Effect of follicular components on meiotic arrest and resumption in horse oocytes

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Two experiments were conducted to evaluate the effect of follicular components on the maintenance of meiotic arrest in horse oocytes. In Expt 1, oocytes were incubated for 24 h with follicular fluid, or with granulosa cells suspended either in medium or in follicular fluid at 25×10^6 cells ml^{-1} . None of the treatments resulted in significant maintenance of the germinal vesicle stage over that of non-suppressive control. Culture with follicular fluid plus granulosa cells resulted in a significantly higher proportion of oocytes at metaphase I compared with controls. In Expt 2, oocytes were divided into those originally having compact or expanded cumuli. Oocytes were cultured with sheets of mural granulosa or sections of follicle wall, or after injection into intact dissected follicles. After incubation, half of the oocytes from each suppressive treatment were matured for 24 h. All three suppressive treatments were effective in maintaining oocytes at the germinal vesicle stage (no significant difference from control oocytes fixed directly after removal from the follicle). However, no treatment maintained normal viability of oocytes, as significantly fewer oocytes were at metaphase II after all the suppression–maturation treatments compared with the maturation control. The highest rate of post-suppression maturation was found in the mural granulosa treatment. Within this treatment, the proportion of oocytes in metaphase II was significantly higher for oocytes with expanded than for oocytes with compact cumuli (31% versus 11%, respectively; $P < 0.05$). Suppression by injection into an intact follicle was associated with a lack of progression to metaphase II during subsequent maturation. These results indicate that follicular fluid alone does not suppress maturation of horse oocytes. Incubation of horse oocytes with follicle wall tissue (sheets of granulosa, follicle wall sections or intact follicles) can effectively suppress maturation but variably impairs the viability of oocytes.

Introduction

In mammalian oocytes meiosis is arrested in the dictyate stage of prophase I. Oocytes are maintained in prophase I (the germinal vesicle stage) until the signal for follicle maturation and ovulation is received, or until the follicle becomes atretic (Rajakoski, 1960; Gougeon and Testart, 1986). Oocytes that are removed from the follicle mature spontaneously, suggesting that follicular components actively suppress maturation in the follicle-enclosed oocyte (Pincus and Enzmann, 1935).

Many studies have attempted to define the meiosis-suppressive factor within the follicle. In mice, follicular fluid alone can maintain oocytes at the germinal vesicle stage, and this action has been attributed to hypoxanthine, a component of mouse follicular fluid (Eppig *et al.*, 1985; Eppig and Downs, 1987). Pig follicular fluid can suppress oocyte maturation in pigs, mice and rats (Tsafiri and Channing, 1975; Tsafiri *et al.*, 1977; Stone *et al.*, 1978). In golden Syrian hamsters, culture of oocytes with follicular fluid will transiently maintain oocytes at the germinal vesicle stage (Racowsky and Baldwin, 1989). Bovine follicular fluid has been shown to suppress meiotic

maturation in mouse oocytes (Sato and Koide, 1984), but is ineffective in suppressing maturation of bovine oocytes, or does so only transiently (Leibfried and First, 1980; Sirard and Bilodeau, 1990a). Granulosa cell monolayers have moderate ability to suppress meiosis of cattle oocytes (Sirard and Bilodeau, 1990b; Kotsuji *et al.*, 1994). This ability is potentiated by co-culture of granulosa cells with theca cells, and further by addition of FSH to the co-culture (Kotsuji *et al.*, 1994). In cattle, culture of oocytes with granulosa cells in suspension is more effective than is culture on monolayers; granulosa cells in medium at 25×10^6 cells ml^{-1} maintained 40% of oocytes in the germinal vesicle (GV) stage after 24 h, and granulosa cells in follicular fluid (10 – 25×10^6 cells ml^{-1}) maintained 70% of oocytes at the germinal vesicle stage (Sirard and Bilodeau, 1990a). Culture of bovine oocytes with follicle hemisections is also effective in maintaining oocytes at the germinal vesicle stage (Foote and Thibault, 1969; Sirard and Coenen, 1993).

