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Reduced Polyspermic Penetration in Porcine Oocytes Inseminated in a New In Vitro Fertilization (IVF) System: Straw IVF[1]

Yong-Hai Li, Wei Ma, Ming Li, Yi Hou, Li-Hong Jiao, and Wei-Hua Wang[2]

State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, People's Republic of China

ABSTRACT

High incidence of polyspermy is still a major problem in the in vitro fertilization (IVF) of porcine oocytes matured in vitro. This study was designed to examine whether embryo cryopreservation straws can be used to conduct IVF in porcine oocytes. The efficiency of this system was further compared with traditional microdrop IVF. Immature oocytes were aspirated from antral follicles and matured in vitro. After maturation, oocytes were inseminated either in straws or in microdrops with frozen-thawed boar spermatozoa. For straw IVF, sperm concentration and the presence of air columns between insemination segment and oil column were examined. Sperm-oocyte binding and cortical granules (CGs) before and after sperm penetration were examined by confocal microscopy. When various sperm concentrations were used for IVF in the straws with air columns, it was found that 5 × 10^6 cells/ml of sperm concentration was the optimal concentration; a high penetration rate (94.0%) and normal fertilization (oocytes with both male and female pronuclei) rate (38.2%) were obtained. Increasing sperm concentration to 10 × 10^6 cells/ml increased polyspermic penetration (61.9%) without affecting sperm penetration (86.9%). Reducing sperm concentration to 1 × 10^6 cells/ml reduced polyspermic penetration (25.6%), but sperm penetration rate (69.9%) was also reduced. When IVF was conducted in the straws with or without air columns, and in the microdrops, it was found that sperm penetration in the straws with air columns (96.5%) was significantly ($p < 0.05$) higher than that in the straws without air columns (81.7%) and in the microdrop (72.9%). However, the incidence of polyspermic penetration in the straws with air columns (34.2%) and without air columns (36.6%) was significantly ($p < 0.05$) lower than that (52.4%) in the microdrops. The number of spermatozoa bound to the oocytes was increased gradually in the straws but not in the microdrops in which more spermatozoa bound to the oocytes soon after insemination. CG exocytosis was more complete and faster in the oocytes inseminated in the straws than in the microdrops. These findings indicate that IVF of porcine oocytes in the straws provides a better condition in which more oocytes are fertilized normally than that in the microdrop IVF.

assisted reproductive technology, fertilization, in vitro fertilization

INTRODUCTION

Porcine embryos produced by in vitro maturation (IVM)/in vitro fertilization (IVF) have been successfully developed to the blastocyst stage [1–4]. However, a high incidence of polyspermy remains a major impediment in pig IVF and often exceeds 50% [5–9]. This rate is significantly higher than that of in vivo matured and fertilized oocytes (<5%) [9, 10]. Under in vivo conditions, it is believed that the oviduct microenvironment participates in the completion of oocytes to block polyspermic fertilization [10]. Some approaches by mimicking in vivo conditions, such as preincubation of spermatozoa [11] or oocytes [12] in cultured oviductal epithelial cells or follicle somatic cells [13] or preincubation of oocytes in collected oviductal fluid [14], have been reported to be able to reduce polyspermic penetration of pig oocytes matured and inseminated in vitro, but a satisfactory IVF system in the pigs has not been established.

Some factors that affect sperm penetration and polyspermy in porcine oocytes have been considered, such as high concentrations of spermatozoa at the site of fertilization during IVF, inadequate IVM of oocytes, and suboptimal insemination conditions [10, 15, 16]. High levels of polyspermy have also been observed in the in vivo-matured oocytes inseminated in vitro [17–21], suggesting that excess spermatozoa and suboptimal IVF conditions are the major reasons for polyspermy in pig oocytes. Reducing sperm number during IVF decreased polyspermic penetration, but it also reduced sperm penetration rates [1, 5, 15, 16, 22, 23]. Optimal numbers of fully capacitated spermatozoa for pig IVF have been examined [1, 5, 24–26], but the results are still not stable and satisfactory. Thus, further development of insemination conditions that stimulate sperm capacitation and maintain the oocyte's ability to block polyspermic penetration is necessary.

