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Detection of Proteins from Porcine Follicular Fluid and Their Effect on the Maturation of Mouse Oocytes in vitro

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= **국문초록** =

돼지 濾胞液內 蛋白質의 檢出과 培養中인 생쥐卵子의 成熟에 미치는 그의 영향에 관하여

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면역학적인 방법을 사용하여 가돈 여포액내의 특이단백질의 존재여부와 이들을 추출하여 난자성숙에 미치는 영향을 조사한 결과 다음과 같았다.

1. 가돈 혈청 및 혈장에는 존재하지 않는 면역학적으로 특이한 두 단백질이 가돈 여포액내에서 검출되었다.
2. 이들 두 단백질은 전기영동적으로 각각 fast alpha-1 globulin과 beta-globulin의 이동성을 나타냈다.
3. 이들 두 단백질은 생쥐 여포난자의 성숙을 억제시켰다.

본 실험의 결과로 미루어 가돈 여포액내에는 난자성숙을 억제시키는 특이단백질이 존재하는 것으로 사료된다.

INTRODUCTION

Foote and Thibault (1969) examined the inhibiting action of the granulosa cells to the bovine oocytes *in vitro* and they assumed that antimeiotic factors may be produced by the granulosa cells. Cho and Lim (1975) succeeded to produce meiotic resumption of bovine follicular oocytes cultured in the dialyzable fraction of the bovine follicular fluid, but not in the medium with non-dialyzable which is mainly composed of macromolecules. The results suggest that antimeiotic factor(s) in the follicular fluid will be present in the form of macromolecules such as proteins or polysaccharides.

Recently, Sato *et al.* (1978) reported that an inhibitor to compensatory ovarian hypertrophy in mice was detected in porcine and bovine follicular fluid. It was also reported that the porcine follicular fluid contained two sorts of globulines; fast and slowly migrating, which show the inhibitory effect on the oocytes maturation (McGaughey, 1975). The evidence that follicular fluid containes some specific proteins was made by the studies of Lorenzen *et al.* (1978) who found some kinds of homrone binding proteins of molecular weight over 10,000 daltons, and oocyte maturation inhibitor, a peptide with a molecular weight of about 2,000 daltons in the porcine follicular fluid.

In general, the search for proteins specific to the follicular fluid using immunological methods has not yet been rewarded, because it was unable to detect any specific antigens in follicular fluids of human, cow and pig (Shivers *et al.;* 1964, Desjardins *et al.;* 1966, Manarang and Menge; 1971, Shalgi *et al.;* 1973).

On the other hand, Herve *et al.* (1968) and Bailey *et al.* (1972) reported that specific antigenic components have been identified in the human ovaries and follicular fluid. Their studies offer interesting possible applications to other species, such as cow or pig.

The purpose of the present experiments is, by applying more effective immunological methods than previously employed, (1) to identify whether unknown macromolecules are present in the porcine follicular fluid, and (2) to examine role of the specific macromolecules detected to the oocytes *in vitro*.

MATERIALS AND METHODS

Experiment 1. *Detection of specific proteins from porcine follicular fluid by immunological methods.*

1. Collection of procine follicular fluid

Pig ovaries were collected at a slaughter house at random stages of her reproductive cycle. Each of Graafian follicle (3-8 mm in diameter) was coarsely isolated from ovaries and washed in saline more than twice. The follicular fluid then was aspirated with a 25 G hypodermic needle and syringe (Dickerson Co.). After aspiration, follicular puncture was done with a sharp needle. The follicular fluid was pooled together into one until reached to 60 ml and centrifuged at 800 x g for 30 minutes at 4°C to remove granulosa cells and debris. After centrifugation, the supernatant was immediately pressure-filtered with a Millipore syringe and stored at -20°C until use.

Blood sample was taken from female pig by cardiac puncture immediately after sacrifice at the slaughter house. Plasma was prepared by centrifugation of the blood in a heparin coated tube at 800 x g for 30 minutes at 4°C. Serum was prepared by blood clotting and centrifugation. The plasma and serum were Millipore-filtered and stored at -20°C or lyophilized for use.

The protein concentrations of follicular fluid, plasma and serum were measured by the method of Lowry *et al.* (1951). For conversion of optical density to mg of protein, a standard curve based on bovine serum albumin (Fraction V, Sigma Chemical Co.) was employed.

