Induction of Cumulus Expansion and Maturation Division of Porcine Oocyte-Cumulus Complexes *in vitro*

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(Received January 13, 1982)

Abstract Porcine oocyte-cumulus complexes were cultured in various media in order to induce cumulus expansion and maturation division *in vitro*. When the complexes were cultured in the basic medium (modified Krebs-Ringer bicarbonate medium), 83.1% of oocytes resumed maturation division and 63.5% reached metaphase II in 48-54 hr, but cumulus expansion did not occur. Resumption of maturation division was not affected by the addition of only 10 i.u./ml PMSG or hCG, but was distinctly inhibited by the addition of 1 or 5% fetal calf serum (FCS). Expansion of cumulus mass did not take place in the medium supplemented with either FCS or gonadotrophin. By contrast, in the medium containing both 1% FCS and gonadotrophin (10 i.u. PMSG or hCG), cumulus expansion was induced in high percentages (86.0-89.1%) and 67.5-78.8% of oocytes reached metaphase II. The proportion of oocytes reaching metaphase II was reduced when 5% FCS was added together with PMSG or hCG. Ultrafiltrated fraction of FCS (molecular weight <10,000) exerted similar effects as whole FCS. It is suggested that gonadotrophin promotes both germinal vesicle breakdown and cumulus expansion in the presence of low molecular component(s) of FCS.

Jpn. J. Zootech. Sci., 53 (7): 480-487, 1982

Mammalian follicular oocyte arrested at diplotene stage is known to be capable of resuming the process of meiosis when liberated from the ovarian follicle and then cultured *in vitro*¹⁻⁴⁾. In the pig, influences of various culture conditions⁵⁻¹²⁾ and follicular components¹³⁻¹⁷⁾ on meiosis have been studied. However, the relationship between nuclear maturation and the changes in the cumulus cells surrounding the oocytes has not been fully explored yet.

The present study was to examine the culture conditions necessary for the induction of cumulus expansion and maturation division of porcine oocyte-cumulus complexes *in vitro*.

Materials and Methods

1) Collection of oocyte-cumulus complexes

Ovaries of prepuberal gilts were obtained from recently slaughtered animals in Towada City abattoir. The ovaries were excised within 20-30 min of the donor's

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death and were transported to the laboratory in warm saline containing 50 $\mu\text{g}/\text{ml}$ streptomycin sulphate and 100 i.u./ml penicillin G potassium at 30–33°C. Oocyte-cumulus complexes (Fig. A) were recovered by puncturing the ovarian follicles (2–5 mm in diameter) with a gauge needle. The complexes from individual animals were pooled in 0.4 ml droplet of medium covered with paraffin oil, then washed twice in fresh droplet and transferred to culture medium. All 94 oocytes examined immediately after the start of culture had an intact germinal vesicle.

2) Culture medium and conditions

The basic medium used throughout this study was a medium for *in vitro* fertilization of mouse oocytes devised by TOYODA *et al.*¹⁸⁾, consisting of NaCl, 119.37 mM; KCl, 4.78 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.71 mM; KH_2PO_4 , 1.19 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.19 mM; NaHCO_3 , 25.07 mM; bovine serum albumin (Fraction V, Wako Pure Chemicals), 4 mg/ml; penicillin G potassium, (100 i.u./ml) and streptomycin sulphate (50 $\mu\text{g}/\text{ml}$). Fetal calf serum (FCS, Gibco) was treated at 56°C for 30 min and was added to the basic medium just before use. Fresh culture medium was prepared in each experiment, sterilized through a Millipore filter (pore size, 0.45 μm) and 0.4 ml of the medium was placed in a Petridish (10×35 mm, Lux) and was covered with 5–6 ml of sterile paraffin oil. The dish was placed in a CO_2 -incubator and kept overnight for equilibration under 5% CO_2 in humidified air at 37°C.

Stock solutions of PMSG (Serotropin, Teikoku Zoki) and hCG (Puberogen, Sankyo Zoki) were prepared by dissolving the gonadotrophins in sterile saline and were stored at –80°C in small aliquots. Four μl of the stock solution was added to 0.4 ml culture medium immediately before the start of culture to reach the final concentration of 0.1, 1.0 or 10 i.u./ml PMSG or 1.0, 10 or 100 i.u./ml hCG, respectively.

