Biochemical Compositions of Follicular Fluid and the Effects of Culture Conditions on the *In Vitro* Development of Pig Oocytes

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ABSTRACT : The aims of this study were, firstly, to analyze the biochemical compositions of serum and follicular fluid (FF) from prepubertal gilts after PMSG (1,000 IU) treatment. The concentrations of total proteins, lipids, cholesterol, glucose and sex hormones (progesterone, P_4; estradiol-17β, E_2; testosterone, T) were measured. Secondary, the effects of porcine FF (pFF) addition (40% and 100%) in IVM media and different culture conditions [Exp. 1: mBMOC-2+20% porcine serum (PS), fresh IVM medium, filtered IVM-conditioned medium, or rabbit oviducts; Exp. 2: mBMOC-2+20%PS or stepwise medium replacement procedures (SMRP) cocultured with or without cumulus cells] on the *in vitro* development (IVD) of porcine oocytes were also examined. Results showed that no significant differences were found in total protein levels between serum and pFF from different sizes (large, >7 mm; medium, ~5-7 mm; small, <3-5 mm) of follicles (75-85 and 49-90 mg/dl; $p>0.05$). Total lipid concentrations remained constant in serum (395-472 mg/dl), and reduced significantly in the pFF from large follicles (287 mg/dl) at 132 h after PMSG treatment when compared to those at other time points (441-480 mg/dl). Basal cholesterol levels in serum and pFF at 12 h were similar (153-161 mg/dl), but increased at 36 h (186-197 mg/dl). Basal P_4 and E_2 levels in serum (0.1 ng/ml and 5.5 pg/ml) were low, but increased from 0.34 ng/ml and 12.13 pg/ml at 24 h to 0.81 ng/ml and 61.70 pg/ml at 98 h, respectively, after PMSG treatment ($p<0.05$). P_4 levels increased linearly in pFF from large follicles during 12 through 132 h (138-1,288 ng/ml). A similar increase was also observed in E_2 levels (22-730 pg/ml) before 60 h post PMSG treatment, and then dropped afterwards (730-121 pg/ml). The development of the oocytes fertilized in 40% pFF-medium was greater than that in 100% pFF-medium group without gonaodtropin addition (31% vs 10%, $p<0.05$). However, both were lower than those in mBMOC-2+20%PS and in rabbit oviducts ($p<0.05$). When cocultured with cumulus cell monolayers, a greater cleavage rate was observed in the group cultured in filtered IVM-conditioned medium than the SMRP group (36% vs 18%, $p<0.05$). A similar phenomenon was also observed in the culture without cumulus cell monolayers (33% vs 19%, $p<0.05$). It is concluded that neither the fresh IVM nor filtered IVM-conditioned medium has positive effect on the IVD of oocytes. Coculture with cumulus cell monolayers and the SMRP were not beneficial to the development of IVF pig oocytes. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 10 : 1403-1411)***

Key Words : Protein, Lipid, Cholesterol, Sex Hormones, IVMFC

INTRODUCTION

The female germ cells grow and are eventually liberated from the follicles under hormonal controls (Kihara et al., 2000). The activity of ovary is characterized by alternative phases of growth and regression between follicular and luteal structures (Barboni et al., 2000). Accumulation of FF is a characteristic feature of maturing follicles in most mammals (Kihara et al., 2000). It has been shown that FF contains proteins, amino acids, sugars, enzymes, mucopolysaccharids, hormones (LH, FSH, prolactin, oestrogens, androgens and progestagens) and salts. (Guraya, 1985; Hafez, 1987). These components play a major role in biochemical and metabolic processes during oocyte maturation (Hafez, 1987). In addition, FF has also been shown to promote sperm motility, capacitation and acrosome reaction (Brüssow et al., 1999). Yanagimachi (1969) reported that large volume of FF is released from mature follicles during ovulation. A portion of this fluid is transported into the oviduct with the cumulus-oocyte complexes. However, it has been estimated that less than 1% of the FF retained in the oviduct after ovulations (Hunter, 1990). The biochemical compositions, properties, and possible physiological roles of FF have been extensively studied (Guraya, 1985). However, changes of the FF composition after gonadotropin treatment have not been critically evaluated.