2. Preparation of antiserum against follicular fluid

The antiserum against porcine follicular fluid was raised in the male rabbits (4 - 5 months old, 2 - 3 kgs). The procedures are as follows: The porcine follicular fluid collected was diluted to 10 mg/ml in protein concentration with saline solution. For the first injection, diluted follicular fluid was emulsified with an equal volume of Freund's complete adjuvant (DIFCO lab.). The rabbits were shaved in the nuchal region, and

both sides of the vertebral column. Two mililiters of the emulsion was injected subcuteneously at ten different sites in the shaved area. It was not injected more than 0.2 ml at any one site. From one week later the identical booster injections (11 times, twice a week) were given, using Freund's incomplete adjuvant (DIFCO lab.) instead. At the fifth day of last injection, blood was taken from rabbits by cardiac puncture, pooled and allowed to clot for 24 hours at 4°C. After clotting, the serum was centrifuged at 1,000 x g for 30 minutes at 4°C. Heat inactivation at 56°C for 30 minutes was performed to remove complements in serum.

3. Absorption of antiserum

To obtain antiserum specific to procine follicular fluid, antiserum absorption with pig female serum was done by the modified method was Sacco and Shivers (1973). Six grams of lyophilized pig female serum was added to 20 ml of antiserum, then it was vigorously shaken for 30 minutes at room temperature and left stand at 4°C for overnight. It was then centrifuged at 1,400 x g for 30 minutes at 4°C. The precipitate was discarded and the supernatant was applied for isolation of the specific antibodies against porcine follicular fluid.

4. Isolation of antibodies from porcine follicular fluid

Ammonium sulfate precipitation was performed by the method of Campbell *et al.* (1974) to isolate the antibodies. Ten ml of saturated ammonium sulfate solution (pH 7.8) was slowly added to 20 ml of the supernatant prepared by the procedures shown in above with stirring. Upon completion of the addition of the ammonium sulfate solution, the suspension was continued stirring for 2-3 hours in order to avoid mechanical trapping of serum components other than gamma globulins in the precipitate, then centrifuged at 1,400 x g for 30 minutes at 4°C. The precipitate was dissolved in 20 ml of borate buffered saline (pH 8.4, buffer 1) to restore the volume of the solution to that of the original supernatant.

The gamma globulin fraction was purified by a second and third precipitation in borate buffered saline (buffer 1) to a final volume of 10 ml. Ammonium sulfate was removed from the precipitate by dialyzing against buffer 1 for three days at 4°C. During the dialysis, buffer I was changed every morning after checking the presence of sulfate ions in dialyzate by the balium chloride test. After dialysis was completed, the solution was centrifuged at 5,000 x g for 30 minutes at 4°C. The solution was stored at -20°C until use after Millipore filtration.

5. Detection of specific proteins from porcine follicular fluid by rocket and crossed immunoelectrophoresis

The procedures for detection of proteins in the porcine follicular fluid were mainly applied by the modified method of Krll (1973). Brief descriptions of methods are as follows:

a. Rocket-immunoelectrophoresis

One gram of agarose powder (Mann Res. Lab.) was added to 100 ml of barbital buffer (pH 8.6, i. s. = 0.005, buffer 2) and boiled gently with frequent stirring. To make an 1.5 mm thick agarose gel, 15 ml of agarose solution was mixed with 0.5 ml of purified antibodies (9.6 mg/ml) in a tube and placed gently on the plate (100 x 100 mm, Kodak) at constant 48°C. The gel was cooled to 10-14°C, then the gel became an 1.5 mm thick. The gel was connected to barbital buffer (pH 8.6, i.s. = 0.02; buffer 3) using filter paper (3-4 layers, Whatman No. 2). 40 μl of samples (5 μg/μl), previously millipore filtered, was applied as rapidly as possible to minimize diffusion and consequent precipitate distortion.

Electrophoresis was performed at constant voltage to 40 volts (4 volts/cm) for 24 hours.

After electrophoresis, the plate was covered with wet filter papers (7-8 layers, Whatman No. 2) and pressed for 1 - 2 hours, then immersed in 0.1 M NaCl solution for 1 hour for deproteinization. After the above procedure was repeated for three times, the gel plate was slowly dried in a warm oven (75°C), fixed in a 3 % picric acid solution for 10 minutes and rinsed with distilled water twice. The fixed gel plate was stained with 0.5 % Coomassie brilliant blue (Sigma) for 10 minutes. Destaining was done in the destaining solution (Ethanol: Acetic acid: Distilled water = 9:2:9).

b. Crossed-Immunoelectrophoresis

To confirm the results obtained by rocket-immunoelectrophoresis, crossed-immunoelectrophoresis except followings; the first dimensional electrophoresis was done at 10 volts/cm for 1.5 hours and the second dimensional electrophoresis was done at 4 volts/cm for 24 hours.

