Effects of Levels and Sources of Follicular Fluid on the *In Vitro* Maturation and Development of Porcine Oocytes

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ABSTRACT : The aims of this study were first to evaluate the effects of different levels (20, 40 and 100%) and sources (follicular size: large, >7 mm; medium, >5-7 mm; small, 3-5 mm) of porcine follicular fluid (pFF) on the *in vitro* maturation (IVM) of porcine oocytes, and the effects of fertilization treatments and different culture conditions on development of fertilized oocytes were also investigated. No differences in the maturation (63.6-76.6%) and cleavage (24.8-34.3%) rates were observed among the 20, 40 and 100% pFF groups (p>0.05). The cleavage rates of oocytes cultured and fertilized in 40% and 100% pFF maturation media were significantly higher than those fertilized in m199-NBCS (51.0-61.2% vs. 12.8-31.8%, p<0.05), regardless of sources of the pFF. When oocytes were fertilized in m199-NBCS followed by culture in rabbit oviducts for 4 days, the cleavage rate in 40% pFF group was better than that in 100% pFF group (46.9% vs. 32.5%, p<0.05). Two oocytes recovered from the oviducts in the 40% pFF group developed to blastocysts after IVC. However, none developed to blastocysts when fertilized in the IVM medium after being transferred to rabbit oviducts. In conclusion, addition of pFF accompanied with gonadotropins (FSH, LH) in IVM medium enhanced maturation and cleavage rates of porcine oocytes. Direct addition of sperm suspension to IVM medium may be an alternative to simplify the fertilization procedures and to reduce the mechanical lesion during manipulation. Furthermore, rabbit oviducts provide a better environment for the *in vitro* fertilized oocyte developing to the morula and blastocyst stages. ***(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 10 : 1360-1366)***

Key Words : IVMFC, Follicular Fluid, Rabbit Oviduct, Pig

INTRODUCTION

The development of mammalian follicles and meiosis of the oocyte are regulated by various factors including hormones. The growth and maturation of the preovulatory oocytes proceed in the antral follicles filled with follicular fluid (Hafez, 1987). Less than 1% of the follicular fluid enters the isthmus after ovulation (Hunter, 1990), which could have an effect on activation, chemotaxis, acrosome reaction of the sperm and prevention of polyspermy of the oocyte during fertilization (Gwatkin and Anderson, 1969; Hansen et al., 1991; Ralt et al., 1991; Lee et al., 1992; Yoshida et al., 1992; Funahashi and Day, 1993). Bovine follicular fluid was found to induce capacitation and acrosome reaction of hamster and bovine sperms (Gwatkin and Andersen, 1969; Fakih and Vijayakumar, 1990). However, boar sperm failed to penetrate the oocyte when coincubated with the oocyte in medium containing porcine follicular fluid (pFF; Baker and Polge, 1976).

Porcine follicular fluid (pFF) obtained from different sizes of follicles inhibits meiotic resumption of pig oocytes and luteinization of granulosa cells *in vitro*. However, the inhibition can be overcome by addition of gonadotropins to culture medium, and development of porcine oocytes after *in vitro* maturation, fertilization and culture (IVMFC) increased significantly (Tsafriri and Channing, 1975; Tsafriri et al., 1976; Richards, 1980; Naito et al., 1988). Gonadotropins induce differentiation and expansion of cumulus cells and meiotic resumption of oocytes accompanied with reduced association of oocytes with cumulus cells prior to ovulation (Motlik et al., 1986; Mattioli et al., 1988). This indicates that pFF contains factors functioning synergistically with gonadotropins in supporting IVMFC of the oocyte. Although culture systems for early pig embryos have been developed, efforts were focused on developing an optimized system for production of good quality oocytes and embryos (Hunter, 2000). To our knowledge, there was no report dealing with fertilizing ability of *in vitro* capacitated boar sperm in pFF or pFF-containing media. In addition, the effect of pFF from different sizes of follicles on the subsequent development of these IVM-derived embryos has not been well defined yet. In this study, pFF of different follicular sizes were tested in the IVM and IVF systems of pig oocytes and the effects of different fertilization treatments on the subsequent development of porcine oocytes were also examined.