Maturation and developmental competence of pig oocytes are influenced by the presence of pFF in the medium and the size of the follicle from which pFF is harvested (Naito et al., 1988, 1989, 1990; Wu et al., 1991; Huang et al., 2001). Although previous study indicated that addition of pFF from small and medium follicles inhibits the *in vitro* maturation (IVM) of porcine oocytes (Tsafriri and Channing, 1975), sperm penetration, normal fertilization and even oocyte maturation rates were improved by the addition of pFF from large follicles (Naito et al., 1988; Yoshida et al., 1990, 1992; Funahashi and Day, 1993ab). Furthermore, male pronuclear formation was greatly enhanced while the oocytes were cocultured with the follicular shells from large follicles. (Ding and Foxcroft, 1992). Our previous study also indicated that porcine

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oocytes had better maturation and cleavage rates when cultured in the medium containing 40% pFF and gonadotropins. Blastocyst formation was only observed when embryos were transferred into rabbit oviducts (Huang et al., 2001).

The objectives of this study were to analyze the biochemical components of pFF including the total proteins, lipids, cholesterol, glucose and the sex hormones (P_4, E_2, T) in both serum and pFF from different follicular sizes after PMSG treatment. The effects of different culture systems, including fresh IVM medium, filtered IVM-conditioned medium, mBMOC-2+20%PS, rabbit oviducts, and cumulus cell coculture, on the development of IVF pig oocytes were also compared in this study.

MATERIALS AND METHODS

Animals and treatments

Thirty seven prepubertal crossbred gilts (DL, 5-6 month-old, 88-112 kg) were used in this study. Following intramuscular injection of PMSG (1,000 IU, Sergona, China Chemical and Pharmaceutic, Taiwan), animals (6/group) were ovariectomized at 0, 12, 36, 60, 84, 108 and 132 h by laparotomy. Blood samples were collected from the anterior vena cava of the animals at 24, 48, 72 and 96 h after PMSG and stored at 4°C for serum preparation.

Preparation of sera and follicular fluids

The pFF was aspirated from the follicles of different sizes (diameter: small, ≤3 mm; medium, 3-7 mm; large, ≥7 mm) and was centrifuged at 1,000×g for 10 min. The supernatant was collected and supplemented with 50 μg/ml of Dibekacin sulfate (Meiji, Japan) and then stored at -20°C. For serum preparation, animals were bled around 3 to 4 pm for four consecutive days after PMSG treatment. Fifteen ml of blood sample were collected from the anterior vena cava of the animals and kept at 4°C for 3 to 4 h. Serum was separated by centrifugation for 10 min at 800×g and stored at -20°C until use.

Measurements of the biochemical components and hormones

The pFF samples and sera were assayed for the concentrations of the total proteins (Lowry et al., 1951), lipids (Falhot et al., 1973), cholesterol (Ellefson and Caraway, 1975) and glucose (Lin et al., 1977) using the methods described in previous studies. As insufficient pFF was collected at 12 h post-PMSG treatment, only samples from the medium and large follicles were analyzed. The levels of P_4, E_2, and T in sera and FF were measured using radioimmunoassay kits purchased from Coate A Co. (USA).

Oocyte collection and IVMFC

Porcine oocytes for *in vitro* maturation, fertilization and culture (IVMFC) were collected from slaughterhouse ovaries. Transportation of the ovaries to the laboratory was carried out in a Dewar flask containing Dulbecco's phosphate-buffered saline (DPBS, Gibco 450-1300) supplemented with 100 IU/ml Penicillin and 100 μg/ml Streptomycin (Penstrep®, Gibco) in a 35°C thermal container. Antral follicles of 2 to 6 mm in diameter were aspirated and cumulus-oocyte-complexes (COCs) were collected in DPBS containing 2% new born calf serum (NBCS). Oocytes with homogenous cytoplasm surrounded by at least 3 layers of compact cumulus cells were used. The procedures for NBCS preparation were described in previous reports (Wu et al., 1991; Huang et al., 2001).

