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***In Vitro* Culture Conditions Using Chemically Defined Media for *In Vitro* Matured and Intracytoplasmically Inseminated Porcine Oocytes**

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Abstract. The present study investigated *in vitro* culture methods [droplet and Well of the Well (WOW)] using semi-defined and defined media [modified porcine zygote medium (mPZM)] and the additional effects of insulin on *in vitro* matured and intracytoplasmically inseminated porcine oocytes. In Experiment 1, *in vitro* matured and intracytoplasmically inseminated porcine oocytes were cultured for 6 days in the following four groups: 1) mPZM-3 (containing bovine serum albumin) + droplet (30 μ l), 2) mPZM-3 + WOW, 3) mPZM-4 (containing polyvinyl alcohol) + droplet, and 4) mPZM-4 + WOW. The culture media (mPZM-3 and mPZM-4) and methods (droplet and WOW) did not significantly affect the cleavage rate, but the blastocyst rate of the oocytes cultured in mPZM-3 was significantly ($P < 0.01$) higher than that of mPZM-4 (20.1 and 9.4%, respectively). The blastocyst rates as percentages of the cleaved oocytes (51.8 and 16.9%) and the hatched blastocyst rate as percentages of the number of blastocysts (12.3 and 2.2%) were also significantly ($P < 0.01$) higher in mPZM-3 compared with those in mPZM-4. There was significant interaction ($P < 0.05$) between the two main factors; the effects of the culture media and methods on the rate of hatched blastocysts as percentages of the blastocysts produced and, the hatched blastocyst rate (20.3%) as percentages of the number of blastocysts produced in mPZM-3 were significantly ($P < 0.05$) higher than in the other groups. In Experiment 2, the additional effects of insulin (100 ng/ml) in mPZM-3 and mPZM-4 media was investigated in the WOW culture system. Insulin addition did not improve cleavage, blastocyst formation, or the number of cells in blastocysts. However, as in Experiment 1, mPZM-3 resulted in a significantly higher blastocyst rate as percentages of the cleaved oocytes than mPZM-4 (33.9 and 18.4%). These results indicate that a chemically defined medium (mPZM-4) needs to be improved to provide more suitable culture conditions for *in vitro* development of *in vitro* matured and intracytoplasmically inseminated porcine oocytes. However, the WOW system may be a useful IVC method for blastocyst development of *in vitro* matured porcine oocytes following ICSI when a semi-defined medium (mPZM-3) is used.

Key words: *In vitro* culture, *In vitro* maturation, Intracytoplasmic injection, Porcine oocytes
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In vitro production (IVP) technologies are comprised of three steps, *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC). Farm animal embryos have been produced using *in vitro*

systems and have been supplied to commercial embryo transfer programs, especially for cattle. However, in pigs, IVP has been limited due to high polyspermy rates (20–60%) and low blastocyst rates (20–30%) following IVM and IVF compared with bovine IVP.

In porcine IVM, serum or follicular fluid (FF) is

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added to the medium to increase the efficiency of IVP [1–5]. Although the use of serum or FF for IVM culture of farm animal oocytes is beneficial for increasing blastocyst rate after IVF, serum [usually, fetal bovine serum (FBS)] and FF contain many unknown substances and there are differences between batches. This has made it difficult to clarify which factor(s) has the greatest effect, not only nuclear maturation, but also on cytoplasmic maturation of immature oocytes, which affects their developmental capacity. Furthermore, adding serum during IVC may have a beneficial effect on embryonic development as it contains energy sources and growth factors [6, 7]. However, serum may cause lipid accumulation in the culturing embryos and may adversely lower cryotolerance after freezing and thawing or after vitrification [8, 9]. In this respect, recent development of chemically defined culture media for IVM and IVC has been intensively studied in the bovine, porcine, and other farm animals. Yoshioka *et al.* [10] have developed a chemically defined culture medium for porcine zygotes [porcine zygote medium-4 (PZM-4)], and production of piglets derived from *in vitro* developed blastocysts fertilized and cultured in PZM-4 has been reported [2]. However, the developmental capacity of embryos derived from IVC using chemically defined media is usually low compared with those of embryos cultured in media containing BSA or FBS [11–14]. Therefore, to improve the frequency and quality of preimplantation embryos produced using defined media, various energy substrates, such as amino acids, insulin, glucose, phosphate, and growth factors, have been added to IVC media [6, 7, 16–19]. The addition of insulin during IVC has improved embryonic development in rat [16] and porcine embryos [19]. Therefore, it would be worthwhile to investigate whether the addition of insulin during IVC using a defined medium such as PZM-4 produces a positive effect on *in vitro* development of porcine oocytes matured *in vitro* with subsequent intracytoplasmic sperm injection (ICSI).