Experiment 2: *Purification of specific proteins from porcine follicular fluid by immunological methods.*

1. Removal of antibody against fibrinogen in antiserum against porcine follicular fluid raised in the male rabbit.

Results of Experiment 1 indicated the presence of specific proteins, including fibrinogen in porcine follicular fluid. It was necessary to remove antibody against fibrinogen in antiserum against porcine follicular fluid for purification of pure forms of specific proteins from porcine follicular fluid. 6.0 g of lyophilized pig female plasma was added to 20.0 ml of purified antiserum against porcine follicular fluid in the male rabbit. Antibodies against pure specific proteins of pig follicular fluid in the mixed solution was isolated by ammonium sulfate precipitation method previously described in Experiment 1.

2. Purification of specific proteins from porcine follicular fluid by immunoadsorbent column chromatography.

The procedures were adopted by modified method of McEntire (1978).

a. Preparation of CNBr-Sepharose 6B

Fifteen milliliters of Sepharose 6B (Gel volume, 8 ml; Sigma) swollen in buffer 1 was washed with 600 ml of cold distilled water using sintered funnel. The washed gel was transferred to the reaction vessel and adjusted to pH 11 ± 0.1 with 2 M NaOH. In well ventilated fume-hood, 2.4 g of finely divided cyanogen bromide (CNBr; Sigma, 300 mg/ml of gel) was added to the gel in in one step. With constant stirring, the reaction was completed within 8-10 minutes. Temperature of the gel solution was constantly kept at 20°C to 4°C with ice flakes, and transferred to the sintered funnel. The gel was washed with 600 ml of cold coupling buffer (0.1 M borate buffer in 0.5 M NaCl, pH 8.0; buffer 5) to remove non-reactive CNBr. After final washing, gel was transferred to beaker containing 15 ml of ice-cold coupling buffer (buffer 5).

b. Preparation of immunoadsorbent column by CNBr-Sepharose 6B

Four milliliters of antiserum against porcine follicular fluid raised in the male rabbit (2.4 mg/ml) was dialyzed against buffer 5 and added to the CNBr-activated Sepharose 6B. The mixed solution was stirred slowly for 2 hours at room temperature. After washing again with buffer 5, 35 ml of blocking buffer (0.1 M glycine in 0.1 M sodium bicarbonate containing 0.5 M NaCl, pH 8.3; buffer 6) was added to the gel and stirred slowly for 2 hours to block unbound reactive CNBr groups. The gel was carefully packed into the 7 x 200 mm chromatography column (Pharmacia Co.). Packed column was thoroughly washed 4 times with alternating aliquots (50 ml) of buffer 5 and washing buffer (0.1 M glycine-HCl buffer in 0.5 M NaCl, pH 4.0; buffer 9). Final bed volume of the column

was 8 ml.

c. Purification of specific proteins from porcine follicular fluid

Two milliliters of porcine follicular fluid (55 mg/ml), previously dialyzed against buffer 5, was applied on the surface of gel in the column and set incubation for 30 minutes at room temperature. After incubation, column was washed with buffer 5 (75-100 ml) until the 280 nm absorbence of the effluent reached blank values. Bound proteins were eluted with elution buffer (0.1 M glycine-HCl buffer in 0.5 M NaCl, pH 2.6; buffer 7). Fractions collected (2 ml/tube) were checked the optical density at 280 nm (Shimadz). After elution, the column was washed thoroughly with the elution buffer (buffer 7) and re-equilibrated with buffer 5 before another sample application. Fractions with 280 nm absorbence were pooled, dialyzed against distilled water, concentrated by lyophilization and kept at -20°C until use.

Identification of purified specific proteins was performed by crossed-immunoelectrophoresis with previously described procedure (Experiment 1; 5 b).