The protocols for IVMFC were based on Cheng (1985) and Huang et al. (2001). Briefly, freshly ejaculated boar semen (sperm-rich fraction) was kept at 18°C for 16 h. Five ml of semen (1×10^9 spermatozoa/ml) were mixed with an equal volume of saline containing BSA Fraction V (1 mg/ml, Sigma, St. Louis, MO, USA) and Dibekacin sulfate (50 μg/ml, Meiji, Japan) 2.5 h before insemination. After centrifugation (200×g, 10 min), the top 5 ml of the supernatant containing spermatozoa were mixed with 5 ml washing medium, and then were centrifuged at 600×g for 10 min. The pellet was washed with another 10 ml of washing medium and centrifuged again (600×g, 10 min). The sperm pellet was harvested and resuspended in 2 ml of capacitation medium, and then stored at 39°C for 90 min. The sperm concentration was adjusted to $0.1\text{-}1\times10^9$/ml. After IVM, the COCs were rinsed three times and then incubated with sperm suspension ($0.1\text{-}1\times10^6$/ml) at 39°C (5% CO_2 in air) for 6 to 8 h. The IVF oocytes were stripped off the cumulus cells and excessive sperms, washed, and transferred to fresh IVC media.

Specific experiments

To test the effects of different culture conditions supplemented with pFF, two independent experiments were performed. In the first experiment (Exp. 1), the influences of culture media or environments on the development of the IVF porcine oocytes were evaluated. The COCs were matured in IVM medium containing 40% or 100% pFF and were inseminated by direct addition of capacitated sperm into the IVM medium. Developmental competence was evaluated after cultured in mBMOC-2+20%PS, fresh IVM, filtrated IVM-conditioned media or in ligated rabbit oviducts as in previous study (Huang et al., 2001).

Based on Exp. 1, the COCs were cultured in 40% pFF IVM medium and inseminated by direct addition of capacitated sperm into the IVM medium. The IVF oocytes were randomly allocated to a 2×2 factorial design (Exp. 2), in which two culture systems (with or without cumulus monolayers) and two medium replacement procedures

[mBMOC-2+20%PS throughout the culture period or stepwise medium replacement procedure (SMRP)]. In the SMRP group, a mixture of the filtered IVM-conditioned medium and mBMOC-2 was used, i.e., 1/3, 2/3 or 3/3 of the filtered IVM-conditioned medium (M-199 based) were gradually replaced by mBMOC-2+20%PS on Day 1, Day 2 and 3, and Day 4 of the culture period, respectively (Huang et al., 2001). The cleavage rate was used as the assessment criterion for developmental competence.

Rabbit oviduct transfer

Female California rabbits (≥6-month-old) were kept individually in the cage for at least 3 weeks before being used as the recipients. The recipient does were treated with 100 IU hCG for induction of ovulations 24 h prior to receiving pig embryos (Ju et al., 1991, 2000). A modified procedure for transferring pig embryos into rabbit oviducts was based on the methods described by Herrmann and Holtz (1985) and Huang et al. (2001). Briefly, the recipients were anaesthetized with ketamine (Ketara®, 25 ng/kg BW, China Chemical and Pharmaceutic) and xylazine hydrochloride (Rompum®, 5.83 mg/kg BW). After abdominal mid-line incision, both oviducts were ligated 0.3-0.5 cm proximal to the utero-tubal junctions. Thirty pig embryos were transferred with 0.01-0.02 ml BMOC-2 medium to each ligated oviduct during 16-20 h post-fertilization. The recipients were sacrificed at 96 h after receiving pig embryos and the embryos were recovered by flushing the dissected oviducts with 10 to 20 ml of mBMOC-2 medium.

Statistical analysis

The differences of pFF compositions within follicular sizes and serum samples were compared using ANOVA. The effects of pFF levels, fertilization treatments and culture conditions on all criteria were compared using Duncan's multiple range test in the Statistical Analysis System (SAS) software.

RESULTS

Biochemical compositions of pFF and serum

Proteins : The concentrations of total proteins in the pFF from different size of follicles are presented in Figure 1. Average protein concentrations are 73.97±9.47, 56.82±7.19 and 59.87±6.86 mg/dl in large, medium and small follicles, respectively.