Different culture conditions, such as four-well dish or micro-drop methods, have been used in many studies on IVC of farm animal embryos. In 2000, Vajita *et al.* [20] developed the Well of the Well (WOW) system for culture of bovine zygotes as a new IVC method. Taka *et al.* [21] were the first to use and report on the effectiveness of the WOW system for porcine oocytes matured in a defined

IVM medium with subsequent ICSI. Different culture dishes with various medium volumes and numbers of ova would be one of the main factors influencing subsequent development [22–27].

Traditional IVF has produced high rates of polyspermic fertilization [2–5, 26] and aneuploidy, especially mixoploidy in subsequently cultured bovine and porcine embryos [28–31]. In order to remove the incidence of polyspermic fertilization in IVF of porcine oocytes, we attempted ICSI of *in vitro* matured porcine oocytes and obtained 20–30% blastocyst rates after IVC [32–34]. Therefore, an attempt of porcine ICSI would contribute to removal of polyspermic fertilization and to production of normal diploid porcine blastocysts *in vitro*.

In the present study, two experiments were conducted to investigate IVC methods (droplet and WOW) using modified PZM-3 [containing bovine serum albumin (BSA)] or PZM-4 [containing polyvinyl alcohol (PVA)] and the additional effects of insulin (100 ng/ml [19]) on the developmental capacity of *in vitro* matured and intracytoplasmically inseminated porcine oocytes.

Material and Methods

Oocyte collection

Porcine ovaries were obtained at a local slaughterhouse and transported to the laboratory in sterile 0.9% NaCl containing penicillin G (Meiji Seika, Tokyo, Japan) and streptomycin (Meiji Seika) at about 37 °C within 3 h of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–6 mm diameter) using an 18-gauge needle attached to a 10 ml disposable syringe. Subsequently, only COCs with a compact cumulus mass of more than three layers were selected for IVM culture. The COCs were washed three times in Hepes-buffered Tyrode's medium with 0.05% (w/v) PVA (TLH-PVA; Sigma Chemical Co., St. Louis, MO, USA).

In vitro maturation

The base medium for IVM was a modified TCM199 (Sigma) supplemented with 3.05 mM glucose (Wako Pure Chemical, Osaka, Japan), 0.91 mM Na-pyruvate (Wako), 10 ng/ml epidermal growth factor (EGF; Sigma), 0.05% (w/v) PVA and 75 mg/l kanamycin (Sigma). The medium was

further supplemented with 100 μ M cysteamine (M-6500, Sigma) or 0.6 mM cystine (Wako).

Selected COCs were washed in an IVM medium, and 23 to 38 COCs per group were transferred into each well of a Nunc four-well multidish (Nunc, Roskilde, Denmark) containing 500 μ l of IVM medium. They were then cultured at 39 C in a humidified atmosphere of 5% CO₂ in air. During the first 22 h, the COCs were cultured in IVM medium supplemented with 10 IU/ml PMSG (Teikoku Hormone Tokyo, Japan) and 10 IU/ml hCG (Teikoku Hormone). Then, the COCs were washed three times in IVM medium without hormones and cultured for an additional 22 h in the same medium.

Oocyte and sperm preparation

After IVM culture, the COCs were denuded of the cumulus cells by repeated pipetting in 0.1% (w/v) hyaluronidase (Sigma) in TLH-PVA. The oocytes were washed three times in TLH-PVA and remained in the same medium until ICSI.

Semen from an individual boar was used for ICSI throughout all the experiments. Frozen pelleted semen [35] was thawed at 39 C and the sperm suspension was placed under 1.2 ml of Dulbecco's phosphate buffered saline (PBS; Gibco-BRL, Grand Island, NY, USA) containing 0.1% PVA (PBS-PVA) in a microcentrifuge tube. This was then incubated for 30 min at 39 C in 5% CO₂ in air for swim-up.

Intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection (ICSI) was performed according to the method of Yong *et al.* [36] with some modifications [32]. Briefly, manipulation was conducted with the aid of a pair of micromanipulators (Leitz, Wetzlar, Germany) under an inverted microscope. After swim-up treatment, 7 μ l of sperm supernatant was collected from the top of the tube and dropped directly onto the lid of a Petri dish (50 mm $\times$ 9 mm; Falcon 1006; Becton Dickinson, Franklin Lakes, NJ, USA). Another 7 μ l drop of TLH-PVA was made adjacent to the drop containing the sperm for oocyte manipulation. The two drops were covered with mineral oil (Sigma). Then, 5–18 oocytes per group were transferred into the drop of TLH-PVA for manipulation. A spermatozoon was aspirated into an injection pipette tail-first and transferred to the drop containing the oocytes. An oocyte was held with its polar body at either the 6 or 12 o'clock

position by an holding pipette. A spermatozoon was injected into the oocyte cytoplasm and mixed with cytoplasmic component with the aid of a mouth-regulated open tube.

In vitro culture

After ICSI, the oocytes were washed three times in 30 μ l droplets of IVC media, a modified PZM-3 (mPZM-3), or PZM-4 (mPZM-4) [10] according to the experimental design. The oocytes were then cultured into 30 μ l droplets of modified PZM-3 containing 3 mg/ml BSA (mPZM-3: semi-defined medium) or modified PZM-4 containing 3 mg/ml PVA (mPZM-4: defined medium) under mineral oil for 6 days at 39 C in 5% CO₂, 5% O₂ and 90% N₂. Both culture media were further supplemented with 2.77 mM myo-inositol and 0.34 mM trisodium-citrate. Cleavage and development to the blastocyst stage were observed on Day 2 (day of ICSI=Day 0) and Day 6, respectively. Blastocysts that developed on Day 6 were fixed to examine their cell numbers.

Determination of blastocyst cell numbers

Blastocysts were washed in 1% (w/v) sodium citrate solution (1% SCS) and placed in the same solution for 20 min on a warming plate. Then, the blastocysts were fixed in fixative solution I (1% SCS:acetic acid: methanol=40:1:1) for 1 min and mounted with a small amount of fixative solution on a glass slide. Thereafter, before the solution was dried, a small amount of fixative solution II (acetic acid:methanol=1:1) was dropped onto the blastocysts for complete fixation. The glass slide was dried and stained with 0.4% Giemsa solution (Sigma) for 30 min. After washing, the number of cells was determined under a compound microscope by counting the number of stained nuclei ($\times$ 400).

Experimental design

Experiment 1: The effects of two main factors (culture media and methods) on cleavage and blastocyst formation were examined in oocytes matured *in vitro* with subsequent ICSI. The culture media were mPZM-3 and mPZM-4, and the culture methods were WOW (1 mm in diameter) in a 4-well multi-dish (Nunc) and a 30 μ l droplet under mineral oil. The WOW system used was constructed according to the methods of Taka *et al.* [21]. Four to 5 intracytoplasmically inseminated

Table 1. Effects of different culture conditions for porcine oocytes matured *in vitro* and with subsequent ICSI (Experiment 1)

Culture medium	Culture method	No. of oocytes cultured	Cleavage (% SEM)	Blastocysts /cultured oocytes (% $\pm$ SEM)	Blastocysts /cleaved oocytes (% $\pm$ SEM)	Hatched blastocysts /blastocysts (% $\pm$ SEM)	No. of blastocysts examined	No. of cells/blastocyst (mean $\pm$ SEM)
mPZM-3	Drop	129	49.9 $\pm$ 6.3	17.9 $\pm$ 3.8	47.4 $\pm$ 9.1	4.4	23	32.2 $\pm$ 2.4
	WOW	135	48.1 $\pm$ 4.8	22.2 $\pm$ 2.7	56.1 $\pm$ 6.9	20.3 $\pm$ 7.3	27	32.5 $\pm$ 2.4
	Total	264	49.0 $\pm$ 3.9	20.1 $\pm$ 2.3 ^a	51.8 $\pm$ 5.7 ^a	12.3 $\pm$ 4.5 ^c	Total	32.3 $\pm$ 1.7
mPZM-4	Drop	129	42.0 $\pm$ 4.9	7.3 $\pm$ 2.1	14.2 $\pm$ 3.9	4.3	9	35.1 $\pm$ 4.7
	WOW	134	48.8 $\pm$ 7.2	11.5 $\pm$ 3.1	19.7 $\pm$ 4.9	0.0	14	35.9 $\pm$ 5.8
	Total	263	45.4 $\pm$ 4.3	9.4 $\pm$ 1.9 ^b	16.9 $\pm$ 3.1 ^b	2.2 ^d	Total	35.6 $\pm$ 3.7
Total	Drop	258	46.0 $\pm$ 4.0	12.6 $\pm$ 2.4	31.3 $\pm$ 5.7	4.3	32	33.2 $\pm$ 2.2
	WOW	269	48.4 $\pm$ 4.2	16.9 $\pm$ 2.3	38.6 $\pm$ 5.3	10.1 $\pm$ 2.6	41	33.7 $\pm$ 2.5

a–b: Values with different superscripts are significantly different ($P < 0.01$).