Ultrafiltration of FCS was carried out with immersible ultrafiltration unit for small volume (molecular weight <10,000; Millipore Co.) at 4°C. The ultrafiltrated fraction was heated at 56°C for 30 min prior to use and added to the basic medium.

Ten to 15 complexes were placed in each droplet of the medium and were cultured for 48–54 hr.

3) Evaluation of cultured oocyte-cumulus complexes

After cultivation for 30 or 36 hr *in vitro*, the states of cumulus cell layers were observed under a inverted microscope and classified as expanding, i.e. individual cells of cumulus were spread apart and were embedded in sticky intercellular substance, or not expanding, i.e. cumulus cells were detaching from the oocyte or were surrounding the oocyte tightly adhering each other. Examination of oocytes was conducted 48–54 hr after cultivation. At the end of culture, the oocytes were mechanically denuded of surrounding cells with finely drawn Pasteur pipette in the medium containing 0.1% hyaluronidase, washed in fresh medium and mounted on a slide under a coverslip. The oocytes were fixed in acetic acid:ethanol (1:3 v/v), stained with 1% acetic orcein and were examined under a phase contrast microscope. The stages of meiotic maturation were classified according to the criteria of HUNTER and POLGE¹⁹⁾. The results were statistically analysed by χ^2 -test.

Results

1) Oocyte-cumulus complexes cultured in the basic medium or in medium containing either gonadotrophin or fetal calf serum

In both the basic medium (control group) and the medium containing either gonadotrophin or FCS, expansion of cumulus was not observed (Table 1). While almost all of the cumulus masses in medium containing FCS were adhering to the bottom of culture dish, those cultured without FCS did not adhere to the dish but floating in the medium.

Table 1. Effects of gonadotrophins (GTH) and fetal calf serum (FCS) on the expansion and maturation division of porcine oocyte-cumulus complexes *in vitro*.

Additions		No. of cumulus examined	Cumulus cell layers		No. of oocytes examined	Maturation division			Deg.
GTH (10 I. U./ml)	FCS (%)		Not expanding	Expanding		G. V.	Promet. I —Telo. I	Met. II (%)	
—	—	148	148	0	148	24	29	94 (63.5) ^a	1
PMSG	—	77	77	0	67	2	29	36 (53.7) ^a	0
hCG	—	35	35	0	34	1	7	26 (76.5) ^a	0
—	1	55	55	0	48	34	3	11 (22.9) ^b	0
—	5	55	55	0	53	48	1	4 (7.5) ^c	0

G. V.: Germinal vesicle, Promet. I: Prometaphase I, Telo. I: Telophase I, Met. II: Metaphase II, Deg.: Degeneration. Significance of difference; a vs b, c: $P < 0.001$, b vs c: $P < 0.05$

Of 148 oocytes cultured in the basic medium, 24 had a germinal vesicle, 29 had the chromosomes from prometaphase I to telophase I of meiotic division, and 94 (63.5%) completed the first meiotic division with extruded first polar body. In the medium containing PMSG or hCG, the number of oocytes reaching metaphase II was not significantly different from that in the basic medium ($P > 0.1$). In contrast, cultures in the medium containing 1 or 5% FCS led to a marked decrease in the percentage of maturing oocytes ($P < 0.001$ as compared with other groups). Furthermore, increasing the concentration of FCS from 1 to 5% resulted in a decrease in the number of oocytes reaching metaphase II (22.9% vs 7.5%) and an increase in the number of those having intact germinal vesicle (71% vs 91%).

2) Cumulus expansion and maturation division in media containing both gonadotrophin and FCS

a) Effects of various concentrations of FCS

As shown in Table 2, when the complexes were cultured in the medium containing only 10 i. u./ml PMSG or hCG, none of the cumulus mass was expanding, but adding FCS to the medium containing either PMSG or hCG resulted in sticky intercellular substance and expanding cumulus mass (Fig. B). The morphological appearance of the expanded cumulus mass resembled those surrounding the oocyte shortly after ovulation (Fig. C).

