Porcine follicular fluid does not inhibit maturation of rat oocytes *in vitro*

A. D. Fleming*, W. Khalil and D. T. Armstrong

M.R.C. Group in Reproductive Biology, University of Western Ontario, London, Ontario, Canada N6A 5A5

Summary. Follicular fluid from small- (1–2 mm) or medium- (3–8 mm) sized pig follicles was collected under conditions designed to minimize possible alteration or degradation of native fluid components. The effects of follicular fluid with or without benzamidine, an inhibitor of proteolytic activity, on oocytes collected 20 or 44 h after PMSG treatment of rats were examined. A follicular fluid fraction of $M_r < 10\,000$ (PM-10 membrane filter) was also tested. Follicular fluid from small- and medium-sized follicles and control medium alone supported maturation of oocytes collected 20 or 44 h after PMSG, but follicular fluid (50%) from medium-sized follicles containing 5·0 mM-benzamidine significantly inhibited oocyte maturation. Comparable inhibition was also observed with medium containing 5·0 mM-benzamidine. The PM-10 filtrate failed to inhibit oocyte maturation as assessed by germinal vesicle breakdown but did significantly inhibit first polar body formation and therefore restricted the extent of maturation. The results indicate that native pig follicular fluid is unable to inhibit the initiation of maturation of rat oocytes *in vitro*.

Introduction

The initiation of oocyte maturation in most vertebrates appears to be under the direction of luteinizing hormone (LH). Whilst the sequence of events subsequent to LH stimulation are understood in considerable detail in some vertebrates, such as amphibians (see Masui & Clarke, 1979, for an extensive review), the regulation of oocyte maturation in mammals is unclear. The spontaneous maturation of isolated oocytes *in vitro* suggested that an inhibitor in follicular fluid may be removed or overcome to permit the initiation of maturation (Pincus & Enzmann, 1935; Chang, 1955). The existence of such an inhibitor was reported in porcine follicular fluid by Tsafriri & Channing (1975) and in hamster and bovine follicular fluid by Gwatkin & Anderson (1976). Tsafriri, Channing, Pomerantz & Lindner (1977) reported that pig follicular fluid inhibited rat oocyte maturation *in vitro*, suggesting that the inhibitory activity was not species-specific. The putative oocyte maturation inhibitor (OMI) has not been identified nor has its reported efficacy in whole fluid or purified fractions exceeded about 50% inhibition of treated oocytes. The existence of an inhibitory activity *in vivo* being responsible for the maintenance of meiotic arrest is possibly more readily accepted than the evidence for it warrants. For example, Jagiello, Graffeo, Ducayen & Prosser (1977) reported inhibitory activity despite demonstration of only low levels of inhibition or even "minimally effective" inhibition. It has been reported that pig follicular fluid does not inhibit

* Present address: Perinatal Biology Program, Cardiopulmonary Department, Southwest Foundation for Research and Education, San Antonio, Texas 89284, U.S.A.

maturation *in vitro* of cow oocytes (Leibfried & First, 1980a) or pig oocytes (Sato & Ishibashi, 1977; Leibfried & First, 1980a; Racowsky & McGaughey, 1982).

We have now examined the effects of pig follicular fluid on the maturation *in vitro* of rat oocytes.