MATERIALS AND METHODS

Oocyte collection

Pig ovaries were collected from a local abattoir. Transportation of the ovaries to the laboratory was carried out in a Dewar flask containing Dulbecco's phosphate-buffered saline (PBS, Gibco 450-1300) supplemented with 100 IU Penicillin mL^{-1} and 100 μg Streptomycin mL^{-1}

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(Penstrep®, Gibco) in a 35°C thermal container. Antral follicles of 2-6 mm in diameter were aspirated and cumulus-oocyte-complexes (COCs) were collected in PBS containing 2% new born calf serum (NBCS). Oocytes with homogenous cytoplasm surrounded by at least 3 layers of compact cumulus cells were selected for experiments. Preparation processes of the NBCS were described elsewhere (Wu et al., 1991).

In vitro maturation (IVM) and nuclear staining

The pFF collected from antral follicles (3-7 mm) of prepubertal gilts ovaries was centrifuged at 1,000×g for 10 min. The supernatant was collected and supplemented with 50 μg mL^{-1} of Dibekacin sulfate (Meiji, Japan) and stored at -20°C until use.

Oocytes were matured for 44 h in 2 mL Medium 199 (M-199) with Earle's salts (Gibco, No. 400-1100) supplemented with 5 IU/mL of LH (Sigma, L-2019), 2.5 μg/mL of FSH (Sigma, F-8001) and 10% pFF. The culture condition was maintained at 39°C and 5% CO_2 in air. After IVM, one third of the oocytes were washed in M-199 and denuded mechanically by repeated pipetting. The oocytes were then fixed in acetic alcohol (acetic acid: alcohol=1:3, v/v) for 24-48 h and stained with 1% (w/v) lacmoid in 45% (v/v) acetic acid. Oocytes that developed to the second meiotic metaphase (M II) or beyond were considered as mature.

In vitro fertilization (IVF) and culture (IVC)

The IVF protocol was based on Cheng (1985). Briefly, freshly ejaculated boar semen (sperm-rich fraction) from 3 healthy boars was kept at 18°C for 16 h with 50 μg/mL Dibekacin sulfate (Meiji, Japan). Some 2.5 h before fertilization, 5 mL of 1×10^9/mL spermatozoa were mixed with an equal volume of saline containing 1 mg/mL of BSA Fraction V (Sigma, St. Louis, MO, USA) and 50 μg/mL Dibekacin sulfate (pH=7.2). After centrifugation at 200×g for 10 min to sediment clots of gelatinous portions or particles, the top 5 mL of the supernatant containing spermatozoa were mixed with 5 mL of the same washing medium, then centrifuged at 600×g for 10 min. The supernatant was discarded and the sperm pellet was resuspended in 10 mL of washing medium and again centrifuged at 600×g for another 10 min. The sperm pellet was resuspended in 2 mL of capacitation medium (M-199 + 0.91 mM Na-pyruvate + 2.92 mM Ca-lactate + 3.05 mM glucose + 12% NBCS) at pH 7.8 and then kept for 90 min at 39°C. Sperm concentration was adjusted to 1×10^8 to 1×10^9/mL.

After IVM, the COCs were washed three times prior to transferring into a 35-mm plastic petri dish containing 2 mL of m 199-NBCS (capacitation medium + 2.01 mM caffeine at pH 7.4). The pre-incubated sperm suspension was added to give a final concentration of 1×10^5 to 1×10^6 /mL.

After incubation with spermatozoa for 6-8 h at 39°C and 5% CO_2 in air, the oocytes were removed from the cumulus cells and excessive sperm, washed and transferred to fresh mBMOC-2 + 20% porcine serum (PS, Day 0)+0.4% BSA (mBMOC-2 plus) for 96 h. The medium was renewed every 24 h.

Specific experiments

Experiment 1 : To determine the effect of pFF on IVMFC of porcine ooctyes, COCs were randomly allocated to the basal maturation medium containing 20, 40 or 100% pFF and the sperm was directly inseminated into m199-NBCS medium.

Experiment 2 : Based on the result of experiment 1, the COCs were randomly allocated to the maturation media containing 40 or 100% pFF to evaluate the effects of pFF sources on the development of IVF oocytes. The pFF was collected from follicles with different diameters, i.e., large (L, >7 mm), medium (M, >5~7 mm) and small (S, 3~5 mm). After IVM, oocytes were inseminated in m 199-NBCS or by direct addition of capacitated sperm into the original maturation medium. Development of the oocyte was examined 96 h after insemination.