c–d: Values with different superscripts are significantly different ($P < 0.05$).

oocytes were introduced into the WOW system, and 10–15 oocytes were placed in a droplet for IVC. Cleavage (Day 2 after ICSI) and development to the blastocyst stage (Day 6 after ICSI) were compared for the two factors and to examine their interaction.

Experiment 2: The effects of insulin addition to mPZM-3 or mPZM-4 media during IVC were investigated. The dose (100 ng/ml) of insulin (Sigma, 15523 from the porcine pancreas) was adapted from the report by Lee *et al.* [19], which showed that the dose of insulin significantly increased the blastocyst rates in porcine IVM and IVF studies. The ICSI-oocytes were cultured in the WOW system (1 mm in diameter) using mPZM-3 or mPZM-4 media with or without the addition of the insulin dose. Cleavage (Day 2 after ICSI) and development to the blastocyst stage (Day 6 after ICSI) were compared for the two factors (the presence or absence of insulin and culture media) and to examine their interaction.

Statistical analysis

Factorial designs comprised of two culture media (mPZM-3 and mPZM-4) and the two culture methods (the drop and WOW systems) in Experiment 1 (12 replicates) and the two culture media and insulin addition status (with or without) in Experiment 2 (10 replicates) were used to analyze the main effects and their interactions. All percentage data on cleavage and development to blastocysts was subjected to arcsine transformation and was analyzed by two-way ANOVA for analysis of variance and Fisher's PLSD test using the StatView program (Abacus Concepts, Berkeley,

CA, USA). The mean cell numbers per blastocyst were analyzed by two-way ANOVA, and differences among the groups were analyzed using Turkey-Kramer multiple comparison tests.

Results

Experiment 1

The effects of the two culture media (mPZM-3 and mPZM-4) and two culture methods (the drop and WOW systems) on cleavage, blastocyst development, and mean cell number per blastocyst are shown in Table 1. The proportions of cleaved embryos were not significantly different between the two culture media (49.0 and 45.4% for mPZM-3 and mPZM-4, respectively; $P = 0.575$) or between the two culture methods (46.0 and 48.4% for drop and WOW systems, respectively; $P = 0.695$). However, the mean blastocyst rate (20.1%) as a percentage of the cultured oocytes in the mPZM-3 group was significantly ($P < 0.01$) higher than that (9.4%) of the mPZM-4 group. The two culture methods did not affect blastocyst development (12.6 and 16.9% for the drop and WOW systems, respectively; $P = 0.168$). The mPZM-3 medium resulted in significant differences ($P < 0.01$) in the blastocyst rate as a percentage of the cleaved oocytes (51.8 and 16.9%) and the rate of hatched blastocysts to the produced blastocysts (12.3 and 2.2%) when compared with those of the mPZM-4. There was significant interaction ($P < 0.05$) in the rate of hatched blastocysts as a percentage of the blastocysts produced between the two main factors,

Table 2. Effects of insulin addition (100 ng/ml) under different culture conditions for porcine oocytes matured *in vitro* and with subsequent ICSI (Experiment 2)