In the oviducts, motile spermatozoa that have been deposited to the cervix canal swim to the site of fertilization, the ampullary-isthemic junction in which only very active sperm can penetrate the oocytes. The oviduct isthmus serves as a sperm reservoir and regulates the number of sperm reaching the site of fertilization [27]. Thus, the number of spermatozoa at the site of fertilization is well controlled and limited and ensures that most oocytes are fertilized normally (monospermy). However, during IVF, high concentrations of spermatozoa are usually introduced to microdrops in which oocytes have been placed. Hence, the oocytes are exposed to an excess number of spermatozoa, which causes simultaneous sperm penetration into an oocyte and results in polyspermic fertilization, even in a condition that the oocytes have the ability to block the polyspermic penetration. In addition, dead spermatozoa are also introduced to the microdrops, and the dead spermatozoa

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[2]Correspondence: Wei-Hua Wang, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Science, Beijing 100080, China. FAX: 86 10 62650042;
e-mail: wangweihua11@yahoo.com

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may release some detrimental factors that influence the oocyte's function. A technology that can mimic the oviducts to control spermatozoa during IVF may provide a better approach to reduce polyspermic penetration without affecting overall sperm penetration rates. In the present study, to mimic the oviduct, we performed IVF in embryo cryopreservation straws and examined whether such a method can be used to inseminate porcine oocytes in vitro. In this new IVF system, the experiments were designed to allow only motile spermatozoa to swim to the site of fertilization and then fertilize the oocytes. The efficiency was further compared with the traditional microdrop IVF.

MATERIALS AND METHODS

Media and Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. The basic medium used for maturation of oocytes was tissue culture medium 199 (with Earle salts; Gibco, Invitrogen, Gaithersburg, MD) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulphate. This medium was essentially the same as that used by Wang et al. [5, 6] except that calcium lactate was deleted in the present study. Insemination medium was modified Brachett and Oliphant (mBO) medium [28] supplemented with 2 mg/ml BSA, 9 mM $CaCl_2$, 2 mM caffeine-sodium benzoate, and 1 mg/ml casein phosphopeptides (Meijiseika Kaisha, Tokyo, Japan) that was exactly the same as reported by Nagai et al. [29]. Embryo culture medium was North Carolina State University (NCSU) 23 medium [7] containing 0.4% BSA (A-8022).

Oocyte Collection and IVM

Ovaries were collected from prepuberty gilts at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/w) NaCl solution containing 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate at 30–35°C. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles of 2–5 mm in diameter with an 18-gauge needle fixed to a 10-ml disposable syringe. The COCs were washed three times with Hepes-buffered Tyrode's medium containing 0.1% polyvinylalcohol (Hepes-T2-PVA) and three times with maturation medium. Groups of 60 COCs were transferred into 500 μl maturation medium in which 10 ng/ml epidermal growth factor, 10 IU/ml hCG, and 10 IU/ml eCG were added just before culture. The medium had been previously covered with warm paraffin oil in a polystyrene cell culture dish (35 × 10 mm, Nunc, Ruskilde, Denmark) and equilibrated in an atmosphere of 5% CO_2 in air for at least 6 h. These COCs were cultured at 39°C for 44 h in the same condition.

In Vitro Fertilization of Oocytes

Two different systems were used and compared in the present study. The first was the traditional IVF in microdrops. For this, matured oocytes were freed from cumulus cells in the maturation medium containing 0.1% (w/w) hyaluronidase obtained from bovine testis (type I-S, H-3506) and then washed three times with the insemination medium (mBO). Thereafter, groups of 40 oocytes were transferred into a 50-μl droplet of mBO medium covered with paraffin oil. The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination. For IVF, one 0.1-ml frozen semen pellet, made as described by Wang et al. [5], was thawed at 39°C in Dulbecco PBS (DPBS) containing 1 mg/ml BSA (fraction V, A-8022) and antibiotics. The semen was collected from three fertile boars tested in a farm by artificial insemination and mixed together after collection (before cryopreservation). Two different frozen batches of semen were used. After being washed three times, spermatozoa were resuspended with mBO medium containing 2 mM caffeine to give a concentration of 5×10^6 cells/ml, and 50 μl of sample was added to 50 μl of the fertilization drops containing the oocytes. The oocytes and sperm were cocultured for 6 h at 39°C in an atmosphere of 5% CO_2 in air.

The second system was IVF in the straws. Sterile 0.25-ml embryo cryopreservation straws (IMV France Cryo Bio System, Paillette Crista, France) were used in the present study. Oocyte and sperm preparations were exactly the same as that in the microdrop IVF preparations mentioned above. To load the straws, the plugs in the straws were cut off with scissors. One end of the straw was connected to a 1-ml syringe through a hosepipe, and the straws were loaded in the following sequences. In the straw with air columns (Fig. 1A), 1 cm medium with spermatozoa, 5 cm insemination medium, 1 cm medium with 60 oocytes, 1 cm air, and 1 cm paraffin oil were loaded. Then the hosepipe was connected to the other end of the straw, 1 cm air and 1 cm paraffin oil were loaded. In the straw without air columns (Fig. 1B), 1 cm medium with spermatozoa, 7 cm insemination medium, 1 cm medium with 60 oocytes, and 1 cm paraffin oil were loaded. Then the hosepipe was connected to the other end of the straw, and 1 cm paraffin oil was loaded. After being loaded, the straws were gently put on the adhesive tape holders horizontally in a square plate and were incubated for 6 h at 39°C in an atmosphere of 5% CO_2 in air.