Materials and Methods

Immature rats (55–60 g body weight) were obtained from Charles River, Canada, Ltd (St Constant, Quebec, Canada) and housed under 14 h light : 10 h dark (lights on 05 : 00–19 : 00). Immature oocytes were collected 20 or 44 h after injection of PMSG (25 i.u.; Ayerst, Montreal, Quebec) which yielded about 32 developing follicles per female. At autopsy the ovaries were removed to medium containing 0·05% hyaluronidase (ovine type III, Sigma Chemical Co., St Louis, Missouri, U.S.A.). The incubation media used throughout this study were TCM-199 with Earle's Salts (Flow Laboratories, Mississauga, Ontario, Canada) plus 0·002% sodium pyruvate (Sigma) and Leibovitz's L-15 medium (Flow Labs) both supplemented with 0·05% bovine serum albumin (Fraction V, B. grade; Calbiochem. La Jolla, California, U.S.A.). Oocytes were released by puncturing the developing antral follicles ($\simeq$ 0·4 mm at 20 h and 0·8 mm at 44 h) while applying gentle pressure upon the ovary with fine forceps and then washed three times in hyaluronidase-free medium. Oocytes were collected from the ovaries of 3 or 6 females to provide a pool of oocytes for random distribution to control or treatment groups. Care was taken to use a sufficiently large bore pipette in collection and transfer of the oocytes to avoid loss or disturbance of the attachment of the cumulus granulosa cells to the zona pellucida and the oocyte itself.

Oocytes were selected for culture on the basis of a dense complete cumulus investment and the appearance of a single round vitellus within the zona which filled the entire zona cavity (i.e. no perivitelline space at this time). Oocyte normality after culture was assessed after removal of the cumulus by repeated passage of the oocyte–cumulus complex through a fine bore pipette. The criteria for normality were a single round vitellus exhibiting a solid sharp margin and an even non-granular ooplasm which filled the entire zona cavity unless maturation had proceeded through the first polar body formation (PB) thus giving rise to the perivitelline space. Oocytes were transferred to control or treatment conditions within 10 min of collection. Oocytes were incubated for 9–10 h at 37°C in an atmosphere of 5% CO_2 in air (pH 7·35). Each replicate consisted of at least 15 oocytes and 45 or more oocytes were assessed for each condition of culture in all experiments. Experiments were performed with 2 or 3 preparations of the test materials.

Maturation of rat oocytes *in vitro* was assessed by phase microscopy on live whole mounts gently compressed between a microscope slide and a cover-slip supported at the corners by wax droplets. An oocyte with an intact germinal vesicle (GV) was considered immature. Mature oocytes were those in which maturation had proceeded only to germinal vesicle breakdown (GVB) and oocytes in which maturation had proceeded to first polar body formation (PB).

Porcine follicular fluid was obtained from ovaries collected within 20 min of slaughter. Ovaries were freed immediately from the rest of the tract and rinsed with 0·9% (w/v) NaCl at 4°C for transport back to the laboratory. Follicular fluid was aspirated from small follicles (1–2 mm diam.) or from medium-sized follicles (3–8 mm diam.). Fluid was centrifuged at 4°C first at 1000 *g* for 10 min and then at 10 000 *g* for 20 min. Fluid was collected and centrifuged within 3 h of slaughter with all but the first 20 min being at ≤4°C.

Fluids from small- or medium-sized follicles were tested without further treatment (whole fluid) or after addition of the trypsin inhibitor benzamidine (Sigma). Fluid was stored frozen at –60°C under nitrogen and used after only a single thawing. Fluid from medium-sized follicles was also separated immediately after collection with a PM-10 membrane filter (Amicon, Lexington, Massachussets, U.S.A.) to obtain the M_r < 10 000 fraction described previously by Tsafriri, Pomerantz & Channing (1976), Tsafriri *et al.* (1977), Jagiello *et al.* (1977), and Stone, Pomerantz, Schwartz-Kripner & Channing (1978). The filtrate was freeze-dried and stored frozen under

nitrogen at $-60°C$. The filtrate was resuspended just before use in a volume of double-distilled water sufficient to yield a 10-fold concentration of the $M_r < 10\,000$ fraction over that in the original whole follicular fluid. A final dilution of 1 :4 or 1 :9 with medium for culture of rat oocytes yielded $\times 2$ or $\times 1$ concentrations, respectively.

Proportionate data after arcsine transformation were analysed by two-way (unbalanced) analysis of variance. Statistical significance was tested at $P < 0.05$ employing Duncan's New Multiple Range Test. For tabular presentation, group means and their 95% confidence intervals were transformed back to proportions.