Maturation of Porcine Oocyte-Cumulus Complexes

Table 2. Effects of various concentrations of FCS on the expansion and maturation division of porcine oocyte-cumulus complexes *in vitro*

GTH (10 I. U./ ml)	Conc. of FCS (%)	Cumulus cell layers		Maturation division			Deg. •
		Not expanding	Expanding (%)	G. V.	Promet. I —Telo. I	Met. II (%)	
PMSG	0	77	0 (0)	2	29	36 (53.7) ^a	0
	0.1	90	4 (4.3)	6	25	55 (61.8) ^a	3
	1	13	106 (89.1)	1	21	82 (78.8) ^b	0
	5	10	85 (89.5)	33	13	43 (48.3) ^a	0
hCG	0	35	0 (0)	1	7	26 (76.5) ^c	0
	0.1	92	2 (2.1)	9	13	65 (74.7) ^c	0
	1	12	74 (86.0)	13	13	54 (67.5) ^c	0
	5	17	28 (62.2)	29	3	13 (28.9) ^d	0

Significance of difference; a vs b: $P < 0.01$, c vs d: $P < 0.01$

Explanation of the plate

Photomicrographs of porcine oocyte-cumulus complexes using Nomarski optics. All are fresh whole mount preparations.

- A. An oocyte-cumulus complex at the start of culture. The cumulus is not expanded and tightly surrounds the oocyte. Approx. $\times 270$.
- B. An oocyte-cumulus complex after 36 hr of incubation in medium containing 1% FCS and 10 I.U./ml PMSG. The cumulus is expanded and the complex morphologically resembles to the freshly ovulated oocyte (Fig. C). Approx. $\times 140$.
- C. An ovulated oocyte recovered from the gilt which was treated with 750 I.U. PMSG followed 72 hr later with 500 I.U. hCG and killed 47 hr after hCG. The cumulus cells surrounding an oocyte are expanded radially. Approx. $\times 140$.

Cumulus expansion was induced in more than 80% of oocyte-cumulus complexes in the media containing 1 or 5% FCS together with PMSG and also in those containing 1% FCS and hCG, although the percentage was slightly reduced in the medium containing 5% FCS and hCG. The highest percentage of oocytes which reached metaphase II (78.8%) was obtained in the medium containing 1% FCS and 10 i.u./ml PMSG. When the concentration of FCS was increased to 5%, the percentages of oocytes reaching metaphase II were significantly reduced both in media containing PMSG and hCG ($P < 0.01$).

b) Effects of various concentrations of PMSG and hCG

Since the results in Table 2 indicated that 1% was optimal concentration of FCS for cumulus expansion and maturation division in both media containing 10 i.u./ml of PMSG and hCG, further experiments were conducted to examine the effects of various concentrations of gonadotrophins in the medium containing 1% FCS. The percentages of the complexes with expanding cumulus and chromosomes at metaphase II were raised with increasing the concentration of PMSG from 0.1 to 1 i.u./ml, although there was not statistically significant difference in the proportion of expanded cumulus between 1 and 10 i.u.. When the concentration of hCG was increased from 1 to 10 i.u./ml, the percentages of the complexes with expanding cumulus and at metaphase II were increased, whereas further increasing to 100 i.u./ml was not effective.

Table 3. Effects of various concentrations of gonadotrophins on the expansion and maturation division of porcine oocyte-cumulus complexes in the medium containing 1% FCS

GTH	Conc. (I.U./ ml)	Cumulus cell layers		Maturation division			Deg.
		Not expanding	Expanding (%)	G. V.	Promet. I —Telo. I	Met. II (%)	
	0	55	0 (0)	34	3	11 (22.9) ^f	0
PMSG	0.1	63	17 (21.3) ^a	44	16	13 (17.8) ^f	0
	1	1	24 (96.0) ^b	4	6	14 (58.3) ^g	0
	10	13	106 (89.1) ^b	1	21	82 (78.8) ^g	0
hCG	1	20	5 (20.0) ^c	18	3	3 (12.5) ^h	0
	10	12	74 (86.0) ^d	13	13	54 (67.5) ⁱ	0
	100	34	34 (50.0) ^e	14	25	27 (40.9) ^j	0