Experiment 3 : To test the beneficial effects of temporal oviduct incubation following different IVF treatments on the development of porcine oocytes, the COCs were cultured in the medium containing either 40% or 100% pFF and were inseminated in m199-NBCS or in the original maturation medium as in experiment 2. Sixteen to twenty hours after insemination, the 2-cell embryos were transferred to the ligated rabbit oviducts. Development of the embryos was evaluated by flushing the embryos from the oviducts at 96 h after transfer.

Procedures of rabbit oviduct transfer

Female California rabbits over 6-month old were kept individually in the cage for at least 3 weeks before use as the recipients. The recipient does were treated with 100 IU hCG (Ju et al., 1991) for induction of ovulation 24 h prior to receiving pig embryos.

A modified procedure for embryo transfer into rabbit oviducts was based on the methods described by Herrmann and Holtz (1985). 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 an abdominal mid-line incision, both oviducts were ligated at a distance of 0.3-0.5 cm close to the utero-tubal junctions. About 30 pig embryos with 0.01-0.02 mL BMOC-2 medium were transferred to each ligated oviduct 16-20 h post-fertilization. The recipients were sacrificed 96 h after receiving pig embryos and the oviducts were removed and flushed using 10 mL of

mBMOC-2 medium for recovering pig embryos.

Statistical analysis

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

Experiment 1

The effects of the different pFF levels on the IVMFC of porcine oocytes were shown in tables 1 and 2. No significant differences were detected neither in maturation rates (73.5, 76.6 and 63.6%) nor in the cleavage rates (24.8, 34.3 and 30.1%) among 20, 40 and 100% pFF groups (P>0.05). It is evident that large proportions of the embryos were still blocked before the 4-cell stage (table 2). The 2- and 4-cell porcine embryos after IVF in m199-NBCS and IVM medium were shown in fig. 1a and 1b.

Experiment 2

Due to no significant effect of pFF levels found in experiment 1, 40 and 100% pFF levels were selected for this experiment. Different sources of pFF and fertilization treatments were compared and the effects on developmental competence of porcine oocytes were shown in table 3. Both levels (40 vs. 100%) and sources (L, M and S) of pFF did not significantly affect the cleavage rates within the same fertilization treatment or medium. However, the cleavage rates of oocytes fertilized directly in IVM medium ranged from 51 to 61%, which were significantly higher than that in the m199-NBCS treatment groups (13-32%, p<0.05).

Experiment 3

Beneficial effects of temporal incubation of IVF embryos in rabbit oviducts were examined in this experiment. After being fertilized, the 2-cell embryos were selected and transferred to the oviducts of recipient does for 96 h. No significant difference in total cleavage rates was found between the 40 and 100% pFF groups in the oocytes directly fertilized in the IVM medium (p>0.05, table 4). On the contrary, a significant difference between the pFF levels (40% vs. 100%) in the m199-NBCS group was observed (47% vs. 33%, p<0.05), which appeared slightly inconsistent with the result in experiment 2. Morula stage embryos appeared developed better in the 40% pFF groups in both fertilization treatments than those in 100% pFF, and two compact morulae developed into expanded blastocyst in 40% pFF of m199-NBCS treatment groups (figure 1, c-f) after 36 h of further development in rabbit oviducts (table 4).

DISCUSSION

Effects of pFF on IVM and IVC of oocytes

Our previous study showed that gonadotropins (FSH, LH) with follicular fluids improved IVM and IVF of pig

Table 1. Effects of the porcine follicular fluid (pFF) levels in TCM-199 medium on *in vitro* maturation of porcine oocytes

% of pFF in IVM medium [1]	No. of oocytes cultured	Oocyte stage [2]						Maturation rate, % [3]
		GV	Pro M I	Met I	Ana I	Tel I	Met II	
0 *	126	84	12	14	0	2	14	10.7±4.5[a]
20	96	4	11	7	1	3	70	73.5±4.1[b]
40	103	5	12	5	2	0	79	76.6±2.0[b]
100	92	3	10	18	1	1	59	63.6±13.4[b]

* Data adapted from Cheng et al. (1997).