Oocyte maturation, fertilization and post-fertilization development in horses are active areas of research, with possible clinical application (Hinrichs, 1994). However, progress has been slow; for example, no foals have as yet been produced from *in vitro*-matured oocytes. Suppression of meiosis is a

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valuable tool in the study of oocyte maturation, and may also increase the developmental competence of immature oocytes (cytoplasmic maturation) before nuclear maturation proceeds (Sirard *et al.*, 1992). Currently, no information is available on the maintenance of meiotic arrest in horse oocytes. This study was conducted to evaluate the effect of co-culture with follicular components on the suppression of maturation of horse oocytes *in vitro*.

Materials and Methods

General methods

Horse ovaries were collected from a local abattoir, placed in isotonic saline containing 1 mg ticarcillin ml^{-1} (SmithKline Beecham Pharmaceuticals, Philadelphia, PA), and were transported (1 h) at room temperature to the laboratory. Oocytes were collected from follicles by opening the follicle with a scalpel blade and scraping the granulosa cell layer from the follicle with a 0.5 cm bone curette (Hinrichs *et al.*, 1993a). The granulosa cells thus collected were washed into a Petri dish with M199 with Hank's salts and 12.5 mmol Hepes l^{-1} (Gibco Life Technologies, Inc., Grand Island, NY) with 0.1 mg ticarcillin ml^{-1} . The dishes were examined under a dissecting microscope at a magnification of $\times 10$ to locate the oocyte. Oocytes were categorized as having compact or expanded cumuli, or as denuded. Oocytes denuded of cumuli at the time of collection were not used in this study.

For chromatin evaluation, the oocytes were stripped of cumulus by pipetting in 1% (w/v) hyaluronidase or a 0.25% (v/v) solution of trypsin in Hank's salt solution without CaCl_2 , MgCl_2 or MgSO_4 (Gibco Life Technologies, Inc.). The denuded oocytes were fixed in buffered formol saline. Fixed oocytes were placed on a slide and covered with a drop of 3:1 glycerol:PBS containing 5 μg Hoechst 33258 ml^{-1} and 2.5 mg sodium azide ml^{-1} . The oocytes were examined using a fluorescent microscope in conjunction with a G365 nm exciter filter. The chromatin was classified into one of four stages: GV (germinal vesicle stage: oocytes having a condensed chromatin mass or a fluorescent nucleus, Hinrichs *et al.*, 1993b); MI (diakinesis to metaphase I); MII (anaphase I to metaphase II); or degenerating (chromatin spread throughout cell, abnormal chromatin or no chromatin visible).

Experiment 1

Oocytes were collected from all follicles of less than 30 mm diameter. Only compact cumulus oocytes were used in this experiment. Oocytes were assigned to one of five treatment groups as follows. (1) Direct control: oocytes were denuded and fixed within 30 min of removal from the follicle. (2) Non-suppressive control: oocytes were placed in 35 mm Petri dishes with 3 ml modified M199. (Modified M199 was composed of M199 with Earle's salts and 10% neonatal calf serum (Gibco Life Technologies, Inc.) with 25 μg gentamycin ml^{-1} and 1.25 μg Fungizone ml^{-1} (Gibco Life Technologies, Inc.)) The dishes were incubated at 38°C in a humidified atmosphere of 5% CO_2 in air for 24 h, after which the oocytes were denuded and fixed for evaluation. (3) Follicular fluid:

