Inhibitory Effects of the Porcine Follicular Fluid on Estradiol and Progesterone Secretion by Cultured Rat Granulosa Cells

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Abstract

The effect of porcine follicular fluid on estradiol and progesterone secretion was examined using a rat granulosa cell culture with FSH and testosterone in the medium. Follicular fluids from small (<5 mm) and large (>6 mm) follicles (SFF1, LFF1) were treated with charcoal, and then fractionated by filtration through an Amicon XM-50 and an PM-10 membrane. The addition of 25% SFF1 and LFF1 into the culture system significantly inhibited estradiol and progesterone secretion ($P < 0.005$). These inhibitory activities were observed in PM-10 retentates (10,000–50,000 MW) and filtrates (<10,000 MW) of SFF1 and LFF1. The addition of XM-50 filtrates (<50,000 MW) of SFF1 and LFF1 caused a dose-dependent inhibition of estradiol and progesterone secretion. The dose-response relationship between the filtrates and estradiol secretion was linear with a significant correlation coefficient. The addition of the filtrates exerted no inhibitory effect on the growth of the cells cultured. XM-50 filtrate of LFF1 from a batch with a low ratio of small/large follicles showed a lower inhibitory activity on estradiol secretion than that of LFF1, while the inhibitory activities in both filtrates on progesterone secretion were almost equivalent. These results suggest that the follicular fluid of small porcine follicle contains nonsteroidal regulators capable of inhibiting estradiol and progesterone secretion by cultured rat granulosa cells, and that the estradiol secretion inhibitor activity decreases in the fluid of large follicle while the progesterone secretion inhibitor activity does not decrease in it.

Changes in the capacity of granulosa cells to metabolize aromatizable androgens to estrogens have been suggested to be a physiologically significant mechanism for determining the fate of individual follicles to mature and ovulate or to undergo atresia (McNatty *et al.*, 1979; Hillier *et al.*, 1981). However, what factors control the changes in steroidogenic capacity of granulosa cells remains essentially unknown.

It has recently been proposed that such nonsteroidal factors as progesterone secretion stimulator (Stewart *et al.*, 1982; Ledwitz-Rigby *et al.*, 1982; Osteen and Channing, 1983), progesterone secretion inhibitor (Hillensjö *et al.*, 1980, 1983; Channing *et al.*, 1982), estrogen secretion stimulator (Ledwitz-Rigby, 1983) and estrogen secretion inhibitor (Kling *et al.*, 1983; diZerega *et al.*, 1983) found in follicular fluids of various species may participate in the regulation of the fate of individual follicles by modifying the

steroidogenic capacity of granulosa cells. However, the biochemical and physiological characteristics of such factors are not established yet.

We previously reported that porcine follicular fluid suppresses estrogen and progesterone secretion by cultured rat granulosa cells (Kigawa, 1984). The present study was designed and projected to extend it in further detail.

Materials and Methods

1. Animals

Immature female Wistar rats 28 days old were subcutaneously injected with 10 IU of PMSG to obtain as homogeneous and mature granulosa cells as possible, and sacrificed 24 h after injection.

2. Culture of Granulosa Cells

The rats were killed by decapitation. The ovaries were removed immediately, shelled from their bursae, and dissected free of oviducts and adhering fat. The follicles were pierced with a fine needle and gently pressed to release granulosa cells into the medium. Cell suspension was filtrated through a platinum-mesh (80 mesh) to remove tissue debris and oocytes, and again filtrated through a platinum-mesh (150 mesh) to obtain homogeneous cell suspension. Granulosa cells were collected by centrifugation at 800 rpm for 5 min, resuspended in medium, counted in a haemocytometer, and diluted to a concentration of approximately $1-3 \times 10^6$ viable cells/ml. Viable cells were determined by a trypan blue dye exclusion method. Culture was made for 4 days in monolayer in 1 ml of medium in Limbro multiwells (No. 76-053-05) at 37°C in 5% CO₂/95% air in high humidity. During the first 48 h culture the cells were allowed to attach to the wells in the control medium, and during the second 48 h culture, test period, the cells were cultured in the test medium containing hormones (FSH and testosterone) and porcine follicular fluid as described in the Results. The culture medium used was a combination of TC 199 medium and 10% FCS with 50 IU/ml penicillin, 50 µg/ml streptomycin and 0.6 µg/ml Fungizone. At the end of the culture, the media were centrifuged, and supernatants were stored at -35°C until the

assay of estradiol and progesterone was made. The cells attached to the culture wells were released from the wells through incubation with a phosphate buffer solution containing 0.5% trypsin and 0.02% EDTA at 37°C for 15 min. These cells were then counted using a haemocytometer. The capacity of granulosa cells to secrete progesterone and estradiol was shown as the amount of steroids secreted per 10⁴ cells per test period of 48 h, or as the amount of steroids released per well per 48 h when the cells could not be released by trypsin-EDTA treatment because of the firm attachment to the well.