3. Identification of purified specific proteins by polyacrylamide gel electrophoresis

Analytical disc gel electrophoresis was carried out on 7 % polyacrylamide gel by modified methods of Davis (1964). The running gel consisted of 7 % acrylamide (Sigma), 0.186 % bis-acrylamide, 0.025 M Tris-HCl (pH 8.9), 0.05 % ammonium persulfate, and 0.0025 % tetraethyl methylenediamine (TEMED). After mixing and deaeriation, the solution was transferred into an 8 x 100 mm column up to 80 mm height, covered with overlay solution (20 % ethanol in 0.38 M Tris-HCl, pH 8.9) and allowed to polymerize for 2 hours. Spacer gel consisted of 4 % acrylamide, 0.106 % bis-acrylamide, 0.015 M Tris-HCl (pH 6.7), 0.05 % ammonium persulfate and 0.0015 % TEMED. Spacer gel solution was pipetted over the polymerized running gel up to 10 mm height, covered with overlay solution and allowed to polymerize for two hours. About 100 μg of samples with bromophenol blue (0.01 %), as tracking dye, were loaded on each gel. A current of 2 mA/gel tube was applied until the dye front had traveled the spacer gel and 5 mA/gel was loaded when the dye was in running gel. Reservoir buffer (buffer 8) used was 0.01 M Tris-glycine (pH 8.3). The gels were stained with 0.5 % coomassie brilliant blue solution for 30 minutes, fixed with 12.5 % trichloroacetic acid for 30 minutes and then destained in the destaining solution. The gels were preserved in 10 % acetic acid solution.

Experiment 3: *Effect of specific proteins purified from porcine follicular fluid on the maturation of mouse follicular oocytes in vitro.*

Three to four weeks old female virgin mice (A strain) were killed by cervical dislocation and the ovaries were removed acceptically and placed in 5 ml of the operation medium in a sterilized culture dish and washed several times. Follicular occytes were released by puncturing the follicles of the ovaries with a sharp needle under a dissecting microscope (Wild Co., M5A). Only those oocytes with an intact germinal vesicle and free of cumulus cells were used as material for culture.

The culture of follicular oocytes was performed with Brinster's paraffin drop method (1963). Usually 5 drops of the medium were placed in each culture dish (Falcon, #1008) and equal numbers of oocytes were implanted to the culture medium with a finely drawn Pasteur pipette. The culture dish was placed in an incubator kept at 37°C and supplied with 5 % CO_2 in fully moistened air. All glasswares and instruments used in culture work were sterilized

by an autoclave or a hot air oven (160°C), and media were Millipore-filtered before use.

To observe the effect of specific proteins of porcine follicular fluid on the germinal vesicle of mouse oocytes *in vitro*, follicular oocytes were cultured in five different media as follows: (a) SECM (Standard Egg Culture Medium supplemented with 0.4 % bovine serum albumin) mixed with elution buffer, which was dialyzed against deionized water for 3 days to remove glycine-HCl and then against SECM for 2 days to equilibrate before adding to the media, (b) same as *a* but mixed with an equal amount (150 μg/ml) of pig female plasma, (c) same as *a* but mixed with pig follicular fluid (150 μg/ml), (d) same as *a* but mixed with purified specific proteins (fast migrating specific protein and slowly migrating specific protein, 150 μg/ml), which was dialyzed like elution buffer, and (e) same as *d* but with the specific proteins heated at 80°C for two minutes before mixture.

After incubation, the oocytes were fixed in acid-alcohol and stained with 0.5 % acid-lacmoid for examination of the nuclear phases.

RESULTS

Result of Experiment 1: *Detection of specific proteins from porcine follicular fluid*

The protein concentrations of pig follicular fluid, plasma and serum were measured by the methods of Lowry *et al* (1951), using bovine serum albumin as a standard. The protein concentration of follicular fluid (54.06 ± 2.31 mg/ml) was always lower than those of plasma (68.13 ± 3.24 mg/ml) and of serum (66.16 ± 3.02 mg/ml).

Figures 1 and 2 show the rocket-immunoelectrophoresis plate in which antisera absorption with pig female serum was reacted with pig female plasma, pig female serum, and porcine follicular fluid as antigens. The presence of one precipitin in pig female plasma, three precipitins in porcine follicular fluid can be detected in contrast to absence of any in pig female serum. It was found a line of identity with the single precipitin formed in plasma and follicular fluid. This precipitin is assumed to be fibrinogen. Rocket-immunoelectrophoretic analysis of antiserum absorption with pig female serum

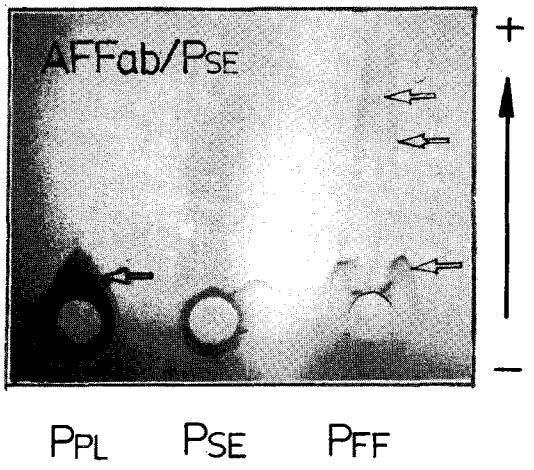

Figure 1. Rocket-Immunoelectrophoresis in 1 % agarose gel mixed with AFFab/P_{SE} (P_{PL} : Pig female plasma, P_{SE} : Pig female serum, P_{FF}: Pig follicular fluid, AFFab/P_{SE} : Pig follicular fluid-rabbit antiserum absorbed with pig female serum).