follicular fluid was aspirated from follicles less than 30 mm in diameter, using a 12 ml all-plastic syringe (Air-tite, Vineland, NJ) and a 2.5 cm 18 gauge needle. The follicle was then opened and the granulosa scraped as described above. Only fluid from follicles having an oocyte with a compact cumulus and having healthy granulosa cells (smooth and evenly granular, without signs of expansion) was used. Follicular fluid from two to three follicles was selected, pooled and filtered through a 0.2 μm pore millifilter (Acrodisc, Gelman, Ann Arbor, MI) to sterilize it and ensure that it was free of granulosa cells. Gentamycin was added to a final concentration of 25 μg ml^{-1} . Oocytes were placed into 3 ml of follicular fluid in a 35 mm Petri dish and incubated as described above. (4) Granulosa cells: granulosa cells scraped from follicle walls were selected from follicles less than 30 mm in diameter that contained an oocyte with a compact cumulus, and healthy granulosa cells. The granulosa cells from two to three follicles were selected, suspended in modified M199 and centrifuged for 3–4 min at 800 g. The pellet was resuspended with 0.5 ml Hank's solution without CaCl_2 , MgCl_2 or MgSO_4 and pipetted vigorously with a 1.5 mm (o.d.) Pasteur pipette to break up clumps of cells. Larger clumps of cells were removed with a pipette. The cells were recentrifuged at 800 g for 3–4 min and repipetted, then a final centrifugation was performed. Cells were then resuspended in 100 μl modified M199. The concentration of cells was calculated using a hemocytometer and the cells were resuspended to a final concentration of 25×10^6 cells ml^{-1} using modified M199. Oocytes were placed into 200 μl droplets of the final preparation under mineral oil, and incubated as described above. (5) Granulosa cells with follicular fluid: granulosa cells and follicular fluid were collected as described above. Preparation of granulosa cells and culture were performed as for treatment with granulosa cells alone except that the final resuspension and dilution to 25×10^6 were performed using follicular fluid, not modified M199.

A proportion of oocytes was directly fixed on each collection day. The remaining oocytes were randomly selected for treatments to be performed. One treatment was performed on each collection day, and all oocytes collected on that day were cultured together. Each treatment was replicated three times; if there were fewer than 30 analysable oocytes in the group, another replicate was performed. The results for treatments having more than 50% degenerating oocytes were not included in the analysis. The proportion of oocytes maintained in the germinal vesicle stage and the proportion in MII in each treatment group was compared with that for controls using Fisher's exact test.

Experiment 2

Oocytes were collected from all visible follicles. Both compact and expanded cumulus oocytes were used in this experiment; each treatment was performed for each cumulus type. Oocytes were assigned to one of five treatment groups as follows. (1) Direct control: oocytes were denuded and fixed after removal from the follicle. (2) Maturation control: oocytes were placed in 200 μl droplets of modified M199 containing 5 μg FSH ml^{-1} (Schering Corp. USA, Kenilworth, NJ) and incubated at 38°C in a humidified atmosphere of 5% CO_2 in air

Table 1. Percentage of horse oocytes in each maturation classification, according to treatment (Expt 1)

Treatment	<i>n</i>	Germinal vesicle stage	Diakinesis to metaphase I	Anaphase I to metaphase II	Degenerating
Direct control	32	69	13	0	19
Non-suppressive control	50	26	20	20	34
Granulosa cells	36	36	14	8	42
Follicular fluid	28	25	32	14	29
Granulosa cells in follicular fluid	40	25	45*	8	23

Proportions of oocytes in germinal vesicle stage in all treatments were significantly different from direct control ($P < 0.05$).

*Significantly different from non-suppressive control ($P < 0.05$).