3. Preparation of Porcine Follicular Fluid

Ovaries were obtained from gilts around puberty at a local slaughter-house. Both the fluids from small (<5 mm in diameter) and large (>6 mm in diameter) follicles were separately collected by aspiration with a 22-gauge needle, and centrifuged at 10,000 g, 0°C for 30 min. Follicular fluids from ovaries collected for one week were pooled. The supernatants were mixed with 10% (wt/vol) dextran-coated charcoal, shaken at 4°C for 15 min, and centrifuged at 10,000 g, 0°C, for 60 min to remove the steroids contained in them. This procedure was repeated 5 times. The remaining charcoal was removed from the fluid by filtration through 0.22 micron Millipore filters. Following the charcoal treatment each follicular fluid was filtrated through an Amicon XM-50 membrane (with a molecular weight exclusion limit of 50,000 daltons) to produce two fractions, XM-50 retentate (>50,000 MW) and XM-50 filtrate (<50,000 MW). XM-50 filtrates of small (SFF1) and large follicular fluid (LFF1) were further fractionated by Amicon PM-10 membrane (with a molecular weight exclusion limit of 10,000 daltons) filtration into two fractions, PM-10 retentate (10,000-50,000 MW) and PM-10 filtrate (<10,000 MW). Each fraction was lyophilized and stored at -80°C until analysis was done. The medium designated 25% SFF1 contained 1 part of SFF1 to 3 parts of medium. The medium designated 100% XM-50 filtrate of SFF1 contained lyophilizate corresponding to 1 ml of the filtrate in 1 ml of medium, etc. For the preliminary characterization of active material(s), the XM-50 filtrate of SFF1 was heated in a water bath at 60°C for 30 min or treated with trypsin. Trypsin treatment was done as follows; XM-50 filtrate was mixed with TPCK-trypsin solution (mg/mg protein), incubated

at 25°C for 5 h, and adjusted to pH 2.0 with 1N HCl. After refiltration through an Amicon XM-50 membrane the filtrate was desalted through a PD-10 column (2×10 cm, Pharmacia) using 0.01 N CH₃COOH, and then lyophilized.

4. Hormones

Ovine NIH-FSH-S8 and NIH-LH-S20 were donated by the Hormone Distribution Office, National Institute of Health. Testosterone was obtained from Sigma Chemical Co. Ltd. and PMSG from Teikokuzoki Co. Ltd.

5. Steroid Assay

¹²⁵I-labeled antibody radioimmunoassay kits for estradiol-17β and progesterone were obtained from Commissariat A L'Energie Atomique (Italy) and Daiichi Radioisotope Laboratory (Japan), respectively. RIA was performed without a prior extraction after dilution of the culture medium with distilled water. The estradiol antiserum cross-reacts 0.2% with estrone, 0.002% with progestins and 0.002% with androgens. The progesterone antiserum cross-reacts 0.3% with pregnenolone and 20α-OH-progesterone, 0.2% estradiol and 0.002% with androgens. All the samples for the same experimental series were assayed in the same assay system. The mean intrassay coefficient of variation for the culture medium samples was 15.9% for estradiol and 9.3% for progesterone. The sensitivity of the assays was approximately 1.3 pg/tube for estradiol and 30 pg/tube for progesterone.

6. Statistics;

Statistics difference was determined by analysis

of variance. A P value less than 0.05 was considered significant.