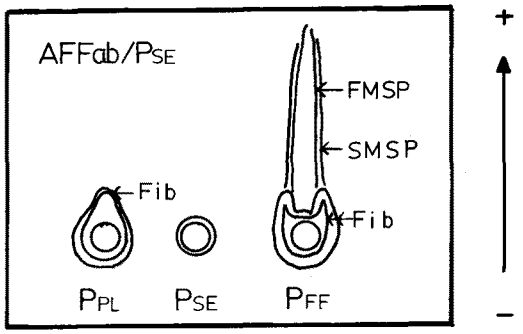

Figure 2. Diagram of rocket-immunoelectrophoresis shown in the figure 1 (Fib; fibrinogen, FMSP; fast migrating specific protein, SMSP; slow migrating specific protein).

detected the presence of two precipitins from porcine follicular fluid, which were absent in both plasma and serum. Staining with coomassie brilliant blue indicated them proteins. These two precipitins were named Fast migrating specific protein (FMSP) and Slow migrating specific protein (SMSP) by their electrophoretic mobilities (Fig. 2).

Crossed-immunoelectrophoresis of antiserum absorption with pig female serum with pig female plasma, serum and follicular fluid (Figs. 3 and 4) also confirmed the presence of two specific proteins in porcine follicular fluid, as well as fibrinogenlike molecule in both follicular fluid and plasma.

These results indicate that porcine follicular fluid contains at least two specific proteins, which were immunogenic to rabbits.

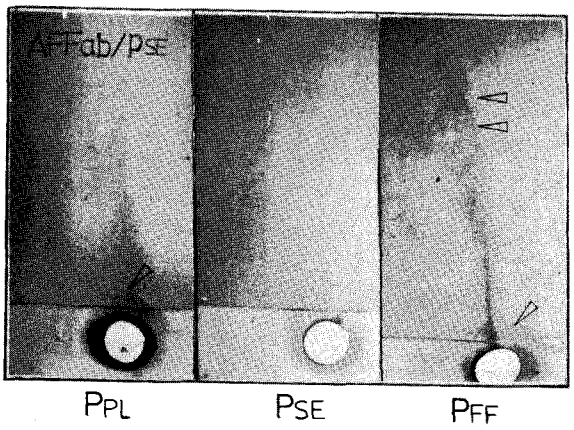

Figure 3. Crossed-Immunoelectrophoresis in 1 % agarose gel mixed with AFFab/P_{SE} in 2nd dimension (P_{PL}; pig female plasma, P_{SE}; pig female serum, P_{FF}; pig follicular fluid, AFFab/P_{SE}; pig follicular fluid-rabbit antiserum absorbed with pig female serum).

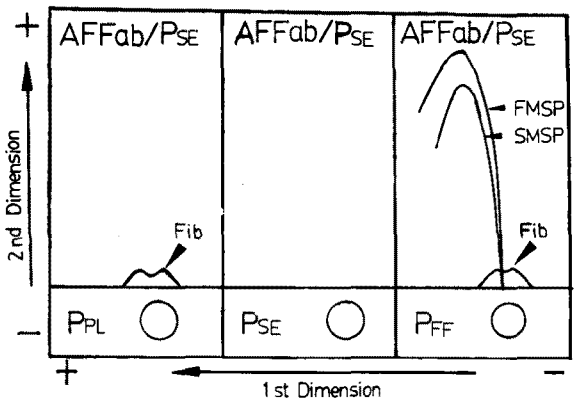

Figure 4. Diagram of Crossed-Immunoelectrophoresis shown in the figure 3 (Fib; fibrinogen, FMSP; fast migrating specific protein, SMSP; slow migrating specific protein).