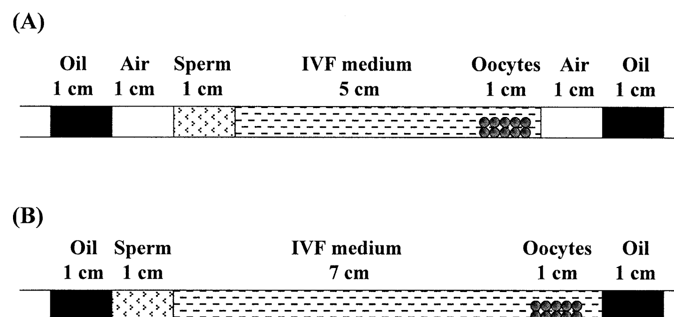

FIG. 1. Diagram of IVF of porcine oocytes in embryo cryopreservation straws. **A**) air columns were added between the oil and fertilization medium columns. **B**) No air column was added.

Assessment of Sperm Penetration, Pronuclear Formation, and Early Embryo Development

Six hours after insemination, a part of the oocytes was washed four times in NCSU 23 and then cultured for another 10 h (16 h total after IVF) for examination of sperm penetration according to the methods reported previously [6]. Briefly, oocytes were fixed in acetic acid:alcohol (1:3) for 48 h, stained with 1% (w/v) orcein for 5 min and examined under a phase-contrast microscope at 400× magnification. If an oocyte had both male (only one) and female pronuclei, this oocyte was considered as a normal fertilized oocyte. Subgroups of oocytes were cultured in 500 μl of NCSU 23 in a four-well culture plate for 48 h for examination of embryo development. Only embryos at 2- to 4-cell stages with same size of blastomeres containing nucleus were counted according to the methods reported previously [30]. Fragmented embryos were not counted in the present study.

Assessment of Sperm-Egg Binding

At 0.5, 1, 1.5, 2, 2.5, and 3 h after insemination, oocytes were removed from straws or microdrops, and the loosely binding spermatozoa were removed completely by pipetting. After being washed three to four times in PBS-0.1% PVA, oocytes were stained with 10 μg/ml *bis*-benzamide (Hoechst 33342) in PBS-0.1% PVA for 5 min, mounted on slides, and examined under a fluorescence microscope. The number of sperm bound to the zona pellucida was counted.

Assessment of Cortical Granules (CGs)

At 3, 4, 5, and 6 h after insemination, oocytes from straws and microdrops were fixed with 3.7% (w/v) paraformaldehyde in PBS-0.1% PVA for 30 min at room temperature and then washed three times in PBS-0.1% PVA, each for 5 min. Oocytes were treated with 0.5% (w/v) Triton X-100 in PBS-0.1% PVA for 5 min and then washed twice in PBS-0.1% PVA for 5 min. Then oocytes were incubated in 100 μg fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA)/ml in PBS-0.1% PVA for 30 min. After FITC-PNA staining, oocytes were washed three times in PBS-0.1% PVA and then stained with 10 μg/ml propidium iodide in PBS-0.1% PVA for 2 min. After being washed twice in PBS-0.1% PVA, oocytes were mounted on slides with antifade solution. In the control groups, oocytes after maturation or culture in mBO medium for 6 h without insemination were also fixed for CG staining. Finally, these oocytes were evaluated by laser confocal microscopy [7, 9].

Experimental Design

Experiment 1 was designed to examine the effect of sperm concentrations on the penetration of oocytes inseminated in the straws with air

TABLE 1. Penetration of porcine oocytes with different concentrations of frozen-thawed spermatozoa in straws.*

Sperm concentration	No. of oocytes examined	No. of oocytes reaching MII (%)	No. of oocytes penetrated (%)	No. of polyspermic oocytes (%)	No. of normal fertilized oocytes† (%)
10×10^6	137	120 (87.6 ± 3.1)	113 (86.9 ± 2.2)[a]	70 (61.9 ± 2.0)[a]	30 (26.5 ± 2.8)[a]
5×10^6	130	117 (90.0 ± 2.9)	110 (94.0 ± 2.0)[a]	38 (34.5 ± 4.2)[b]	42 (38.2 ± 2.3)[b]
1×10^6	135	123 (91.1 ± 1.8)	86 (69.9 ± 3.4)[b]	22 (25.6 ± 3.5)[b]	24 (27.9 ± 2.0)[a]