Results

1. Steroid Concentrations in Follicular Fluid

The concentraion values for estradiol and progesterone in SFF1 and LFF1 are shown in Table 1. The values are the mean for two batches. The concentration of each progesterone and estradiol was high in original SFF1 and LFF1, but the charcoal treatment brought about a decline in these concentrations. The decline of estradiol concentration was sharper than that of the progesterone concentration. The fifth charcoal extraction removed 99.5% of estradiol in SFF1 and 96.8% of it in LFF1, while it removed only 60.5% of progesterone in LFF1 and 89.4% of it in SFF1. The estradiol and progesterone concentrations in the PM-10 retentates (10,000–50,000 MW) and filtrates (<10,000 MW) of SFF1 and LFF1 were at an undetectable level, but estradiol and progesterone were markedly concentrated in the XM-50 retentates (>50,000 MW) of SFF1 and LFF1.

2. Estradiol and Progesterone Secretion by Cultured Rat Granulosa Cells

During the first 48 h culture granulosa

Table 1. Estradiol and progesterone concentrations in porcine follicular fluids

	Small follicular fluid		Large follicular fluid	
	Estradiol (pg/ml)	Progesterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
Original	5,257	1,307	1,125	782
Charcoal treat. 2 times	22	330	58	378
Charcoal treat. 5 times	27	138	36	231
XM-50 retentate (>50,000 MW)	455	9,713	126	1,718
PM-10 retentate (10,000–50,000 MW)	ND	ND	ND	ND
PM-10 filtrate (<10,000 MW)	ND	ND	ND	ND

Original; supernatant of follicular fluid after centrifugation. Charcoal treat. 2 times or 5 times; fluid treated 2 times or 5 times with charcoal. XM-50 retentate; obtained by filtration through an Amicon XM-50 membrane of follicular fluid treated 5 times with charcoal. PM-10 retentate and PM-10 filtrate; obtained by filtration through an Amicon PM-10 membrane of XM-50 filtrate of follicular fluid. ND; nondetectable. Values represent the mean of two batches.

Fig. 1. Effect of FSH, LH and testosterone on estradiol and progesterone secretion by cultured rat granulosa cells

Granulosa cells from PMSG-pretreated immature rats were cultured in medium containing testosterone and FSH or LH for the second 48 h culture. Each bar represents the mean $\pm$ SD of five cultures.

cells from PMSG-pretreated immature rats were allowed to attach to the culture wells, and during the second 48 h culture, test period, androgens and gonadotropins were added into medium alone or in combination with these hormones. Dose of both testosterone (0.5 μ M) and gonadotropins (0.1 and 1.0 μ g/ml) added were chosen according to the report by Dorrington *et al.* (1975). The addition of both testosterone and FSH or LH caused a significant increase in progesterone and estradiol secretion and the increase was dependent on dose of FSH and LH (Fig. 1). These results indicated that granulosa cells from preovulatory follicles of PMSG-pretreated immature rats actively

secrete estradiol and progesterone when grown *in vitro* in the presence of testosterone and FSH.

3. Effect of Porcine Follicular Fluid on Estradiol and Progesterone Secretion by Cultured Rat Granulosa Cells

The effect of porcine follicular fluid on estradiol and progesterone secretion was examined using a culture system in which the granulosa cells from PMSG-pretreated immature rats were cultured during the second 48 h in the presence of FSH and testosterone to augment estradiol and progesterone secretion. SFF1 and LFF1 were added into the culture system at a dose of

25%. Since SFF1 and LFF1 contained a measurable amount of progesterone in themselves, the net amount of progesterone secreted into the medium by granulosa cells was calculated by subtracting the progesterone contained in 25% SFF1 and LFF1 from the total progesterone measured in medium after the culture ended. In contrast to this, a very small amount of estradiol contained in SFF1 and LFF1 was negligible in the calculation of net estradiol secreted into the medium by the cells. XM-50 retentates of SFF1 and LFF1, in which a large amount of estradiol and progesterone were concentrated were not used for the assay of biological activities. PM-10 retentates (10,000–50,000 MW) and filtrates (<10,000 MW) of SFF1 and LFF1 were added into the culture system at a dose of 50%, since estradiol and progesterone were undetectable in them. Different batches of follicular fluids were used in different experiments.