Results of Experiment 2: *Purification of specific proteins from porcine follicular fluid*

A typical elution profile for 2.0 ml of follicular fluid, plasma and serum in the column of CNBr-Sepharose 6B immunoadsorbent chromatography is shown in Fig. 5. As shown in the figure, porcine follicular fluid has specific proteins fraction curve in the elution buffer, but pig plasma and serum have not any protein fraction curve. This also confirms the fact that porcine follicular fluid has specific

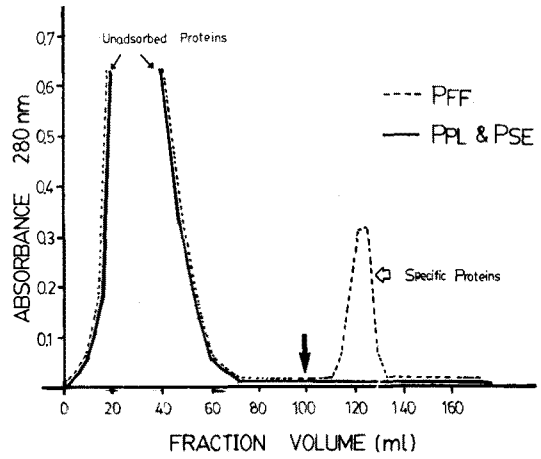

Figure 5. Purification of specific proteins by immunoadsorbent chromatography. Each sample (2.0 ml) was loaded into the column and allowed to incubate for 30 minutes at room temperature. Two milliliters of fractions were collected at a flow rate of 50 ml/hr. Large black arrow indicates change to elution buffer and white arrow indicates specific proteins fraction in P_{FF}.

proteins, which are absent in plasma.

Most fractions of specific proteins were eluted within 40 ml after changing to elution buffer (pH 2.6). Inclusion of the incubation period (30 minutes) prior to washing resulted in incrdasing yield of fractions.

Purity of specific proteins purified was shown in Fig. 6. The immunoelectrophoretic pattern of specific proteins, which were purified by immunoadsorbent chromatography, formed a identity with those precipitins as shown in Figs. 1, 2, 3, and 4. Two precipitins corresponding to Fast and Slow migrating specific proteins are shown, indicating the absence of fibrinogen-like molecule.

To compare where these specific proteins are located in the total protein patterns of follicular fluid, plasma, serum and precipitates of combination of follicular fluid and antiserum absorption with pig female plasma, analytical polyacrylamide gel electrophoresis are shown in Figs. 7 and 8. The electrophoretic mobilities of two proteins, stained with coomassie brilliant blue, were identified by densitogram.

The fast migrating protein represents electrophoretically fast alpha-1 globulin or post albumin mobility and the slow migrating protein represents beta- or slow alpha-2 globulin mobility (Figs. 8 and 9).

The bands of the fast and slow migrating proteins are also detected in the precipitate of mixture of follicular fluid and antiserum absorption with pig female plasma. It was observed that the proteins pattern of the follicular fluid seems to be similar with that of plasma, but different from serum. This observation coincides with other's (Edwards, 1974).

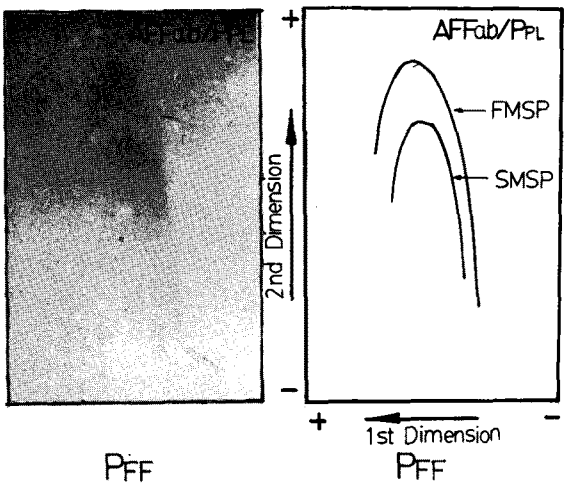

Figure 6. Identification of purified specific proteins by crossed-immunoelectrophoresis.

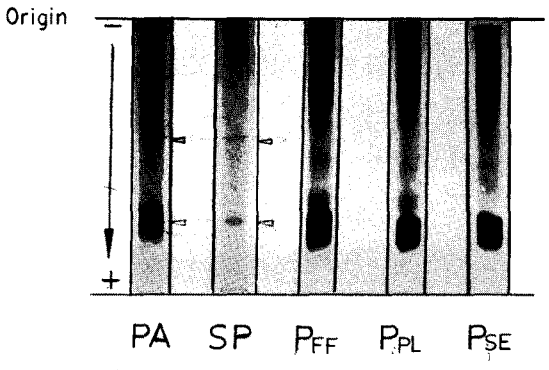

Figure 7. Analytical polyacrylamide gel electrophoresis of purified specific proteins.
PA ; precipitates of P_{FF} + AFFab/P_{PL} dissociated at pH 2.6
SP ; purified specific proteins