for 24 h, after which the oocytes were denuded and fixed for evaluation. (3) Mural granulosa: sheets of mural granulosa cells scraped from follicle walls were selected from follicles less than 30 mm in diameter that contained an oocyte with a compact cumulus and healthy granulosa cells. Sheets of granulosa from two to three follicles were obtained. Three to four sheets, with a total area of approximately 5 mm², were washed in modified M199 and then placed in a 50 µl droplet of modified M199 under oil. Oocytes were placed into the droplets and incubated as described above. (4) Follicle wall: sections of follicle walls were taken from follicles less than 30 mm in diameter, after opening the follicle with a scalpel but before scraping the mural granulosa. After the remainder of the follicle was scraped, wall sections from follicles that contained healthy granulosa cells and an oocyte with a compact cumulus were selected. One follicle wall section of approximately 5 mm² was placed in a 50 µl droplet of modified M199 under oil. Oocytes were placed into the droplet and incubated as described above. (5) Intact follicle: follicles less than 30 mm in diameter were dissected intact from the ovary and as much connective tissue as possible was removed without rupturing the follicle. When all oocytes intended for injection into the follicle were collected, they were aspirated into a glass pipette with a bore just large enough to allow the cumulus-intact oocytes to move through it without injury. A hole was made in the follicle wall with a 25 gauge needle; the tip of the pipette was introduced through the hole and the oocytes were ejected into the follicle. The intact follicle was placed in 50–150 ml of modified M199 (depending on the size of the follicle) in a 250 ml sterile plastic container (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) and incubated as described above. After the incubation period, the oocytes were recovered by placing the intact follicle into a 100 mm Petri dish and slicing the follicle open with two scalpel cuts at 90° from one another. The contents of the follicle were then searched for oocytes using a dissection microscope at a magnification of $\times 10$ – $\times 70$.

For oocytes in the suppression treatment groups (mural granulosa, follicle wall and intact follicle) after the period of incubation, half of the oocytes were fixed immediately for evaluation, and the other half of the oocytes were transferred into 200 µl droplets of modified M199 with 5 µg FSH ml⁻¹ to determine their ability to mature after the suppressive treatment. These oocytes, designated as suppressed/matured oocytes, were incubated for 24 h, then denuded and fixed for evaluation.

Each treatment was replicated four times. In this experiment, the effects of the suppressive treatments were evaluated by comparing the proportion of oocytes at the germinal vesicle stage with that for direct controls, and the proportion of oocytes in MII with that for direct controls, within each cumulus group, by Chi square analysis. Non-suppressive controls were not used so that more oocytes were available for treatment. Fisher's exact test was used for comparisons with less than five expected for any cell. The ability of oocytes to resume meiosis after treatment was evaluated by comparing the proportion of oocytes in MII in the suppression/maturation groups with that for the maturation controls and to that found immediately after the respective suppressive treatment.

Results

Experiment 1

In the incubated groups with granulosa cells alone and with granulosa cells plus follicular fluid, the granulosa cells settled from suspension during the incubation period, forming a sheet of cells at the bottom of the microdroplet. The oocyte–cumulus complexes adhered to the granulosa cells, so that the oocytes had to be dissected free with needles to collect them from the microdroplet after culture.

None of the suppressive treatments was successful in maintaining meiotic arrest, as the proportion of oocytes in the germinal vesicle stage was significantly lower for all treatments than that for the direct control ($P < 0.05$; Table 1). The proportions of oocytes at the germinal vesicle stage for the suppressive treatments were not significantly different from the non-suppressive control group ($P > 0.1$). Although there was no significant difference in the proportion of oocytes at MII between the non-suppressive controls and each of the three treatment groups, there were significantly more oocytes at MI in the granulosa cell–follicular fluid treatment group than in the non-suppressive control group ($P < 0.05$).

In five of the 18 total replicates performed, more than 50% of oocytes were found to be degenerating after culture; the average percentage of degenerating oocytes in these treatments was 69%. These replicates were not included in the analysis of results.

Fig. 1. Photomicrograph of horse oocytes after injection into an intact horse follicle, and incubation for 24 h *in vitro*. The transferred oocyte-cumulus complexes have become confluent with the granulosa of the follicle. Scale bar represents 200 μ m.