Lipids : Total lipid concentrations in the pFF are shown in Figure 2. No significant differences were observed among large, medium and small follicles ($p>0.05$). It decreased slightly from 12 to 84 h (445.5 to 356.4 mg/dl) and then increased from 108 to 132 h (373.1 to 428.5 mg/dl) after PMSG injection in small follicles. A similar trend was observed in medium follicles, which increased from 12 to 36 h (328.7 to 509.3 mg/dl) and decreased from 60 to 132 h (431.4 to 376.2 mg/dl) after PMSG treatment. However, in large follicles, it remained constant from 36 through 108 h (480.6 to 456.9 mg/dl), and then significantly decreased to 286.9 mg/dl at 132 h after PMSG treatment ($p<0.05$).

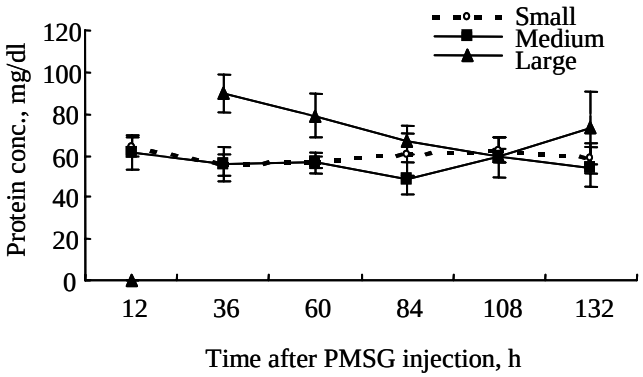

Figure 1. Changes of total protein concentrations of follicular fluids derived from different sizes of follicles in prepubertal gilts after PMSG injection (1,000 IU). A missing datum occurred in the large follicle group at 12 h after PMSG treatment. (Small: follicle diameter<3 mm; Medium: follicle diameter ~3-7 mm; Large: follicle diameter>7 mm).
[a,b] Indicates statistically significant differences in large follicle group ($p<0.05$).

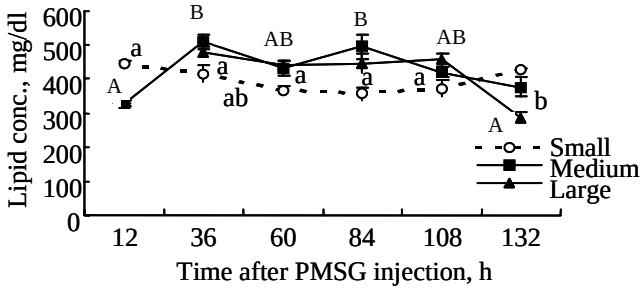

Figure 2. Changes of total lipid concentrations of follicular fluids derived from different sizes of follicles in prepubertal gilts after PMSG injection (1,000 IU). A missing datum occurred in the large follicle group at 12 h after PMSG treatment. (Small: follicle diameter<3 mm; Medium: follicle diameter~3-7 mm; Large: follicle diameter>7 mm).
[A,B] Indicates statistically significant difference in large follicle group ($p<0.05$).
[a,b] Indicates statistically significant difference in medium follicle group ($p<0.05$).

Cholesterol and glucose : Changes of total cholesterol and glucose concentrations in pFF after PMSG treatments are shown in Figures 3 and 4, respectively. No significant differences in total cholesterol were found within different collection time points in all follicular categories (small:

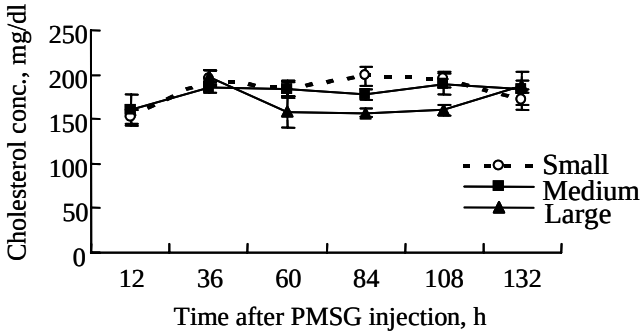

Figure 3. Changes of cholesterol concentrations of follicular fluids derived from different sizes of follicles in prepubertal gilts after PMSG injection (1,000 IU). A missing datum occurred in the large follicle group at 12 h after PMSG treatment. (Small: follicle diameter <3 mm; Medium: follicle diameter ~3-7 mm; Large: follicle diameter >7 mm). No significant difference was detected among groups (p>0.05).