The addition of 25% SFF1s from two batches (A and B) caused a significant decrease ($P < 0.005$) in estradiol secretion, but did not cause a decrease in progesterone secretion (Table 2). This finding suggesting

Table 2. Effect of small porcine follicular fluid on estradiol and progesterone secretion by cultured rat granulosa cells

Batch	A	B
Estradiol secretion (pg/10 ⁴ cells/48 h)		
Control	518.4 ± 46.6	99.7 ± 30.8
SFF1	97.1 ± 14.1*	69.0 ± 5.8*
PM-10 retentate (10,000–50,000 MW)	133.4 ± 21.1*	55.2 ± 13.3*
PM-10 filtrate (<10,000 MW)	167.7 ± 25.6*	64.4 ± 9.8*
Progesterone secretion (ng/10 ⁴ cells/48 h)		
Control	11.0 ± 0.8	3.6 ± 0.9
SFF1	15.3 ± 2.3	9.1 ± 1.6
PM-10 retentate (10,000–50,000 MW)	3.0 ± 0.4*	1.2 ± 0.2*
PM-10 filtrate (<10,000 MW)	2.1 ± 0.3*	1.2 ± 0.3*

SFF1; small follicular fluid treated 5 times with charcoal. PM-10 retentate and PM-10 filtrate; obtained by filtration through an Amicon PM-10 membrane of XM-50 filtrate of SFF1. The effect of follicular fluid on estradiol and progesterone secretion was examined using a rat granulosa cell culture system. SFF1, and PM-10 retentate and filtrate were added into the culture system at dose of 25% and 50%, respectively. Values represent the mean ± SD of five cultures. *; $P < 0.005$ vs control.

Table 3. Effect of large porcine follicular fluid on estradiol and progesterone secretion by cultured rat granulosa cells

Batch	A	C	D
Estradiol secretion (pg/10 ⁴ cells/48 h)			
Control	518.4 ± 46.6	308.1 ± 47.1	172.0 ± 43.8
LFF1	38.9 ± 7.0*	63.4 ± 12.4*	168.9 ± 29.5
PM-10 retentate (10,000–50,000 MW)	—	180.7 ± 30.7*	192.9 ± 43.6
PM-10 filtrate (<10,000 MW)	60.5 ± 15.8*	115.1 ± 10.1*	179.2 ± 49.2
Progesterone secretion (ng/10 ⁴ cells/48 h)			
Control	11.0 ± 0.8	19.4 ± 0.9	4.8 ± 0.8
LFF1	1.4 ± 0.5*	2.8 ± 0.2*	2.0 ± 0.2*
PM-10 retentate (10,000–50,000 MW)	—	8.1 ± 0.8*	3.2 ± 0.8**
PM-10 filtrate (<10,000 MW)	1.0 ± 0.3*	2.2 ± 0.2*	3.9 ± 0.7

LFF1; large follicular fluid treated 5 times with charcoal. PM-10 retentate and PM-10 filtrate; obtained by filtration through an Amicon PM-10 membrane of XM-50 filtrate of LFF1. The effect of follicular fluid on estradiol and progesterone secretion was examined using a rat granulosa cell culture system. LFF1, and PM-10 retentate and filtrate were added into the culture system at dose of 25% and 50%, respectively. Values represent the mean ± SD of five cultures. *; $P < 0.005$ vs control. **; $P < 0.05$ vs control.

that 25% SFF1s had no inhibitory effect on progesterone secretion was probably brought about by the overestimation of net progesterone by adding progesterone left in SFF1s after charcoal extractin. The addition of PM-10 retentates and filtrates of the same SFF1s (A and B) at a dose of 50% resulted in a significant decrease in both estradiol and progesterone secretions ($P < 0.005$) (Table 2). As shown in Table 3, 25% LFF1s from two batches (A and C), and 50% PM-10 retentates and filtrates of the same LFF1s added into the culture system caused a significant decrease in both estradiol and progesterone secretions ($P < 0.005$). However, 25% LFF1, and 50% PM-10 retentate and filtrate from a batch (D) showed no

inhibitory effect on estradiol secretion, but showed a significant inhibitory effect on progesterone secretion, except 50% filtrate.

4. Dose Effect of Porcine Follicular Fluid Inhibition of Estradiol and Progesterone Secretion by Cultured Rat Granulosa Cells

A dose effect of porcine follicular fluid inhibition of estradiol and progesterone secretion by cultured rat granulosa cells was examined using XM-50 filtrates ($< 50,000$ MW) of SFF1 and LFF1, since both PM-10 retentates (10,000–50,000 MW) and filtrates ($> 10,000$ MW) of SFF1 and LFF1 showed inhibitory effects on estradiol and progesterone secretion. The XM-50 filtrate of SFF1 was added into the culture system at