Results

The percentage of oocytes of normal appearance after culture was $\geq 90\%$ for all control and treatment groups except the $M_r < 10\,000$ filtrate at 1 :4 dilution which was 62%.

As shown in Table 1, whole follicular fluid had no effect upon oocyte maturation *in vitro* in comparison to controls, but 50% follicular fluid containing 5·0 mM-benzamidine significantly inhibited GVB. The PM-10 filtrate at $\times 2$ concentration significantly increased GVB and concomitantly decreased PB. Higher concentrations of the filtrate were toxic to the oocytes presumably due to the osmotic effects of the salt content of this low molecular weight concentrate of porcine follicular fluid.

Fluid from small and medium follicles had no inhibitory effect upon the maturation of oocytes collected only 20 h after PMSG (Table 2). Moreover, these oocytes matured as well as those collected 44 h after PMSG (Tables 1, 3 & 4).

Table 1. Maturation *in vitro* of rat oocytes collected 44 h after PMSG treatment and incubated in medium alone or in various preparations of fluid from medium-sized pig follicles (FFM)

Treatment	No. of replicates	No. of oocytes examined	Oocytes with GV*	Oocytes with GVB*	Oocytes with PB*
Controls	4	136	17·6 (8·0–30·0)[a]	36·0 (11·6–56·1)[ab]	44·4 (21·3–68·9)[bc]
FFM 100%	3	63	13·0 (9·1–17·3)[a]	68·4 (51·4–83·2)[bc]	17·6 (2·4–31·8)[ab]
FFM 50%	3	54	11·3 (9·8–12·2)[a]	46·0 (31·3–61·1)[abc]	41·3 (25·6–58·0)[abc]
FFM$_{benz}$ 50%†	3	58	66·9 (34·4–92·3)[b]	24·5 (2·3–59·9)[a]	6·1 (1·3–14·2)[a]
FFM$_{filt}$ × 2‡	4	59	11·9 (0·6–25·2)[a]	78·0 (72·1–83·3)[c]	9·1 (0·4–21·2)[a]
FFM$_{filt}$ × 1	3	46	12·5 (3·3–26·4)[a]	69·9 (62·1–77·1)[bc]	16·3 (10·3–23·4)[abc]

* Values are the group mean proportions of oocytes with the 95% confidence interval in parentheses. Different superscripts within the same column indicate statistically different subsets ($P < 0.05$).
† 50% FFM in medium containing 5·0 mM-benzamidine, an inhibitor of proteolytic enzymes.
‡ The freeze-dried $M_r < 10\,000$ filtrate resuspended first in distilled water to yield a 10-fold concentrate over whole fluid and finally diluted 1 :4 or 1 :9 with medium to give a $\times 2$ or $\times 1$ concentration, respectively, in oocyte cultures.

Table 2. Maturation *in vitro* of rat oocytes collected only 20 h after PMSG treatment and incubated in medium alone (controls) or in 100% fluid from medium- (FFM) or small-sized (FFS) pig follicles

Treatment	No. of replicates	No. of oocytes examined	Oocytes with GV (%)	Oocytes with GVB (%)	Oocytes with PB (%)
Controls	4	91	20·7 (2·0–41·3)	34·2 (16·8–54·2)	43·9 (38·9–49·0)
100% FFM	3	53	25·8 (0·0–61·2)	29·0 (20·7–38·1)	44·4 (24·7–65·1)
Controls	4	63	14·4 (0·5–30·8)	37·9 (23·8–53·1)	47·4 (24·1–71·3)
100% FFS	3	46	8·0 (0·0–18·7)	37·2 (16·2–61·2)	54·2 (31·8–75·8)

Values are the group mean proportions with the 95% confidence intervals in parentheses. There were no statistically significant differences in each column.