Medium	Insulin	No. of oocytes cultured	Cleavage (% SEM)	Blastocysts /cultured oocytes (% $\pm$ SEM)	Blastocysts /cleaved oocytes (% $\pm$ SEM)	Hatched blastocysts /blastocysts (% $\pm$ SEM)	No. of blastocysts examined	No. of cells/blastocyst (mean $\pm$ SEM)
mPZM-3	+	107	61.9 $\pm$ 6.5	19.3 $\pm$ 5.2	32.5 $\pm$ 5.7	27.5 $\pm$ 10.8	20	29.2 $\pm$ 2.1
	–	107	57.7 $\pm$ 7.0	18.1 $\pm$ 3.4	35.3 $\pm$ 8.0	2.0	20	33.4 $\pm$ 3.4
	Total	214	59.8 $\pm$ 4.7	18.7 $\pm$ 3.1	33.9 $\pm$ 4.8 ^a	14.9 $\pm$ 5.8	Total	31.3 $\pm$ 2.0
mPZM-4	+	107	52.8 $\pm$ 5.8	14.4 $\pm$ 3.9	26.4 $\pm$ 8.3	8.6 $\pm$ 5.7	15	33.9 $\pm$ 4.0
	–	107	43.0 $\pm$ 6.6	6.2 $\pm$ 3.4	10.3 $\pm$ 5.2	1.2	6	32.8 $\pm$ 4.8
	Total	214	48.0 $\pm$ 4.4	10.3 $\pm$ 2.7	18.4 $\pm$ 5.1 ^b	6.9 $\pm$ 3.7	Total	33.6 $\pm$ 3.1
Total	+	214	57.4 $\pm$ 4.3	16.9 $\pm$ 3.2	29.4 $\pm$ 5.0	18.1 $\pm$ 6.2	25	31.2 $\pm$ 2.1
	–	214	50.5 $\pm$ 5.0	12.2 $\pm$ 2.3	23.0 $\pm$ 5.5	3.6 $\pm$ 2.6	26	33.1 $\pm$ 2.8

a–b: Values (10 replicates) with different superscripts are significantly different ($P < 0.05$).

the culture media and methods. Namely, a significantly ($P < 0.05$) higher hatching rate (20.3%) of produced blastocysts was observed with mPZM-3 using the WOW system, whereas no hatched blastocysts were observed with mPZM-4 using the WOW system. Both the culture media (32.3 and 35.6 for mPZM-3 and mPZM-4, respectively; $P = 0.376$) and culture methods (33.2 and 33.7 for drop and WOW systems, respectively; $P = 0.902$) did not affect the number of cells per blastocyst produced, which ranged from 32 to 36 cells.

Experiment 2

The effects of insulin addition during IVC using the two culture media on cleavage, blastocyst development, and mean cell number per blastocyst produced are shown in Table 2. There were no significant differences in the mean rates of cleaved oocytes or the blastocyst rates as a percentage of the cultured oocytes between the groups with or without the addition of insulin (cleavage = 57.4 and 50.4%, respectively, with $P = 0.382$; blastocyst = 16.9 and 12.2%, respectively, with $P = 0.271$) or between the two culture media (cleavage = 59.8 and 48.0%, respectively, with $P = 0.138$; blastocyst = 18.7 and 10.3%, respectively, with $P = 0.053$). However, the mean blastocyst rate as a percentage of the cleaved oocytes (33.9%) in the mPZM-3 group was significantly ($P < 0.05$) higher than that (18.4%) of the mPZM-4 group, whereas no significant difference was found between groups with or without the addition of insulin (29.4 and 23.0%, respectively; $P = 0.388$). In the mPZM-4 group,

insulin addition resulted in more than two-folds increase in the blastocyst rates as percentages of both the cultured and cleaved oocytes, but there were no significant differences. The blastocysts produced in mPZM-3 medium with the addition of insulin hatched at a rate of 27.5%, although there were no significant differences in the rates of hatched blastocysts as percentages of the blastocysts produced between groups with or without the addition of insulin (18.1 and 3.6%, respectively; $P = 0.110$) or between the two culture media (14.9 and 6.9%, respectively; $P = 0.376$). Furthermore, insulin addition (31.2 and 33.1, respectively; $P = 0.456$) and the culture media (31.3 and 33.6, respectively; $P = 0.518$) did not affect the number of cells per blastocyst produced, which ranged from 29 to 34 cells.

Discussion

The aims of the present study were to investigate the effects of two main IVC conditions (the drop and WOW systems) using semi-defined (mPZM-3) and defined (mPZM-4) media [10] in Experiment 1 and the additional effects of insulin in Experiment 2 on the developmental capacity of *in vitro* matured and intracytoplasmically inseminated porcine oocytes. An attempt of ICSI would remove the incidence of polyspermic fertilization occurring in traditional porcine IVF and produce normal diploid porcine blastocysts *in vitro*.