153 to 198 mg/dl; medium: 161 to 190 mg/dl; large: 157 to 197 mg/dl). It appeared that a slightly reduction in glucose concentrations was observed at 132 h after treatment (83.9, 79.3 and 82.3 mg/dl for small, medium and large follicles, respectively) when compared to other time points (86-90, 86-91 and 83-90 mg/dl, respectively).

The concentrations of proteins (78-85 mg/dl), lipids (395-472 mg/dl), cholesterol (155-200 mg/dl), and glucose (83-88 mg/dl) in serum are not changed during the sampling period (Table 1).

Sex hormones : The levels of steroid hormones including P_4 and E_2 in serum are also presented in Table 1. It showed that an increasing level of both hormones was detected during the first 4 days after treatment (P_4, 0.09-0.81 ng/ml; E_2, 5.5-67 pg/ml). Progesterone, E_2 and T concentrations in the pFF from medium to large follicles are shown in Table 2. The levels of P_4 increased steadily from 12 h (138 ng/ml) to 108 h (814 ng/ml) after PMSG injection. A 10-fold increase was observed at 132 h after treatment (138.4 vs. 1,287.8 ng/ml). However, E_2 and T levels were fluctuated. E_2 concentrations reached a plateau (611-730 pg/ml) during 60-84 h post-PMSG and then dropped to the lower levels (121-139 pg/ml) during 108 to 132 h, which is similar to the level before 36 h after PMSG treatment. The testosterone concentration at 12 h in pFF was 20.3 ng/ml. It decreased to 9.5 ng/ml at 60 h and increased to 41.6 ng/ml at 84 h and then was down again to the lowest level (5.8 ng/ml) at 132 h post-PMSG injection.

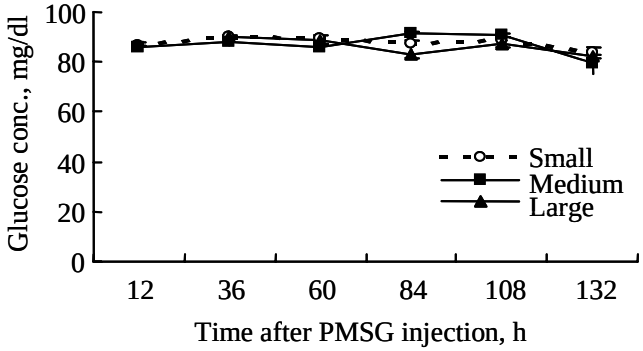

Figure 4. Changes of glucose concentrations of follicular fluids derived from different sizes of follicles in prepubertal gilts after PMSG injection (1,000 IU). A missing datum occurred in the large follicle group at 12 h after PMSG treatment. (Small: follicle diameter<3 mm; Medium: follicle diameter~3-7 mm; Large: follicle diameter>7 mm). No significant difference was detected among groups (p>0.05).

Specific experiment 1: The effects of culture conditions

Results of this experiment (Exp. 1) are shown in Table 3. After insemination by direct addition of sperm in 40% pFF medium, cleavage rates of the oocytes cultured in rabbit oviducts (70%) and mBMOC-2 (59%) were significantly higher ($p<0.05$) than those cultured in fresh IVM medium and filtered IVM-conditioned medium as well as those in the 100% pFF groups. Similar cleavage rates were found in the oocytes cultured in mBMOC-2 and rabbit oviducts in both the 40% and 100% pFF groups ($p>0.05$), of which the cleavage rates were all greater than 50% and 70% in the mBMOC-2 and oviduct transfer groups, respectively. There were no significant differences between embryos cultured in fresh IVM medium and filtered IVM-conditioned medium regardless of the levels (40% and 100%) of pFF addition (31 vs 23% and 10 vs 15%, respectively, $p>0.05$). These results indicated that IVM-derived IVF pig oocytes cultured in mBMOC-2 and rabbit oviducts were better than those cultured in the fresh or filtrated IVM-conditioned medium.

Specific experiment 2: The stepwise medium replacement and cumulus cell coculture

The development of the fertilized oocytes is significantly greater in the filtered IVM-conditioned media than that in the SMRP group (33 and 36% vs 17 and 19%, $p<0.05$), regardless of cocultured with or without cumulus cell (Table 4). Therefore, no significant influence was detected in the cumulus cell cocultured in term of cleavage rates.