Experiment 2

At the time of recovery of oocytes, in the intact follicle group, in most cases, the granulosa cell layer of the follicle separated easily from the follicle wall and could be removed intact. The cumulus of the transferred oocytes appeared to have become confluent with the granulosa layer of the follicle, and it was not possible to determine under the dissection microscope which was the native oocyte of the follicle and which had been transferred to the follicle (Fig. 1). Overall, 117 oocytes were recovered from 11 follicles; thus, 11 of the recovered oocytes (9.4%) could have been the native oocyte of the host follicle. The cumulus type of the native oocyte was unknown, but could be estimated to have an equal chance of being expanded or compact (Hinrichs *et al.*, 1993a). Therefore, approximately half of the time, the native oocyte would have had a different cumulus type than the introduced oocytes. This represents less than 5% of all oocytes evaluated and was not factored into the results. Recovery rate (oocytes recovered from the follicle/oocytes transferred, plus one per follicle for the native oocyte) was 117 of 158 (74%).

There was a significant difference between expanded and compact cumulus oocytes in distribution among the chromatin configuration groups for both direct and maturation controls ($P < 0.001$). In the direct controls, this was reflected in a significantly higher proportion of expanded than compact cumulus oocytes in MI (15% versus 3%; $P < 0.001$), and a significantly lower proportion of expanded than compact cumulus oocytes that were degenerating (15% versus 29%; $P < 0.001$). In the maturation controls, significantly more expanded than compact cumulus oocytes reached MII (60% versus 37%; $P < 0.001$) and the expanded cumulus oocytes had

a significantly lower proportion of degenerating oocytes (16% versus 40% for compact oocytes; $P < 0.001$).

All three suppressive treatments were effective in compact cumulus oocytes. No compact oocytes in any suppressive treatment progressed to MII (Table 2). The proportion of compact oocytes maintained in the germinal vesicle stage for treatments with intact follicles and mural granulosa cells was not significantly different from the proportion of oocytes in the germinal vesicle stage in the direct controls ($P > 0.05$); however, the proportion of oocytes maintained in the germinal vesicle stage in the follicle wall treatment group was significantly lower than that for controls (49% versus 68%; $P < 0.05$), and the proportion of oocytes from the follicle wall group in MI tended to be higher than that for controls (10% versus 3%; $P = 0.12$).

For the expanded cumulus oocytes, treatment with intact follicles and mural granulosa cells effectively suppressed maturation (Table 3). The proportion of oocytes reaching MII in these two treatment groups was not significantly different from that for direct controls ($P > 0.05$); however, treatment with follicle wall resulted in a significantly higher proportion of oocytes in MII than was present in controls (18% versus 1%; $P < 0.01$). The proportion of oocytes at the germinal vesicle stage in the three treatments was not significantly different from that for direct controls ($P > 0.05$).

The proportion of compact cumulus oocytes in MII in the treatment groups incubated with mural granulosa cells or follicle wall and then matured tended to be higher than that for the mural granulosa and follicle wall groups immediately after suppression (Table 2). However, the proportion of compact cumulus oocytes in MII in the group incubated in an intact follicle and matured was not different from that

Table 2. Percentage of compact horse oocytes (those originally having compact cumuli) in each maturation classification, according to treatment (Expt 2)

Treatment	<i>n</i>	Germinal vesicle stage	Diakinesis to metaphase I	Anaphase I to metaphase II	Degenerating
Direct control	130	68	3	0	29
Maturation control	134	14	8	37	40
Follicle wall	45	49 ^b	9	0	42
Follicle wall plus maturation	35	26	11	9 ^a	54 ^c
Mural granulosa	39	54	0	0	46
Mural granulosa plus maturation	27	15	0	11 ^a	74 ^c
Intact follicles	33	58	3	0	39
Intact follicles plus maturation	31	58	0	3 ^a	39

^aThe proportion of metaphase II oocytes in each treatment group matured after suppression is significantly different from the maturation control group ($P < 0.05$). ^bProportion of oocytes at germinal vesicle stage is significantly different from the direct control group ($P < 0.05$). ^cProportion of degenerating oocytes significantly different from the direct control group ($P < 0.05$).