Table 3. Maturation *in vitro* of oocytes collected in the constant presence of 100% fluid from small-sized pig follicles (FFS) and incubated in medium alone (controls) or in 100% or 50% FFS

Treatment	No. of replicates	No. of oocytes examined	Oocytes with GV (%)	Oocytes with GVB (%)	Oocytes with PB (%)
Oocytes collected 20 h after PMSG					
Controls	3	52	20·2 (5·9–33·4)	26·7 (20·6–33·2)	52·3 (33·7–70·5)
50% FFS	3	45	12·7 (7·2–19·5)	36·6 (23·2–51·0)	50·3 (30·0–70·7)
100% FFS	3	59	15·8 (3·9–33·6)	24·7 (17·5–32·7)	57·8 (40·1–74·5)
Oocytes collected 44 h after PMSG					
Controls	3	100	11·0 (4·7–19·6)	34·5 (33·2–35·9)	53·8 (45·6–61·9)
50% FFS	3	94	9·8 (9·3–10·2)	32·2 (20·9–44·8)	57·6 (44·9–69·9)
100% FFS	3	102	5·5 (4·0–7·2)	30·0 (12·0–51·9)	61·1 (43·0–82·7)

Values are the group mean proportions with the 95% confidence intervals in parentheses.
There were no statistically significant differences in each column.

Table 4. Oocytes collected 44 h after PMSG treatment and incubated in medium alone (controls) or in medium containing 5·0 or 2·5 mM-benzamidine

Treatment	No. of replicates	No. of oocytes examined	Oocytes with GV (%)	Oocytes with GVB (%)	Oocytes with PB (%)
Controls	4	58	9·3 (0·5–21·0)[a]	50·0 (35·2–64·8)[a]	39·9 (34·1–46·0)[b]
5·0 mM-benzamidine	4	85	38·2 (23·8–53·8)[b]	60·2 (42·2–76·9)[a]	0·4 (0·0–3·4)[a]
2·5 mM-benzamidine	4	48	20·5 (8·3–36·4)[ab]	68·8 (46·7–83·6)[a]	14·2 (0·1–22·9)[a]

Values are the group mean proportions with the 95% confidence intervals in parentheses.
Different superscripts within the same column indicate statistically different subsets ($P < 0.05$).

To test the possibility that the oocytes underwent a commitment to maturation during the 10-min collection period in the absence of porcine follicular fluid, oocytes were released from their follicles into 100% follicular fluid which had been collected from small follicles no more than 1 h earlier. Oocytes were then washed in a second dish of 100% follicular fluid and incubated in 100% or 50% follicular fluid or washed and incubated in medium alone. Oocytes collected 20 or 44 h after PMSG still matured as well as those in the control group despite constant exposure to follicular fluid (Table 3).

The effects of benzamidine upon oocyte maturation are shown in Table 4. The percentage of oocytes with a GV was significantly increased and of those with a PB was significantly decreased.

Discussion

Porcine follicular fluid had no inhibitory effect upon rat oocyte maturation *in vitro* and it could therefore be an excellent medium or supplement for oocyte maturation *in vitro*. This is in agreement with the earlier work of Edwards (1965), Sreenan (1970), Hunter, Lawson & Rowson (1972), Sato & Ishibashi (1977), Leibfried & First (1980a) and Racowsky & McGaughey (1982). Maturation in the presence or absence of porcine follicular fluid was independent of the time after PMSG (20 or 44 h) at which the oocytes were collected and the diameter of follicles (1–2 mm or 3–8 mm) from which the fluid was obtained. Multiple preparations of follicular fluid or filtrate obtained from many different follicles, ovaries and females discounts the possibility that an abnormal ovary or female could have been responsible for the absence of inhibitory activity. An inhibition of oocyte maturation was only seen when the follicular fluid contained benzamidine. This protease inhibitor was included with one preparation of follicular fluid to preserve potential OMI activity since Tsafriri *et al.* (1976) reported that OMI was sensitive to trypsin (proteolytic degradation). The observed inhibitory effect was probably due to the added inhibitor, benzamidine (Table 4), and not

to an endogenous inhibitor protected from proteolytic degradation by benzamidine. Fluids or filtrates of identical origins but lacking benzamidine did not inhibit maturation. Jeffrey (1977) and Kishimoto, Clark, Kondo, Shirai & Kanatani (1982) have demonstrated that oocyte maturation in invertebrates is inhibited by protease inhibitors.