DISCUSSION

Changes of pFF compositions after PMSG stimulations

There are two possible sources of the FFs. The viscous primary fluid is secreted by the grannulosa cells during folliculogenesis. A maturing follicle contains secondary fluid, which mainly transudes from the blood (Espey and Lipner, 1994). Therefore, protein concentrations in the pFF from large follicles were similar to that in the serum. The

Table 1. Biochemical compositions of prepubertal gilt sera collected at different time courses after PMSG injection[1)]

Hours after PMSG injection	No. of gilts	Progesterone (ng/ml)	Estradiol-17β (pg/ml)	Proteins (mg/dl)	Lipids (mg/dl)	Cholesterol (mg/dl)	Glucose (mg/dl)
0	37	0.09±0.01	5.50±1.84	78.09±3.47	454.29±32.58	154.83±13.90	87.63±0.82
24	30	0.34±0.07	12.13±0.22	75.39±4.90	394.94±50.13	174.16±13.53	87.89±0.79
48	25	0.52±0.06	41.23±3.09	75.42±5.14	429.70±41.81	200.34±16.34	87.11±0.85
72	20	0.70±0.04	67.43±3.09	85.12±6.55	431.46±42.27	170.63±18.88	86.87±1.12
96	14	0.81±0.28	61.70±2.21	75.68±6.55	472.20±28.48	183.22±13.29	83.34±2.86

1) The gilts were subjected to one dose of PMSG (1,000 IU) injection.

Table 2. Changes of steroid hormones concentrations in the follicular fluid of the prepubertal gilt at different time courses after PMSG injection[1)]

Hours after PMSG injection	No. of gilts	Progesterone (ng/ml)	Estradiol-17β (pg/ml)	Testosterone, T (ng/ml)
12	7	138.44±6.65	22.02±0.93	20.33±0.48
36	5	242.79±12.67	122.87±4.54	10.67±0.54
60	5	356.93±6.09	730.40±21.41	9.50±0.12
84	6	642.27±42.38	611.60±12.94	41.63±0.08
108	7	814.20±61.34	139.03±15.00	10.33±0.33
132	7	1,287.78±63.29	121.27±2.18	5.80±0.86

1) The gilts were subjected to one dose of PMSG (1000IU) injection.

Table 3. The effect of porcine follicular fluid addition and culture conditions on the development of *in vitro* matured and fertilized porcine oocytes

Treatment		No. of oocytes	No. of oocytes recovered	% of the embryo recovered	Embryonic cell stage				% of oocytes cleaved[3)]
% of pFF in IVM medium[1)]	Culture condition after IVF[2)]				1	2-4	5-8	Morula	
40	mBMOC-2+20%PS	190	-	-	78	78	34		59.2±5.5^{ab}
	Fresh IVM medium	178	-	-	122	48	8		30.8±8.9^{c}
	IVM-conditioned medium[2)]	170	-	-	131	32	7		23.2±3.7^{cd}
	Rabbit oviduct	199	76	38.2	24	27	14	11	70.5±3.2^{ab}
100	mBMOC-2+20%PS	192	-	-	90	66	36		53.1±1.8 ^{b}
	Fresh IVM medium	151	-	-	136	15			10.2±2.4^{d}
	IVM-conditioned medium[2)]	85	-	-	71	13	1		15.4±5.4^{cd}
	Rabbit oviduct	188	67	35.6	18	15	23	11	73.1±0.1^{a}

1) Basal IVM medium: TCM-199+FSH (2.5 μg/ml)+LH (5 IU/ml).
Each treatment contains FSH (2.5 μg/ml) and LH (5 IU/ml).

2) In filtrated (0.2 μm millipore) original IVM medium.