For *in vitro* culture of porcine zygotes or embryos, microdrops of various medium volumes

(10 to 100 μ l) [2, 11, 37] and a four-well system [7, 25, 38] have been used. As a new culture method, Vajita *et al.* [20] developed the WOW system. The WOW system, which is created at the bottom of a four-well dish, provides a more constant and suitable microenvironment for embryos. The open conditions of the WOW system may also provide an appropriate method for nutrition and dilution of metabolized toxic products during IVC. Taka *et al.* [21] first attempted the WOW system for porcine oocytes matured *in vitro* using a chemically defined medium with subsequent ICSI and found that the WOW system (1 mm in diameter) resulted in a significantly higher blastocyst rate (24.6%) compared with the drop (30 μ l: 12.9%) and four-well (0.5 ml: 14.8%) systems. Experiment 1 was therefore conducted to compare the effects of the culture conditions (drop: 30 μ l; WOW: 1 mm in diameter) using semi-defined (mPZM-3 containing BSA) and chemically defined media (mPZM-4 containing PVA). The present results showed that blastocyst formation with mPZM-4 was significantly lower than with mPZM-3, although cleavage and the number of cells per blastocyst produced were not significantly different between the two media. On the other hand, the WOW system resulted in a significant effect on the rate of hatched blastocysts as a percentage of the produced blastocysts when mPZM-3 was used. Recently, Okada *et al.* [7] obtained a 49% porcine blastocysts and 19% hatched blastocysts using PZM-3, which was changed on Day 2 after IVM and IVF, and by adding 10% FBS on Day 4 of IVC. Observation of hatched porcine blastocysts is rare in porcine IVP studies, especially in a chemically defined IVC medium. In the present study, the WOW system using semi-defined medium (mPZM-3) resulted in a significantly higher rate of hatched blastocysts (20.3%) as a percentage of the produced blastocysts among the four groups, although the droplet and WOW methods did not have any significant effects. These results show a significant interaction between the two main factors examined, the culture media (mPZM-3 and mPZM-4) and culture methods (the drop and WOW systems). In this respect, the WOW system using mPZM-3 seems to be superior to the droplet system, but not with mPZM-4, which needs improvement of the components of the medium. The present results did not fully confirm the results reported in a previous study using mPZM-3 [21]. However, it

seemed that the WOW system may be a useful IVC method for blastocyst development of *in vitro* matured porcine oocytes following ICSI when a semi-defined medium such as mPZM-3 or North Carolina State University-23 [39] is used.

In Experiment 2, the concentration of insulin (100 ng/ml) was adapted from the report of Lee *et al.* [19], who demonstrated the beneficial effects of the insulin concentration on the *in vitro* developmental potential of porcine oocytes and embryos. There are many other reports in which the addition of insulin [16, 19] and various growth factors, such as insulin-like growth factor-I (IGF-I), IGF-II, and epidermal growth factor (EGF) [15, 17, 18] into IVC medium stimulated development to the blastocyst stage. In the present study using the WOW system (1 mm in diameter), the blastocyst rates were similar in mPZM-3 medium with and without the addition of insulin. On the other hand, the rate of blastocyst development was two-fold higher when insulin was added to mPZM-4 medium, although no significant difference was revealed. The use of a chemically defined IVC medium (mPZM-4) containing insulin resulted in blastocyst rates (14% and 26%, respectively) as percentages of the cultured oocytes and of the cleaved embryos following ICSI. These blastocyst rates are comparable to those derived from IVC using BSA-containing media following IVF [1, 4, 5].

In porcine IVP, two main problems to be overcome are 1) prevention of polyspermic fertilization and 2) improvement of developmental capacity to the blastocyst stage [26]. If traditional IVF is replaced with ICSI, the incidence of polyspermic fertilization would be removed. It may be claimed that ICSI produces lower blastocyst rates, but the ICSI technologies have been intensively improved in farm animals, especially for porcine studies, and in our laboratory, we have obtained 20–30% rates of blastocyst development [32–34]. Furthermore, a previous attempt at porcine ICSI provided higher than normal numbers of diploid embryos and lower numbers of mixoploid embryos than IVF [31]. Therefore, more research should be concentrated on improving the developmental capacity of porcine oocytes matured *in vitro*, possibly using a defined IVM culture medium [32, 33] with subsequent ICSI [32–34], and on development of a defined IVC culture medium such as PZM-4 [2, 10].

In conclusion, the present results indicate that a

chemically defined medium (mPZM-4) needs to be improved to provide a more suitable culture conditions for *in vitro* development of *in vitro* matured and intracytoplasmically inseminated porcine oocytes; however, the WOW system may be a useful IVC method for blastocyst development of *in vitro* matured porcine oocytes following ICSI when a semi-defined medium (mPZM-3) is used.

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