The ability of the PM-10 filtrate to restrict the extent of maturation at the PB stage is similar to that achieved with lysophosphatidylserine which inhibited PB in rat oocytes at a concentration of 100 µg/ml (Fleming & Armstrong, 1983). The PM-10 filtrate as a concentrate of the low molecular weight components of follicular fluid must include lipids but their nature and amounts are unknown.

It is unlikely that the failure of porcine follicular fluid to inhibit rat oocyte maturation in the present work is the result of a commitment to maturation having occurred during the 10-min collection period when oocytes were not exposed to follicular fluid. Oocytes that were constantly exposed to 100% follicular fluid during collection and incubation (Table 3) matured as well as control oocytes or oocytes exposed to follicular fluid only during incubation (Tables 1, 2 & 4). Dekel & Beers (1980) showed that a commitment to maturation *in vitro* required 30–45 min as assessed by the sensitivity of rat oocytes to inhibition of maturation by cAMP. Moreover the oocytes collected under the conditions described in the present work were sensitive to inhibition by benzamidine (Table 4).

Our results differ from those of Tsafriri *et al.* (1977) who reported that porcine follicular fluid did inhibit rat oocyte maturation. Our collection of follicular fluid was complete by 3 h after slaughter with all but the first 20 min being carried out at $\leq 4°C$. In contrast, in the report by Tsafriri & Channing (1975) and subsequent reports from these investigators, ovaries were maintained at 'room temperature for about 2 h' before the start of collection of fluid with collections completed some hours later. It is possible that during the lengthy period at room temperature alteration or degradation of native fluid component(s) leads to production of OMI. Liu, Pomerantz & Channing (1982), for example, have reported that fragments of FSH exhibit OMI activity. If OMI activity is demonstrable only by adherence to a specific collection procedure and alteration of native follicular components, OMI may be of some pharmacological interest but of questionable physiological importance.

The limited efficacy of OMI despite purification was explained by Channing *et al.* (1982) as the result of some oocytes having been stimulated to mature *in vivo* before collection and therefore insensitive to inhibition by OMI *in vitro*. If some of the follicles and enclosed oocytes in the present work had matured in response to a stimulus received *in vivo* despite exposure to OMI *in vitro*, one could have expected to see evidence for this by the appearance of some cumulus expansion. Cumulus expansion *in vitro* is dependent upon gonadotrophic stimulation (Dekel & Kraicer, 1978; Dekel, Hillensjo & Kraicer, 1979; Eppig, 1980), unlike oocyte maturation *in vitro*. Expansion of cumulus-intact oocytes collected before the expected time of LH stimulation *in vivo* and cultured in the absence of gonadotrophins *in vitro* was not observed in the present work nor has it been reported by other investigators.

Spontaneous maturation *in vitro* of isolated oocytes may be stimulated by conditions of the collection and incubation system that either mimic or circumvent the in-vivo stimulus. The disruption of the membrana granulosa cell connections to the cumulus granulosa cells of the cumulus–oocyte complex, for example, may provide a stimulus to maturation. Biggers & Powers (1979) and Powers (1982) have suggested that a maturational stimulus may be transduced to the oocyte via changes in membrane permeability and potential precipitated by changes in cell–cell connections of the cumulus granulosa cells. Maintenance of contact *in vitro* between cumulus granulosa cells of the cumulus–oocyte complex and mural granulosa of the complete follicle wall from hemisected follicles will inhibit oocyte maturation *in vitro* (Foote & Thibault, 1969; Leibfried & First, 1980b). Additional changes in the environment of the oocyte upon liberation *in vitro* include changes in pH, pCO_2 or pO_2. Together these may be a sufficient stimulus to induce maturation *in vitro* without postulating the removal of an inhibitor.

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