3) Mean±SEM, data without common superscripts (a, b, c, d) in the same column indicate statistically significant differences ($p<0.05$).

similarity was also observed in the FF derived from medium and small follicles and its concentration increased with the growth of the follicles (McGaughey, 1975). It is suggested that proteins in the FF were the transudates of the blood, which has been generally accepted (Burr and Davis, 1952). It was also reported by Meinecke and Meineckes-Tillmann (1998) that total amino acid content was significantly less in ovarian vein than that in pFF sampled at 72 h after PMSG injection, but no differences were detected when sampled at 36 h after treatment. The increased amino acid concentrations corresponded to the significant rises of urea, ammonia and most amino acids during the pre-ovulatory phase but the concentrations of histidine, lysine, taurine, threonine and tryptophane. In this regards, there may be functional blood-follicle barriers existing between the capillaries and the theca interna, which could be regulated by the rates of transudation and metabolism of specific amino acids (Meinecke and Meineckes-Tillmann, 1998). The increasing lipid concentrations in pFF from medium follicles during 12 to 36 h after PMSG treatment may be associated with the activity of steroidogenesis of the early follicles. Most gilts

Table 4. The effects of coculture with cumulus cell monolayers and stepwise medium replacement procedures on the development of IVM/IVF-derived porcine oocytes[1)]

Culture condition after IVF[2)]	Culture medium[2)]	No. of oocytes	Embryonic cell stage			Cleavage rate[3], %
			1	2-4	5-8	
With fibroblast	mBMOC-2+20%PS	139	88	45	6	36.4±4.3[a]
	SMRP	137	112	22	3	17.8±5.0[bc]
Without fibroblast	mBMOC-2+20%PS	174	117	53	4	33.3±3.4[a]
	SMRP	151	121	29	1	19.2±3.3[b]

[1)] Basal IVM medium - 40% pFF+TCM-199 100% +FSH (2.5 μg/ml)+LH (5 IU/ml).

[2)] Cocultured with or without cumulus monolayers.

[3)] SMRP: Stepwise medium replacement procedure, i.e., mBMOC+20% PS: IVM-conditioned medium=1:2 for Day 1, 2:1 for Day 2 and 3 and 100% IVM-conditioned medium for Day 4 after IVF, respectively.

[4)] Mean±S.E.M.

[a, b, c] Data without common superscripts in the same column indicate statistically significant differences ($p<0.05$).

ovulated at 132 h after PMSG stimulations, and the decreased concentration of lipids in unovulated large follicles indicated follicle degeneration. However, lipids concentrations in small follicles remained high. The coincidence of the decreasing lipids levels in the follicles and serum during 24 to 48 h after PMSG treatment suggested serum lipids are transferred into the FF for steroidogenesis during the growth of the follicles. Serum cholesterol concentrations as well as medium and small follicles rose from 24 to 108 h could be due to the increased cholesterol synthesis by PMSG stimulations. On the contrary, the decreased cholesterol concentrations in large follicle may be attributed to the dilution by the FF or conversion of cholesterol to steroid hormones, such as E_2 or P_4, during steroidogenesis. Moreover, glucose levels in the serum and FF remained constant before and after PMSG treatment, which suggested that glucose may not be a direct substrate involving follicular steroidogenesis.

It has been generally agreed that serum E_2 is a reflection of follicular E_2 levels. In this study, serum E_2 concentrations reached the peak level during 72 to 96 h after PMSG treatment, and the E_2 level in pFF reached the plateau from 60 through 84 h (Table 2). This slightly earlier increase in timing perfectly matched previous results. In the pFF, the testosterone concentrations decreased at 60 h post-PMSG treatment (Table 2), which may be due to E_2 synthesis using testosterone as the precursor. It is not clear why the testosterone concentration transiently increased at 84 h and then decreased, although this phenomenon was also reported by Ainsworth et al. (1980). Our data suggested that the activity of follicular steroidogenesis was enhanced by PMSG stimulations. The P_4 level increased steadily after treatment and the highest level was detected in the pFF at 132 h. Since P_4 stimulates the activity of collagenase in the theca cells, which may thinner follicular wall and ovulation may be triggered (Rondell, 1970). Therefore, inhibition of P_4 synthesis can result in the inhibition of ovulation (Lipner and Greep, 1971). The E_2/P_4 ratio, decreases from 20 to 0.15, is another important regulator for initiation of ovulations (Gerard et al., 1979). A similar result was observed in this study during 60 through 108 h after PMSG treatment. The LH surge might occur at 84 h after PMSG treatment, because P_4 level increased abruptly and E_2 level decreased simultaneously, which lowered the E_2/P_4 ratio. It is interesting that, at the meanwhile, testosterone levels in the pFF also temporally increased and then decreased, although the reason is not clear. Yamada and Kawai (1997) demonstrated that ovulation usually occurred between 32 to 48 h after hCG injection. The hormonal levels, i.e. E_2 and testosterone, decreased in peripheral circulations, ovarian veins, and pFF as the time of ovulation approached. In addition, P_4 level increased remarkably after the onset of ovulations. It has been proposed that prostaglandins (PG) take part in the rupture of follicular wall during ovulations (Yamada and Kawai, 1999). Therefore, ovulations can be blocked by a PG synthesis inhibitor, indomethacin, by which follicular steroidogenesis is not affected.