Significance of difference; a vs b: $P < 0.001$, c vs d, d vs e: $P < 0.001$, c vs e: $P < 0.02$, f vs g: $P < 0.01$, f vs h: $P > 0.5$, f vs j, h vs j: $P < 0.05$, i vs j: $P < 0.01$, f vs i, h vs i: $P < 0.001$

3) Effects of ultrafiltrated fraction (UF) of FCS on cumulus expansion and maturation division

In the complexes cultured in the basic medium supplemented with only UF, cumulus expansion was not observed (Table 4). The percentages of the oocytes that

Maturation of Porcine Oocyte-Cumulus Complexes

Table 4. Effects of ultrafiltrated fraction (UF) of FCS on the expansion and maturation division of porcine oocyte-cumulus complexes *in vitro*

Additions		Cumulus cell layers		Maturation division			Deg.
GTH (10 I. U./ml)	UF ^a (%)	Not expanding	Expanding (%)	G. V.	Promet. I —Telo. I	Met. II (%)	
—	1	50	0(0)	24	9	12(26.1)	1
	5	44	0(0)	30	7	6(14.0)	0
PMSG	1	5	59(92.2)	13	19	29(46.0) ^d	2
	5	2	63(96.9)	37	20	8(12.3) ^e	0
hCG	1	9	35(79.5) ^b	16	5	21(50.0) ^f	0
	5	1	54(98.2) ^c	26	11	14(27.5) ^g	0

a: Molecular weight <10,000. Significance of difference; b vs c: $P < 0.01$, d vs e: $P < 0.01$, f vs g: $P < 0.05$

reached metaphase II were 26.1% and 14.0% in the medium containing 1% or 5% UF, respectively. More than half of the oocytes examined had a germinal vesicle at the end of culture. On the other hand, when both gonadotrophin (PMSG or hCG) and UF were added to the basic medium, expansion of cumulus was induced in almost all of the complexes cultured, the number of oocytes with a germinal vesicle was remarkably reduced ($P < 0.001$) and higher proportions of oocytes reached metaphase II. In the medium containing 5% UF and gonadotrophin, cumulus expansion occurred as efficiently but resumption of the meiosis was distinctly lower as compared with 1% UF.

Discussion

The present investigation indicates that maturation division accompanied with cumulus expansion which is characteristic to the *in vivo* matured oocytes can be induced *in vitro* if the complexes were cultured in the medium containing both gonadotrophin (PMSG or hCG) and FCS. It is clear that component(s) of FCS in addition to gonadotrophin is essential for the induction of cumulus expansion but not for maturation division *in vitro*, since cumulus mass did not expand but meiosis was resumed in the medium containing only PMSG or hCG. As the result obtained here is in agreement with the observations in the mouse²⁰⁻²²⁾ and rat^{23,24)}, it is considered that conditions needed for induction of cumulus expansion *in vitro* is essentially the same for the different species. MEINECKE and MEINECKE-TILLMANN¹²⁾ has reported that expansion of porcine cumulus mass took place only in the medium containing PMSG but not FSH or hCG. The basic medium used in their experiment was TCM-199 supplemented with glutamine, ascorbic acid, Na-pyruvate and bovine serum albumin and did not contain serum.

EPPIG²⁰⁾ has shown that mouse cumulus expansion *in vitro* was dependent upon the presence of some high molecular weight ($> 10,000$) component of serum. In the experiments reported here, expansion of cumulus was induced in the medium containing

UF of FCS and gonadotrophin. From this result, it is inferred that effective factor(s) in FCS for the cumulus expansion of porcine oocyte-cumulus complex has a molecular weight of less than 10,000 and is stable to heat.