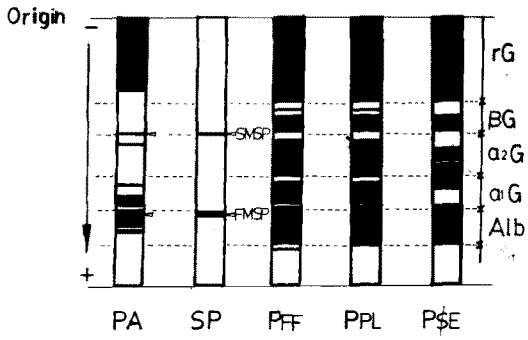

Figure 8. Electrogram of figure 7.
Alb ; albumin, α1G ; alpha-1 globulin, α2G ; alpha-2 globulin
βG ; beta-globulin, bG ; gamma-globulin

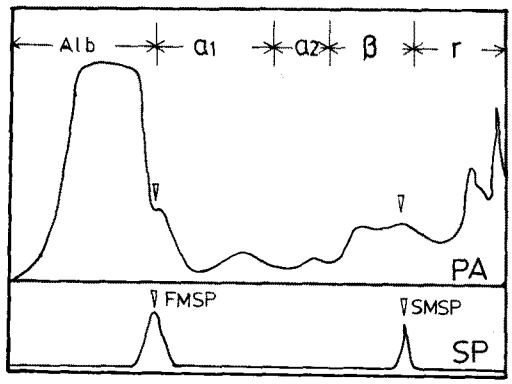

Figure 9. Densitogram of PA and SP to compare the presence of FMSP & SMSP.

Results of Experiment 3: *Effect of specific proteins purified from porcine follicular fluid on the maturation of mouse oocytes in vitro*

To observe the effect of the specific proteins on the maturation of mouse oocytes in *vitro,* experiments were carried out and the results are summarized in Table 1. As the table shows, more than 90 % of the oocytes showed break-down of their germinal vesicle in the media containing various components including heated specific protein within five hours. However, in the medium with non-heated protein, only less than 60 % of them started meiotic resumption. Thus, it is aparant that the specific proteins found in the follicular fluid acts as an inhibiting factor to the meiotic maturation of the oocytes.

DISCUSSION

In general, the total mount of protein in the follicular fluid was less than that in the follicular fluid was less than that in the plasma or in the serum as the findings by other investigators (Shivers *et al*; 1964, McGaughey and Daniel; 1973). Also there have been many attempts to demonstrate any sort of specific

Table Effect of purified specific proteins added to the SECM on the germinal vesicle break-down of the mouse oocytes cultured for 5 hours *in vitro*

Culture medium	Experiment			Total	GVDB %*	Inhibition %**
	I	II	III		Mean±S.D.	Mean ± S.D.
SECM + EBD[a]	28/30	29/32	26/28	83/90	92.26±1.44	0.00
SECM + PPL[b]	28/29	28/30	24/26	80/85	94.06±2.21	- 1.95±2.21
SECM + PFF[c]	32/37	25/30	20/26	77/93	82.24±4.87	10.84±5.47
SECM + SP[d]	21/33	21/30	12/26	54/89	59.92±12.34	34.95±14.03
SECM + (SP)[e]	42/44	32/34	32/36	106/114	92.81±3.47	- 0.61±4.30

* ; No. of oocytes GVBD / No. of oocytes cultured.

** ; Relative % of GVBD inhibition to EBD[a] as control.
The percent inhibition of oocyte maturation was calculated as follows:

$$\% \text{ inhibition} = 100 \times \frac{(\% \text{ maturation of control} - \% \text{ maturation of sample})}{\% \text{ maturation of control}}$$

a ; Elution buffer dialyzed against SECM to use as control.

b ; 150μg/ml of pig female plasma.

c ; 150μg/ml of pig follicular fluid.

d ; 150μg/ml of purified specific proteins.

e ; 150μg/ml of purified specific proteins heated at 80°C for 2 mins.

proteins present only in the follicular fluid obtained from the mammals such as human, pig and cow, although it is generally inferred by the investigators that the follicular fluid is derived *via* selective transudation from plasma and is devoid of any specific macromolecule which might be related with a role in follicle growth and maturation (Beck and Sheldon; 1972). These assumptions are strongly acceptable because of the presence of fibrinogen in the follicular fluid (Manarang-Pangan and Menge; 1971, Desjardins *et al.*; 1966, Shivers et al.; 1964, Shalgi *et al.;* 1973, Edwards *et al.;* 1974). Non the less, it has not been possible to discount the theoretical assessment that the granulosa cells may produce specific proteins in the follicle. The possibility has been raised by the fact that the antigens might exist in follicular fluid, and they are not precipitated by antibodies in the normally employed immunological tests (Manarang-Pangan and Menge; 1971).