It has been demonstrated in the laboratory rodents and primates that the vascular endothelial growth factor (VEGF) is one of the key angiogenic factors regulating ovarian vascularization. Biosynthesis of this factor was controlled by gonadotropins (Barboni et al., 2000). Therefore, it is possible that PMSG stimulates folliculogenesis of ovarian follicles through production of VEGF in this study.

Different culture conditions influence the development of the IVM-derived pig embryos

The achievement of modern biotechnologies such as embryo splitting, nuclear transfer and transgenesis relies on successful in vitro production (IVP) of viable embryos (Petters et al., 1990). It had been a bottleneck in establishing an adequate IVP system for pig oocytes and embryos. The early stage (1 to 2 cell) porcine embryos, regardless of derived *in vivo* or *in vitro*, seldom developed beyond the 4-cell stage in culture, although some of the 4 cell embryo could form blastocyst *in vitro* (Wright, 1977; Pope and Day, 1977; Davis and Day, 1978; Linder and Wright, 1978). Recently, culture of fertilized porcine

embryos with oviductal epithelia (White et al., 1989), oviductal fluid (Archibong et al., 1989), in dissected mouse (Krisher et al., 1989) or rabbit oviducts (Allen et al., 1976; Agrawal et al., 1983; Sirard and Lambert, 1986; Wu et al., 1991) or in simplified culture media (for review see Reed et al., 1992; Petters and Wells, 1993) appeared being beneficial to overcome the 4 cell block in pig embryos. Although several culture systems for the successful development of pig embryos from 1 cell to the blastocyst stage have been developed, many efforts are still focused on the improvement of a complete system for efficient IVMFC (Hunter, 2000; Coy et al., 1999; Long et al., 1999; Machaty et al., 1998).

Because less than 1% of pFF is retained in the oviducts after ovulations (Hunter, 1990), the development of embryos mainly depends on the secretions from the oviducts. It has been shown that the mKRB medium containing 25% oviductal fluid supported the development of 1- to 2- cell pig embryos into the blastocyst, while a negative result was reported when the embryos were cultured only in the oviductal fluid (Archibong et al., 1989). It is proposed that the oviductal fluid might contain inhibitory factors to arrest the development of embryos and these factors could not be easily removed *in vitro* without oviductal cells. Mixtures of oviductal fluid with culture medium could dilute such inhibitors (Archibong et al., 1989). The oviduct provides some factors beneficial to the early development of embryos and has been served as a temporary incubator for the inadequate IVC systems (Allen et al., 1976; Agrawal et al., 1983; Fukushima and Fukui, 1985; Sirard and Lambert, 1986; Wu et al., 1991; Huang et al., 2001). Our results also showed that temporally incubation of the embryo in the oviductal tissue was beneficial (Table 3), which is consistent with our previous study (Wu et al., 1991; Huang et al., 2001).

Porcine oviductal epithelia alone or accompanied with fibroblastic monolayers improved blastocyst rates of early porcine embryos (White et al., 1989). However, our data showed no significant improvement when the embryos were cocultured with cumulus monolayers. This discrepancy might be resulted from different origins or preparations of fibroblastic monolayers or less competent IVM/IVF oocytes used. When compared to mBMOC-2 medium, neither freshly prepared IVM medium nor filtered IVM-conditioned medium or any other treatments was optimal for culturing the IVF porcine oocytes. The metabolites or some deleterious components released by the oocytes during IVM could negatively affect the development of the IVM/IVF-derived pig oocytes.

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