[1] *In vitro* maturation medium: pFF + TCM-199 + FSH (2.5 μg/ml) + LH (5 IU/ml).

[2] GV: germinal vesicle; Pro MI: prometaphase I; Met I: metaphase I; Ana I: anaphase I; Tel I: telophase I; Met II: metaphase II.

[3] Mean ± S.E.M.; data without common superscripts (a, b) in the same column indicate statistically significant differences (p<0.05).

Table 2. Effects of porcine follicular fluid (pFF) levels in the maturation medium on the development of *in vitro* fertilized porcine oocytes

% of pFF in IVM medium [1]	No. of oocytes cultured	Embryonic stage			Total cleavage rate, % [2]
		1	2-4	5-8	
0 *	96	84	11	1	12.4±3.0[a]
20	198	149	41	8	24.8±1.2[b]
40	199	135	50	14	34.3±3.9[b]
100	178	123	39	16	30.1±3.1[b]

* Data adapted from Cheng et al. (1997).

[1] In vitro maturation medium: pFF + TCM-199 100% + FSH (2.5 μg/ml) + LH (5 IU/ml).

[2] Mean ± S.E.M.; data without common superscripts (a, b) in the same column indicate statistically significant differences (p<0.05).

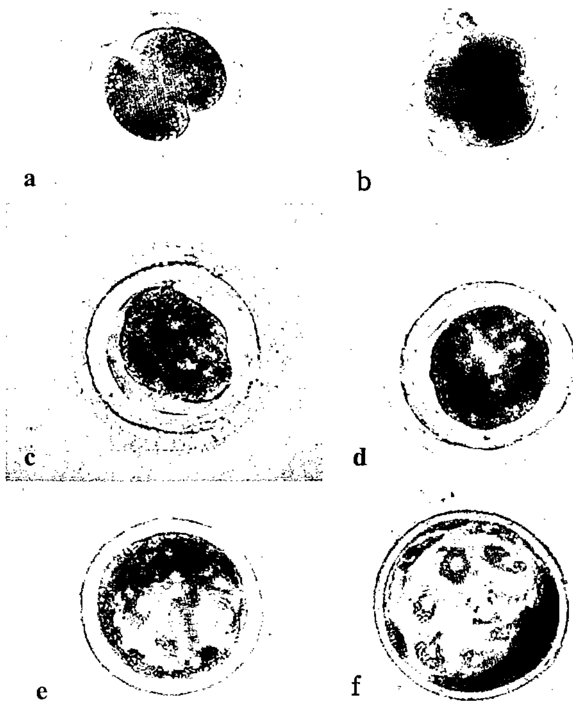

Figure 1. Development of pig embryos at different stages after *in vitro* fertilization. (a) 2-cell stage (b) 4-cell stage (c) A morula stage embryo developed after being cultured in rabbit oviducts, by which an early (d), late (e) and expanding blastocyst (f) formed after another 6, 24 and 36 h of *in vitro* culture, respectively. (400×)

oocytes (Wu et al., 1991) as well as in other reports (Naito et al., 1988, 1989, 1990; Galeati et al., 1990). Although steroids such as estradiol (E_2) is essential for oocyte maturation, no addition of steroids is required in this study due to high level of E_2 in the pFF (Guraya, 1985). Studies on IVM of pig oocytes by coculture with either follicular cells or follicular fluids (Niwa, 1993; Sirard et al., 1993) suggested that follicular cells secrete factors that play a crucial role in supporting oocyte cytoplasmic maturation (Hunter, 2000). In experiment 1, 20, 40 and 100% pFFs were used to improve maturation rate of oocytes. No significant differences in all criteria were detected in different levels of pFF groups (table 1), although it appeared a slightly lower maturation rate in the 100% pFF group (64 vs. 74-77%, $p>0.05$). The slightly increase of premature oocytes maybe a reflection of higher oocyte maturation inhibitors (OMI) in the pFF (Yoshida et al., 1992).