Herve *et al.* (1968) showed the immunologic detection of various components in human follicular fluid. They found two antigens in agar gel diffusion, or immunoelectrophoresis using antisera against human follicular fluid. Shivers *et al.* (1964) once failed to find specific proteins in the pig follicular fluid. However, the present study succeeded to show at least two kinds of specific proteins in the pig follicular fluid like as shown in human one (Manarang-Pangan and Menge; 1971). Thus, the specific proteins are known to act as an immunogenic substance.

It is assumed that inhibitor(s) present in specific proteins is not identical with the oocyte maturation inhibitor (OMI) in pig follicular fluid found by Tsafriri *et al.* (1976) or by Stone *et al.* (1978). Because OMI seems to be polypeptide with a molecular weight of 2,000 daltons, it cannot act as an immunogen without aid of hapten carrier molecules. Furthermore, OMI is present in the dislyzable fraction in follicular fluid. Compared to OMI, however, the specific proteins are immunogenic and present in the non-dialyzable fraction of follicular fluid. It has been already suggested that the non-dialyzable fraction of bovine follicular fluid has the antimeiotic factor(s) (Cho and Lim; 1975). It is assumed that the inhibitor(s) of specific proteins upon oocyte maturation appears to come from the granulosa cells, because it has been reported that granulose cells and granulosa cell extract exhibit the inhibitory action upon oocyte maturation (Tsafriri and Channing; 1975a and b, Tsafriri *et al.* 1976).

Mcgaughey (1975) reported that; (1) a high proportion of fluid from small pig follicules (3-4.5 mm) exhibits a prominent fast migrating alpha globulin band in electrophoresis, compared with a high proportion of fluids from large pig follicles (5-10 mm) exhibiting a prominent slow-migrating alpha globulin band, (2) fluids from small follicles and some large follicles support the oocyte maturation *in vitro,* whereas some other fluids from large follicles inhibit the oocyte maturation.

Furthermore, it was observed by electrophoretically in our preliminary experiments that as the follicles grow, the amount of proteins corresponding electrophoretically to FMSP decreases, whereas that to SMSP increases. With these facts, it is presumed that SMSP seems to have inhibitory activity.

Takikawa (1966) demonstrated that porcine follicular fluid seems to contain estrogen-binding and progesterone-binding proteins. Schwartz and Channing (1977) and Marder *et al.* (1977) exhibited the presence of an inhibitor of serum FSH in the fluid from porcine ovarian follicles, named folliculostatin, shown to be non-steroidal, trypsin sensitive and heat stable at 60°C for 20 minutes, but heat labile at 80°C for 30 minutes, suggesting a molecular weight above 10,000. It was also found that folliculostatin

decreases as the follicles grow. Sato *et al.* (1977 and 1978) have demonstrated the presence of an inhibitor in the non-dialyzable fraction of bovine and porcine follicular fluid which occurs in mice. The electrophoretic mobility of that inhibitor seems to be similar to that of FMSP. Compared to the above facts, it can be assumed that FMSP seems to be hormone-binding or transporting portein.

It is also possible that FMSP or SMSP might act as an inductor or a repressor in controlling the development of follicular oocytes.

An important result of the present study is the purification of inhibitory active protein(s) from porcine follicular fluid.

SUMMARY

It has been already suggested that specific macromolecules in follicular fluid produced by granulosa cells may play a role in suppressing further meiotic maturation of the oocytes. In general, the search for specific macromolecules in follicular fluid using immunological methods has not been rewarding.

These studies were designed, by applying more effective immunological methods than conventionally employed, (1) to identify whether some unknown macromolecules are present in the porcine follicular fluid or not, and (2) to clarify the relationship between the oocytes and the specific macromolecules in the follicular fluid.

The results obtained were as follows;

(1) porcine follicular fluid contained two specific proteins, which were not present in pig plasma and serum.

(2) each of two proteins showed electrophoretically fast alpha-globulin and beta-globulin mobilities.

(3) these proteins seemed to have inhibitory effect on the maturation of mouse oocytes *in vitro*.

From these results, it can be assumed that pig follicular fluid contains specific proteins which seem to be intra-follicular inhibitor(s) of oocyte maturation.

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