Table 3. Percentage of expanded horse oocytes (those originally having expanded cumuli) in each maturation classification according to treatment (Expt 2)

Treatment	<i>n</i>	Germinal vesicle stage	Diakinesis to metaphase I	Anaphase I to metaphase II	Degenerating
Direct control	163	69	15	1	15
Maturation control	152	11	13	60	17
Follicle wall	34	53	15	18 ^b	15
Follicle wall plus maturation	22	18	18	18 ^a	46 ^c
Mural granulosa	53	55	15	4	26
Mural granulosa plus maturation	51	26	28	31 ^a	16
Intact follicles	21	76	5	0	19
Intact follicles plus maturation	17	29	6	6 ^a	59 ^c

^aThe proportion of metaphase II oocytes in each treatment group matured after suppression is significantly different from the matured control group ($P < 0.05$). ^bProportion of metaphase II oocytes is significantly different from the direct control group ($P < 0.05$). ^cProportion of degenerating oocytes is significantly different from the maturation control group ($P < 0.05$).

in the intact follicle group immediately after suppression ($P > 0.1$).

The proportion of expanded cumulus oocytes in MII in the matured mural granulosa group was significantly higher than that for the mural granulosa group immediately after suppression (31% versus 4%, $P < 0.01$; Table 3). The proportions of oocytes in MII in the matured follicle wall and intact follicle groups were not significantly higher than that seen in the follicle wall and intact follicle groups immediately after suppression ($P > 0.1$).

None of the suppression treatments maintained normal viability of oocytes, as the proportion of oocytes reaching MII after post-treatment maturation was significantly lower in all groups than that for the maturation control ($P < 0.01$; Tables 2 and 3). The highest rate of post-treatment maturation for compact and expanded oocytes was found in the matured mural granulosa group (11% and 31% reaching MII, respectively). Within this treatment, the proportion of oocytes in MII was significantly higher for expanded than for compact cumulus oocytes ($P < 0.05$). Subjection of oocytes to suppression/maturation was associated with a significant

increase in the proportion of degenerating oocytes in the follicle wall and mural granulosa treatment groups for compact oocytes and in the follicle wall and intact follicle groups for expanded oocytes ($P < 0.05$).

Discussion

The results of this study indicate that in horses, culture of oocytes with follicular fluid or with granulosa cells in suspension does not maintain meiotic arrest, although incubation with follicular fluid plus granulosa cells may transiently inhibit maturation. Culture of oocytes with sections of follicle wall, sheets of mural granulosa and within intact follicles maintains meiotic arrest, but is associated with a low rate of meiotic resumption after the suppressive treatment. Culture with mural granulosa showed the highest rate of post-suppression resumption of meiosis; however, the proportion of oocytes at metaphase II in this treatment was still significantly lower than that for maturation controls. Oocytes with compact and expanded cumuli reacted differently to the suppression and

maturation treatments. Expanded cumulus oocytes were suppressed at a lower rate by co-culture with follicle wall explants, and expanded oocytes achieved the highest post-suppression maturation rate.

The concentration of granulosa cells used for co-culture in this study was based on findings in cattle that maximum suppression of maturation was achieved with granulosa cells suspended in medium at a concentration of 25×10^6 cells ml^{-1} ; if the cells were suspended in follicular fluid instead of medium, only 10×10^6 cells ml^{-1} were needed (Sirard and Bilodeau, 1990a). In the study reported here, the inability of 25×10^6 cells ml^{-1} in medium to suppress maturation and the partial suppression by granulosa cells plus follicular fluid at this concentration suggests that greater concentrations of granulosa cells may be needed for meiotic suppression in horses.

The granulosa cells used in this study were selected from follicles considered to have the maximum chance of inhibiting maturation. Follicles of less than 30 mm in diameter were selected because at 30 mm in diameter, concentrations of oestrogens and androgens undergo a threefold increase, suggesting the onset of maturation (Kenney *et al.*, 1979). Only follicles that yielded an oocyte with a compact cumulus were used, as oocytes with expanded cumuli are associated with atretic follicles in cattle (Rajakoski, 1960) and horses (Hinrichs, 1991), and the ability of granulosa cells from atretic follicles to suppress maturation may be impaired.

