Taken together, the present observations contrast with our previous study with liberated, cumulus-enclosed hamster oocytes in which tamoxifen exerted a dose-dependent releasing action on forskolin-maintained meiotic arrest and reversed the arresting action of oestradiol in the presence of a low concentration of forskolin; these anti-oestrogenic effects were accompanied by a corresponding reduction in intra-oocyte cAMP (Racowsky, 1985b). These previous findings are consistent with an established role for cAMP in the regulation of oocyte meiotic maturation (reviewed by Thibault *et al.*, 1987), and with the possibility that cAMP-dependent meiotic arrest may result from inhibition of oocyte phosphodiesterase activity by one of several steroids, including oestradiol (Kaji *et al.*, 1987). The lack of modulation by both the oestradiol antagonists and the oestradiol-specific antiserum in the present study may relate to the substantially increased number of cAMP-producing granulosa cells in the follicle as compared with the liberated, cumulus-enclosed oocyte. In the former, the level of intra-oocyte cAMP is undoubtedly regulated by transfer of cAMP from the membrana-cumulus granulosa syncytium via the extensive gap-junctional network which interconnects the somatic cells and the innermost layer of cumulus cells to the oocyte (reviewed by Racowsky *et al.*, 1989). In the latter, the amount of intra-oocyte cAMP is modulated by the level of cAMP within the cumulus oophorus (Racowsky, 1985a). In the physiological system of

the intact follicle, therefore, oestradiol may play a supplementary, rather than primary, role in the regulation of intra-oocyte cAMP, and thus in the maintenance of meiotic arrest. This conclusion is further supported both by the present findings that intrafollicular injection of oestradiol failed to override gonadotrophic stimulation of meiotic resumption (Table 6), and by the previous findings that (1) addition to culture medium of either oestradiol or the aromatase inhibitor, 4-hydroxy-androstenedione, failed to affect markedly the proportion of immature follicle-enclosed sheep (Moor *et al.*, 1980) or rat (Billig *et al.*, 1983) oocytes, respectively, after incubation in the presence or gonadotrophin; and (2) the proportion of maturing oocytes in the perfused rabbit ovary was not significantly affected by cyanoketone, in the presence or absence of oestradiol in the medium (Yoshimura *et al.*, 1987).

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