Fig. 2. Dose response effect of small and large porcine follicular fluids on estradiol and progesterone secretion by cultured rat granulosa cells

sFFF; XM-50 filtrate of small follicular fluid. lFFF; XM-50 filtrate of large follicular fluid. XM-50 filtrates; obtained by filtration through an Amicon XM-50 membrane of small and large follicular fluid. The effect of sFFF and lFFF on estradiol (●) and progesterone (○) secretion was examined using a rat granulosa cell culture system. Numbers of granulosa cells/culture well at the end of experiment are shown by the black triangle (▲).

doses of 0.5%, 5%, 50% and 500%, and the filtrate of LFF1 was added at doses of 1%, 10%, 100% and 1000%. The results are shown in Fig. 2. Estradiol secretion was inhibited by the addition of the filtrates of SFF1 and LFF1 in a dose-dependent manner. The regression line between estradiol secretion and the filtrates was linear. The correlation coefficients for SFF1 filtrate and LFF1 filtrate were -0.9995 ($P < 0.01$) and -0.9742 ($P < 0.05$), respectively. Progesterone secretion was also inhibited by the addition of the filtrates of SFF1 and LFF1 in a dose-dependent fashion, but the regression line between progesterone secretion and the filtrates was not linear. The correlation coefficients for SFF1 filtrate and LFF1 filtrate were -0.9379 ($P < 0.1$) and -0.8461 , respectively. The numbers of granulosa cells per culture well counted at the end of the experiment are shown in Fig. 2. The addition of 0.5%, 5% and 50% SFF1 filtrate, and 1%, 10% and 100% LFF1 filtrate exerted no inhibitory effect on the growth of granulosa cells. The addition of 500% SFF1 filtrate and 1000% LFF1 filtrate exerted a stimulatory effect on the growth of cells.

5. Comparison of Inhibitory Activity on Estradiol and Progesterone Secretion in Small and Large Porcine Follicular Fluid

To test if there was a difference in inhibitory activity on estradiol and progesterone secretion in SFF1 and LFF1, XM-50 filtrates ($< 50,000$ MW) of SFF1 and LFF1 from the same batch were added into the culture system at four doses of 2%, 10%, 50% and 200%. SFF1 and LFF1 used in this experiment were collected from a pool of ovaries appearing to have a low ratio of small/large follicles. The results are shown in Table 4. The XM-50 filtrate of SFF1 showed a dose-dependent inhibitory activity on estradiol secretion, and inhibitory activities of 50% and 200% filtrate were highly significant ($P < 0.005$). In comparison with this, the XM-50 filtrate of LFF1 significantly suppressed estradiol secretion only at a high dose of 200% ($P < 0.01$). The inhibitory activity of SFF1 filtrate on estradiol secretion was significantly higher than that of LFF1 filtrate ($P < 0.01$ at 50% dose, $P < 0.005$ at 200% dose). The XM-50 filtrates of SFF1 and LFF1 showed a dose-dependent inhibition on progesterone secretion with the same potency.

Table 4. Comparison of inhibitory activity on estradiol and progesterone secretion in small and large porcine follicular fluid

Dose	Estradiol secretion (ng/well/48 h)		Progesterone secretion (ng/well/48 h)	
	sFFF	1FFF	sFFF	1FFF
2%	1.34 ± 0.25		34.1 ± 3.6	40.5 ± 5.3
10%	1.42 ± 0.17	1.10 ± 0.44	$25.3 \pm 1.2^*$	$21.1 \pm 1.8^*$
50%	$0.62 \pm 0.16^*$	1.18 ± 0.32	$12.2 \pm 3.7^*$	$1.3 \pm 0.1^*$
200%	$< 0.03^*$	$0.88 \pm 0.13^{**}$	$1.7 \pm 0.1^*$	$11.3 \pm 0.3^*$
Control	1.20 ± 0.13		37.0 ± 2.9	

sFFF; XM-50 filtrate of small follicular fluid. 1FFF: XM-50 filtrate of large follicular fluid. XM-50 filtrates; obtained by filtration through an Amicon XM-50 membrane of small and large follicular fluids from the same batch appearing to have a low ratio of small/large follicles. The effect of the filtrates on estradiol and progesterone secretion was examined using a rat granulosa cell culture system. Values represent the mean $\pm$ SD of five cultures, except three cultures at 200% XM-50 filtrate of large follicular fluid. *; $P < 0.005$ vs control. **; $P < 0.01$ vs control. Value left blank was not determined.