For maximum suppressive effect, contact between the oocyte–cumulus complex and granulosa cells appears to be important. The degree of contact has been shown in hamster oocytes to be strongly related to maintenance of the germinal vesicle (Racowsky and Baldwin, 1989). In cattle, maintenance of the germinal vesicle in oocytes cultured with granulosa cells was reduced, but not eliminated, by embedding the oocytes in agar (Sirard and Bilodeau, 1990a). This suggests that there is a soluble mediator in the suppressive effect of granulosa cells, but that direct contact confers a superior signal. This is probably due to direct transmission of the suppressive agent through gap junctions, which form rapidly between granulosa cells in culture (Larsen and Risinger, 1985). In our study, the oocyte–cumulus complex and granulosa cells adhered tightly to one another in culture, yet granulosa cell co-culture failed to suppress oocyte maturation significantly.

The difference between the results reported here for horses and those reported in cattle may be related to the method of collection of granulosa cells. In the present study, the method of opening the follicle and scraping the granulosa layer, while leaving the basement membrane intact, yielded essentially pure granulosa cells. In the experiments with granulosa cell suspensions in cattle, oocytes, granulosa cells and follicular fluid were obtained by aspirating follicles with a needle and syringe (Sirard and Bilodeau, 1990a). Aspiration results in the collection of ovarian components including granulosa cells, theca, stroma and blood. These additional components may be involved in the suppression of oocyte maturation. It has been shown that theca interacts with granulosa cells to increase suppressive effects in cattle (Kotsuji *et al.*, 1994). In horses, granulosa cell–theca interactions have been shown to affect oocyte maturation *in vitro*, resulting in more complete cumulus expansion than culture with either cell type alone (Okolski

et al., 1993). It is possible that even minor contamination of granulosa cells with theca results in a significant change in suppression compared with that achieved by a pure culture of granulosa cells.

The increased suppressive effect of theca–granulosa cell co-culture may be due to synergism in producing a meiotic-arresting agent, the identity of which is currently unclear. For example, it has been suggested that oestradiol inhibits oocyte maturation (Sirotkin, 1992); if so, manufacture of oestradiol by granulosa cells would be stimulated by androstendione production by theca cells. Furthermore, addition of theca cells inhibits the spontaneous luteinization usually seen in granulosa cells in culture; this may prolong the ability of granulosa cells to produce a meiotic arrestor (Kotsuji *et al.*, 1990, 1994).

The proportion of oocytes that degenerated after treatment may be related to the tissue to medium ratio in the co-culture groups. The length of culture (48 h total) for the suppression/maturation treatments should not have increased the proportion of degenerating oocytes, as oocytes had been previously incubated for up to 65 h without any significant increase in degeneration (Hinrichs *et al.*, in press). In the granulosa co-culture groups, 25×10^6 cells ml^{-1} resulted in a turbid medium, whereas in cattle, this concentration gave a relatively clear follicular fluid after most cells had sedimented (Sirard and Bilodeau, 1990a). In the follicle wall treatment group, the volume of medium and amount of follicle wall tissue used were based on the work of Sirard and Coenen (1993), who used a small droplet (50 μl) to maximize the concentration of factors produced during co-culture of bovine oocytes with one, two or five hemisections of follicles of 2 to 3 mm in diameter. Culture with one hemisection was found to be optimal; culture with two to five hemisections decreased the rate of post-suppression maturation, probably due to a high tissue to medium ratio. On this basis, a tissue section of approximately 5 mm^2 (approximately the tissue volume of one hemisection of a follicle of 2 to 3 mm diameter), within a 50 μl droplet was used in the present study. This was possibly more tissue than in a bovine hemisection, as the section was measured after it had been removed from the follicle and some tissue contraction would have already occurred.