It has been reported that porcine follicular fluid^{13,14,16,17)} and granulosa cells^{13,15)} inhibit the maturation division of porcine oocytes *in vitro* and gonadotrophin counteracts the inhibitory effect of porcine follicular fluid^{14,16,17)}. However, there have been no reports that FCS inhibits the resumption of oocyte meiosis. In this study, it is suggested that inhibitory factor(s) for oocyte maturation in FCS resembles the effective factor(s) for the cumulus expansion because both phenomena were induced by the addition of UF to the basic medium and both activities of FCS involve gonadotrophin. This is in accord with the reported action of gonadotrophin (LH) to the oocyte maturation inhibitor from porcine follicular fluid^{14,16,17)}. It is considered that gonadotrophin induces not only the cumulus expansion but also the resumption of oocyte meiosis *in vitro* as well as *in vivo*. Effects of homologous serum and follicular fluid on porcine oocyte-cumulus complexes are currently being investigated.

The timing of maturation division of porcine oocytes cultured in the basic medium was similar, if not identical, to that reported by MOTLIK⁵⁾, in good agreement with the timing of *in vivo* maturation described by HUNTER and POLGE¹⁹⁾. On the other hand, SATO *et al.*¹¹⁾ have indicated that the rate of maturation division of porcine oocytes *in vitro* was affected by the culture conditions and proportion of oocytes matured to metaphase II became nearly constant after 30 hr of culture in optimal condition. More recently, AINSWORTH *et al.*²⁵⁾ have reported that expansion of the cumulus mass took place 24 hr after hCG injection in the gilts treated with PMSG and hCG. In this study, cumulus expansion was observed 30 hr in culture. Therefore, it seems that the timing of cumulus expansion *in vitro* coincides with the process occurring *in vivo*. As it has been pointed out in several species such as rabbits⁴⁾ that the *in vitro* matured oocytes can be penetrated by spermatozoa but have no ability to change the sperm head into male pronucleus, it would be interesting to know whether *in vitro* matured oocyte-cumulus complex that appears normal should have full fertilizing ability.

Acknowledgements

This research was supported by a grant-in-aid for scientific research No. 556189 from the Ministry of Education, Science and Culture of Japan. The authors wish to thank Miss Mika KUBO, Miss Kimiko MUNEKADO and Mr. Jun NAKURA for their assistance in the experiment and Miss Ruriko SAKURADA for her help in preparing the manuscript.

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体外培養による豚卵母細胞—卵丘細胞複合体の成熟誘起について

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未成熟豚の卵巣から採取した卵丘細胞層に包まれた卵母細胞を種々の培地内で培養し、成熟誘起を試みた。基礎培地で培養された卵母細胞では148個中123個(83.1%)に卵核胞の崩壊が起り、94個(63.5%)が第2成熟分裂中期(Met. II)に達していた。基礎培地に10 I. U./mlのPMSGまたはhCGを添加した区では、それぞれ53.7%(36/67)および76.5%(26/34)がMet. IIに達した。一方、1%または5%牛胎児血清(FCS)添加区では、それぞれ70.8%および90.6%が卵核胞期にとどまっていた。卵丘細胞層の膨化は基礎培地にFCSまたは性腺刺激ホルモンのいずれか一方を添加しても起らなかったが、1%FCSとPMSGまたはhCGが共存する培地では、それぞれ89.1%および86.0%で卵丘細胞層の膨化が認められた。同時に、78.8%および67.5%がMet. IIに

達していた。5%FCSとPMSGまたはhCGの組み合わせでは、卵丘細胞層の膨化がそれぞれ89.5%および62.2%認められたが、Met. IIに達した割合はそれぞれ48.3%および28.9%であった。FCSの代りに、FCSの限外濾過分画(分子量10,000以下)を用いた区においてもほぼ同様の傾向が認められた。これらのことから、1)基礎培地内では卵母細胞の成熟分裂の誘起は可能であるが、卵丘細胞層の膨化は起らないこと、2)性腺刺激ホルモンは、卵核胞崩壊を促進する作用と卵丘細胞層の膨化を引き起す作用を有すること、および3)FCS中には卵丘膨化作用の発現に必要な成分と卵核胞崩壊を抑制する成分が存在し、その両者の成分は分子量10,000以下であることなどが明らかにされた。

日畜会報, 53 (7): 480-487, 1982