Although IVM-derived pig oocytes may be penetrated by sperm under appropriate conditions, oocytes with low pronuclear formation rates and high incidences of polyspermy were observed, and therefore, the development or cleavage rate after IVF was disappointingly low (table 2). This may be resulted from the suboptimal culture system or the IVF procedure per se. Certainly, the *in vitro*-derived oocytes are much less developmental competent than that of *in vivo*-derived oocytes (Hunter, 2000). Motlik and Fulka (1974) transferred *in vitro* matured porcine oocytes to the recipients and inseminated, and found that the male pronuclear formation of the oocytes was lower than those matured *in vivo*. This could be stemmed from incomplete maturation of the nucleus and the cytoplasm, such as low ratios of the dark and light cortical granules (CG), and aging of oocytes leading to the occurrence of polyspermy (Motlik and Fulka, 1974; Cheng, 1985; Cran and Cheng, 1985). Slower movement of the CG to the pericytoplasm of the IVM oocytes (Hyttle et al., 1989) could result in low monospermic fertilization and pronuclear formation following IVF (Nagai et al., 1990).

Effects of direct insemination of capacitated sperm and sources of pFF

Although boar sperm failed to penetrate the oocyte in pFF-medium (Baker and Polge, 1976), follicular fluid from different species can induce sperm capacitation (Gwatkin and Andersen, 1969; Fukui et al., 1983; Fakih and Vijayakumar, 1990). Regardless of levels and sources of pFF, our results suggested that direct addition of capacitated sperm in the IVM medium showed a better cleavage rate than those fertilized in m199-NBCS medium. Less mechanical damages of oocytes during IVF may in turn reduce polyspermy during fertilization. Low pronuclear formation rates and high incidences of polyspermy were also observed in the m199-NBCS medium as those in experiment 2. In our previous study, when oocytes were inseminated directly in the IVM medium, half of the cleaved eggs underwent parthenogenesis due to aging of the oocyte or stimulating factors from the sperm (Huang et al., 1999). Despite of significantly higher cleavage rates in the IVM medium group, it is also possible that a portion of the cleaved embryos were parthenotes in experiment 3 (table 3).

Pig follicles smaller than 0.7 mm in diameter contain incompetent oocytes for meiotic resumption, although some oocytes from follicles between 0.8 and 1.6 mm are capable of being matured *in vitro* (Motlik et al., 1984). Our previous study showed a better development of porcine oocytes obtained from the follicles larger than 3 mm in diameter (Wu et al., 1991). Similarly, when the maturation medium contained FSH and follicular fluid collected from large (>5 mm) and medium (2-5 mm) pig follicles, or from large (10-20 mm) and medium (2-5 mm) bovine follicles achieved similar maturation rates of 66 to 97% (Naito et al., 1990). Our data showed no effect on the maturation and cleavage rates of porcine oocytes when pFF from different sizes of follicles (3-7 mm) were compared (table 3).

Table 3. Effects of different fertilization treatments and porcine follicular fluid (pFF) from different follicular sizes on the development of oocytes following *in vitro* fertilization

Medium for IVF	Medium for IVM [1]	Follicle size [2]	No. of oocytes cultured	Embryonic stage 1	Embryonic stage 2-4	Embryonic stage 5-8	Total cleavage rate, % [3]
IVM medium	40% pFF	L	108	48	35	25	54.3±4.2[a]
		M	115	45	42	28	59.7±8.3[a]
		S	106	43	40	23	59.9±6.6[a]
	100% pFF	L	116	54	36	26	51.0±8.5[a]
		M	99	38	39	22	61.2±3.0[a]
		S	100	40	32	28	60.5±7.6[a]
m199-NBCS	40% pFF	L	101	67	18	16	31.8±16.7[b]
		M	108	95	6	7	16.2±5.5[b]
		S	86	76	1	9	16.3±6.0[b]
	100% pFF	L	95	69	10	16	26.6±7.6[b]
		M	89	80	6	3	22.7±7.5[b]
		S	94	89	3	2	12.8±4.2[b]

[1] In vitro maturation medium: pFF + TCM-199 100% + FSH (2.5 µg/ml) + LH (5 IU/ml).
[2] L: large follicle (> 7 mm), M: medium follicle (5-7 mm), S: small follicle (3-5 mm), [3] Mean ± S.E.M.
[a, b] Data without common superscripts (a, b) in the same column indicate statistically significant differences ($p<0.05$).