While treatment with intact follicles successfully suppressed maturation, it severely limited the ability of oocytes to resume meiosis. This may be due to our inability to maintain horse follicles of large diameter in culture. Cumulus cells in the transferred oocyte–cumulus complex did survive to become confluent with the granulosa cells of the follicle. Culture of intact follicles has been successful in other species (Thibault *et al.*, 1975; Moor and Trounson, 1977). In pigs, oocytes transferred into viable explanted follicles were maintained in meiotic arrest unless gonadotrophins were administered (Meinecke and Meinecke-Tillman, 1982; Fleming *et al.*, 1985). We are particularly interested in the relationship of oocytes to the granulosa of host follicles, as we have transferred immature oocytes into the dominant preovulatory follicles of mares *in vivo*, given gonadotrophin stimulation, and achieved maturation, ovulation, fertilization and development to the blastocyst stage of the transferred oocytes (Hinrichs and DiGiorgio, 1991).

The better performance of expanded cumulus oocytes in the suppression/maturation culture system than that of compact

cumulus oocytes was unexpected. Cumulus expansion is related to follicular atresia in horse oocytes (Hinrichs, 1991; K. A. Williams and K. Hinrichs, unpublished). In Expt 1, only compact cumulus oocytes were used, as it was assumed that maturation in expanded cumulus oocytes would have already begun at the time of collection. However, only a small proportion (7%) of expanded cumulus oocytes was found to be in metaphase I and II at the time of collection, compared with none of the compact cumulus oocytes (Hinrichs *et al.*, 1993a); and overall, the proportion of expanded cumulus oocytes in the germinal vesicle stage was not significantly different from that for compact cumulus oocytes.

For this reason, in Expt 2 the maintenance of compact and expanded cumulus oocytes in suppression by follicular components was examined; the hypothesis was that expanded cumulus oocytes would be more difficult to suppress than were compact cumulus oocytes. While expanded cumulus oocytes in the follicle wall treatment group did enter metaphase II in significant numbers, those in the intact follicle and mural granulosa treatment groups were effectively suppressed. Furthermore, the rate of maturation (31%) for expanded cumulus oocytes in the matured mural granulosa group was significantly higher than that for compact cumulus oocytes in the same group. This may in part be related to faster rates of maturation to MII in expanded than in compact cumulus oocytes, as reported by Hinrichs *et al.* (1993a). However, the high rate of degeneration in the compact cumulus oocytes in this study suggests that expanded cumulus oocytes are more resilient to the suppression/maturation regimen. The higher proportion of degenerating compact compared with expanded cumulus oocytes in the maturation controls was unexpected, as in previous work the proportions of degenerating compact and expanded cumulus oocytes were equivalent (Hinrichs *et al.*, 1993a). A higher proportion of expanded cumulus oocytes would be expected to be degenerating, as they are more likely to come from atretic follicles. Further work with expanded cumulus oocytes has shown that they are also more easily activated than are compact oocytes (Hinrichs *et al.*, in press). The results of these studies suggest that expanded cumulus oocytes gain something while they are within the follicle that allows them to mature more successfully in our culture system than do compact cumulus oocytes. We are currently investigating the physiological differences (chromatin configuration and its relationship to maturation-related proteins) between these two groups of oocytes.

The apparent failure of follicular fluid to suppress oocyte maturation in horses may preclude its use for prolonging the time to onset of maturation during collection of horse oocytes. Willis *et al.* (1991) reported that the time needed for oocytes to reach metaphase II was 15 h, much shorter than the 24–36 h found by other workers (Zhang *et al.*, 1989; Hinrichs *et al.*, 1993a). In the study by Willis *et al.* (1991) however, oocytes were placed in follicular fluid at 39°C during collection, and for transport to the laboratory (4 h), and this time was not included in the time taken for maturation. Our results show that oocytes may start to mature while in follicular fluid. Thus, holding them in follicular fluid during collection, transport and evaluation may result in a shorter observed time from the beginning of culture to metaphase II.

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