6. Effect of Porcine Follicular Fluid Heated and Trypsinized on Estradiol and Progesterone Secretion by Cultured Rat Granulosa Cells:

The XM-50 filtrate of SFFF treated with heat and trypsin was added into the culture system at a dose of 50% (Table 5). The inhibitory activity of the filtrate on estradiol and progesterone secretion was not lost by heat treatment. The trypsin treatment partially inactivated the inhibitory activity on estradiol secretion but the inactivation was not complete. The inhibitory activity of the filtrate on progesterone secretion was not influenced by the trypsin treatment.

Table 5. Effect of small porcine follicular fluid treated with heat and trypsin on estradiol and progesterone secretion by cultured rat granulosa cells

	Estradiol secretion (pg/10 ⁴ cells/48 h)	Progesterone secretion (ng/10 ⁴ cells/48 h)
Control	176.5 ± 20.0	12.69 ± 1.03
sFFF	26.4 ± 5.9*	0.78 ± 0.10*
with heat treat.	79.6 ± 28.1*	0.61 ± 0.07*
with trypsin treat.	131.8 ± 6.0*	2.86 ± 0.56

sFFF; XM-50 filtrate of small follicular fluid. XM-50 filtrate; obtained by filtration through an Amicon XM-50 membrane of small follicular fluid. heat treat; sFFF was treated with heat (60°C, 30 min). trypsin treat.; sFFF was treated with trypsin (mg/mg protein, 25°C, 5 h). The effect of sFFF on estradiol and progesterone secretion was examined using a rat granulosa cell culture system. SFFF was added into the culture system at a dose of 50%. Values represent the mean ± SD of four cultures. *; P < 0.005 vs control.

Discussion

In the present experiments, follicular fluid from small porcine follicles (<5 mm) treated with charcoal inhibited estradiol secretion by cultured rat granulosa cells.

The granulosa cells here isolated from preovulatory follicles of PMSG-pretreated immature rats were cultured by adding FSH and testosterone to augment estradiol and progesterone secretion. The inhibitory activity on estradiol secretion was also detected in the filtrate (<50,000 MW) of small follicular fluid obtained by filtration through an XM-50 membrane, estradiol and progesterone of which had been completely removed by this procedure. Although exogenous progesterone was reported to suppress estradiol secretion by rat granulosa cells cultured with FSH and testosterone (Fortune and Vinecent, 1983), our results indicate that the inhibition by the filtrate of estradiol secretion is not due to the inhibition by progesterone contained in follicular fluid. As shown in Fig. 2, the inhibition by the XM-50 filtrate of estradiol secretion was dose-dependent, and the dose-response relationship between the filtrate and estradiol secretion was negatively linear with a significant correlation coefficient ($P < 0.01$). The possibility that the inhibitory activity of the filtrate is dependent upon the cytotoxic effect of the filtrate on cultured granulosa cells may be denied by the fact that granulosa cells cultured with the filtrate grew in the same way as the cells cultured without the filtrate. These results indicate that follicular fluid from small porcine follicles contains nonsteroidal regulator(s) capable of inhibiting estradiol secretion by cultured rat granulosa cells.

It was impossible to determine the molecular weight of this inhibitor by a membrane filtration method, since two fractions obtained by filtration through a PM-10 membrane of the XM-50 filtrate of small follicular fluid, PM-10 retentate (10,000–50,000 MW) and filtrate (<10,000 MW), inhibited estradiol secretion with the same potency. The inhibitory activity was not decreased by heat treatment, but was partially inactivated by trypsin treatment. Extraction with both ether and charcoal did

not decrease the inhibitory activity (data not shown). These results may suggest that the inhibitory activity is probably heat-stable protein, not lipid in nature.