Table 4. Effect of porcine follicular fluid (pFF) and fertilization treatments on the development of porcine embryos after transferred into rabbit oviducts

Medium for IVF[1]	Medium for IVM	No. of embryos transferred	No. of oocytes recovered	% of embryos recovered	Embryonic stage 1	2-4	5-8	Morula	Blastocyst [2]	Total cleavage rate, % [3]
IVM medium	40% pFF	150	116	77.3	67	25	9	15	0	43.6±4.1[ab]
	100% pFF	120	84	70.0	52	21	6	5	0	37.8±5.3[ab]
m199-NBCS	40% pFF	153	114	74.5	61	31	10	10	2	46.9±4.8[a]
	100% pFF	116	105	90.5	71	18	11	5	0	32.5±2.8[b]

[1] *In vitro* maturation medium: pFF + TCM-199 + FSH (2.5 µg/ml) + LH (5 IU/ml).
[2] *In vitro* culture for 6~36 h after being flushed from rabbit oviducts, [3] Mean ± S.E.M.
[a, b] Data without common superscripts (a, b) in the same column indicate statistically significant differences ($p<0.05$).

Beneficial effects of oviduct transfer

Due to the development of in vitro culture system, early embryos from many species had overcome the cell block, which has been related to the gene expression of early embryos during the maternal-zygotic transition (Prather and First, 1988). Once passed the block stage, embryos developed to the blastocyst or to term after embryo transfer (Rexroad and Powell, 1988; Fukui and Ono, 1989). Due to the nature of pig oocytes and embryos, development of in vitro-derived pig oocytes to good quality blastocysts has not been well established (Pope and Day, 1977; Davis and Day, 1978; Petters and Wells, 1993; Hunter 2000). Rabbit oviducts have been served as a temporary and movable incubator to bypass the inadequate *in vitro* system for embryo culture (Allen et al., 1976; Agrawal et al., 1983; Fukushima and Fukui, 1985; Sirard and Lambert, 1986; Wu et al., 1991). Therefore, in experiment 3, we transferred cleavage stage embryos derived from different protocols into rabbit oviducts. Results showed that some oocytes after IVM and IVF then cultured in rabbit oviduct developed to morula or blastocyst stages (table 4 and figure 1), which is consistent with our previous study (Wu et al., 1991). In comparison to the results in experiments 2 and 3 (tables 2 and 3), rabbit oviducts are beneficial in supporting further development of pig embryos. Most interestingly, rabbit oviducts seem to exert an interaction between different IVF procedures and pFF contents in culture media (table 4). For the cleavage rates as shown in table 4, no significant differences were found between 40 and 100% pFF group in the IVM medium (44 vs. 38%), whereas in the m199-NBCS treatment group, a significant lower cleavage rate was observed in the 100% pFF group (33%) compared to that in the 40% pFF group (41%). However, the low blastocyst rate may be attributed to inadequacy of the *in vitro* culture

system for pig embryos and, possibly, the nature of the reproductive tract and coating of the mucin, which specifically secreted by rabbit oviducts, might also limit further development of the xenotransplanted pig embryos in rabbit oviducts.

Hunter (1990) reported that less than 1% of the follicular fluid enters the isthmus in gonadotropin-treated gilts during ovulations. Therefore, the development of early embryos could mainly depend on oviductal secretions. Archibong et al. (1989) cultured the *in vivo* derived early porcine embryos with oviduct fluid and mKRB at a ratio of 1:3, by which a beneficial influence on development of the embryos to the blastocyst was found. However, no such effect was observed when cultured with oviduct fluid alone. It suggested that the oviduct fluid may contain factors which would inhibit blastocyst formation. These factors could not be removed without the oviduct epithelium. It is possible that combination of oviduct fluid with mKRB medium could dilute or inactivate these factors (Archibong et al., 1989). However, it is still not clear about what exact molecules are in the oviducts and how it works to benefit embryo development.

In conclusion, addition of pFF together with gonadotropins in culture systems increases maturation and cleavage rates of pig oocytes, and direct addition of *in vitro* capacitated sperm into the maturation medium not only simplify the IVF procedures, but also enhance development of pig oocytes and embryos. Rabbit oviducts may be served as a short-term incubator for pig embryos to bypass the cell block and to produce quality embryos in the regular IVC system.

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