As shown in Table 2, charcoal-treated follicular fluid from large porcine follicles (>6 mm) and the two fractions obtained by filtration through a PM-10 membrane of the XM-50 filtrate showed variable inhibitory effects on estradiol secretion compared with small follicular fluid and the two PM-10 fractions. The large follicular fluid and two PM-10 fractions from a batch showed the potent inhibitory activity, while those from another batch showed no inhibitory activity. Each time follicular fluids were collected in a pool, porcine ovaries at puberty had different ratio of small/large follicles. We supposed that a pool of large follicular fluids from ovaries with a low ratio of small/large follicles might have only a slightly inhibitory effect on estradiol secretion. Results supporting this supposition were obtained (Table 4); the XM-50 filtrate of large follicular fluid from a batch appearing to have a low ratio of small/large follicles showed inhibitory activity only when added at a higher dose, while the XM-50 filtrate of small follicular fluid from the same batch inhibited estradiol secretion at a lower dose and the inhibition was dose-dependent. It is, therefore, suggested that the estradiol secretion inhibition activity in follicular fluid of developing antral follicles decreases depending upon the maturational state of the follicles, the higher the maturational state of the follicles, the lower the inhibitory activity of follicular fluid. However, it is possible that the concentration of inhibitor is low in a pool of follicular fluid with a high ratio of atretic/nonatretic large follicles. The possibility may be possibly denied by the fact that the XM-50 filtrate of large follicular fluid with a low inhibitory activity on estradiol secretion has a highly inhibitory effect on progesterone secretion.

There are several reports on nonsteroidal intrafollicular substance(s) capable of regulating estrogen secretion by granulosa cells *in vitro*. In agreement with our results, Kling *et al.* (1983) extracted a proteinous substance from porcine follicular fluid from medium follicles (3–5 mm) using ammonium sulfate precipitation and Orange A dye-matrix chromatography, which inhibited an increase in estradiol secretion by porcine granulosa cells from medium follicles incubated in the presence of LH/FSH and androstenedione. The same inhibitory activity was detected in the human follicular fluid (diZerega *et al.*, 1983). In contrast to this, Ledwitz-Rigby (1983) reported that charcoal-treated fluid from large porcine follicles (6–12 mm) enhanced estradiol secretion by granulosa cells from small porcine follicles (1–2 mm) cultured with FSH and testosterone. Whether porcine follicular fluid contains both an inhibitor and a stimulator of estradiol secretion or either remains to be determined.

In the present experiments, in addition to the estradiol secretion inhibition activity, the XM-50 filtrates of small (<5 mm) and large (<6 mm) follicular fluids have been demonstrated to exert an inhibitory effect on progesterone secretion by rat granulosa cells cultured with FSH and testosterone. As shown in Fig. 2, the inhibition by both filtrates was dose-dependent, but the dose-response relationship between the filtrates and progesterone secretion was not linear as far as tested. The inhibitory activity on progesterone secretion was observed in the two PM-10 fractions, PM-10 retentate and filtrate, of small and large follicular fluids. These results indicate that follicular fluids from small and large porcine follicles contain nonsteroidal regulators(s) capable of inhibiting progesterone secretion by rat granulosa cells cultured with FSH and testosterone. The inhibitory activity has been suggested to be possibly heat-stable and resistant to trypsin digestion.

Whether the estradiol secretion inhibitor and the progesterone secretion inhibitor in porcine follicular fluid described here are different substances or a single substance acting via a different pathway is now unclear. However, the finding that the inhibitory effect on estradiol secretion in the XM-50 filtrate of large follicular fluid is weaker than that in the filtrate of small follicular fluid while the inhibitory effects on progesterone secretion in both small and large follicular fluids are almost equivalent appears to indicate that the two inhibitors are not the same substance.

There are some contradictory reports with regard to the effect of follicular fluid on progesterone secretion by granulosa cells *in vitro*. Hillensjö *et al* (1980) reported that a low molecular weight fraction (<1,000 MW) obtained by PM-10 membrane filtration and Sephadex G-25 column chromatography of a pool of porcine follicular fluids from small, medium and large follicles produced inhibition of basal progesterone secretion by cultured cumulus granulosa cells from medium (3–5 mm) follicles. In contrast to this, charcoal-treated fluid from large (6–12 mm) porcine follicles was reported to enhance basal progesterone secretion by cultured porcine granulosa cells from small follicles (Stewart *et al.*, 1982). The identification and purification of the progesterone secretion inhibitor are needed to explain the contradiction.

In conclusion, our results may suggest that although the granulosa cells in small antral follicles, if placed in a milieu containing FSH and aromatizable androgens, are capable of secreting both estradiol and progesterone, the cells are restrained by the follicular fluid inhibitor from secreting estradiol and the disappearance of inhibitor in follicular fluid stimulates them to secrete estradiol, while progesterone secretion by the cells is continuously suppressed by another inhibitor during the whole process of follicular development to secure the efficient

estradiol secretion needed to stimulate the growth of the follicles.

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