* Values within parentheses are mean ± SD.
† Oocytes with one male and one female pronucleus were designated normal fertilized oocytes.
[ab] Values with different superscripts within the same column are significantly different ($P < 0.05$); experiment was repeated six times.

columns. Sperm concentrations were adjusted to 10×10^6, 5.0×10^6, and 1×10^6 cells/ml in mBO medium after being thawed and washed in DPBS. Because sperm density varied in the different segments of the straws, the concentrations in the present experiment were those before loading. Experiment 2 was designed to compare the fertilization of oocytes in two systems: traditional microdrop IVF and straw IVF. In the straw IVF, as shown in Figure 1, the presence or absence of air columns was also compared. Sperm concentration used in this experiment was 5×10^6 cells/ml (before loading). Experiment 3 was designed to evaluate CG exocytosis and sperm-egg binding at various times after insemination in both straws and microdrops.

Statistical Analysis

Six replications for examination of oocyte nuclear maturation, fertilization, and development were conducted. All percentage data were subjected to arc sine transformation before statistical analysis. Data were analyzed by ANOVA and shown mean ± SD in tables.

RESULTS

Effects of Sperm Concentration on Penetration of Oocytes Inseminated in the Straws With Air Columns (Experiment 1)

As shown in Table 1, 5×10^6 cells/ml of sperm concentration was the optimal concentration in the straw IVF system. The actual average sperm concentration in the straw was about one seventh of 5×10^6 cells/ml but different densities were present at different segments. The highest density was in the sperm column and the lowest was in the oocyte column. Higher penetration rate (94.0%) and normal fertilized oocyte rate (38.2%) were obtained when oocytes were inseminated at 5×10^6 cells/ml ($p < 0.05$). When sperm concentration was increased to 10×10^6 cells/ml, polyspermic penetration (61.9%) was also increased without increasing sperm penetration rate (86.9%). Although reduced polyspermic penetration (25.6%) was observed when sperm concentration was reduced to 1×10^6 cells/ml, it also reduced sperm penetration rate (69.9%). Thus, the number of normal fertilized oocytes was also reduced (27.9%).

Comparison of Microdrop IVF and Straw IVF (Experiment 2)

As shown in Table 2, the proportions of oocytes penetrated by spermatozoa were significantly ($P < 0.05$) higher in the straw IVF (81.7%–96.5%) than in the microdrop IVF (72.9%). Addition of air columns in the straws gave the highest sperm penetration rate (96.5%). However, the polyspermic rates (34.2%–36.6%) of oocytes inseminated in the straws were significantly ($P < 0.05$) lower than that (52.4%) of oocytes inseminated in microdrops. As a result, the proportions of oocytes with both male and female pronuclei, named as normal (monospermy) fertilized oocytes, were significantly ($P < 0.05$) higher in the straw IVF (32.1%–38.2%) than in the microdrop IVF (24.2%), and no difference was observed in the straws with and without air columns. However, as shown in Table 3, the cleavage rate (35.7%) of putative zygotes from the oocytes inseminated in the straws with air columns were significantly ($P < 0.05$) higher than that of oocytes inseminated in the straws without air columns (24.6%) and in microdrop IVF (20.8%).

Sperm Binding and CG Exocytosis (Experiment 3)

In straw IVF, spermatozoa swam toward the oocytes after the straws were loaded and spermatozoa were observed around the oocytes 0.5 h after insemination. Although the motility of sperm used in the present study was about 50%, more than 90% of spermatozoa around the oocytes were motile when the straws were directly observed under a microscope. As shown in Figure 2, the numbers of sperm bound to the oocyte were gradually increased in the straw IVF and reached the highest at 1.5 h after insemination and then maintained this level. However, in the microdrop IVF, spermatozoa were observed around the oocytes soon after insemination, and it was found that spermatozoa moved in all directions in the microdrops; both motile and dead spermatozoa were observed around the oocytes. The number of spermatozoa that bound to the oocytes was significantly more during the first hour after insemination in the microdrops than in the straws. Although the numbers of spermatozoa bound to the oocytes were lower in the straws during the first hour than those in the microdrops, more spermatozoa were found to bind to the oocytes 1.5 h after insemination and the rates were maintained thereafter.

CGs were distributed under plasma membrane in the oocytes after maturation (Fig. 3A) or after culture for 6 h in the insemination medium (Fig 3B). No sign of CG exocytosis and no zona pellucida staining was observed in the control oocytes (Fig. 3, A and B). When oocytes were ex-

TABLE 2. Penetration of porcine oocytes inseminated in microdrops and straws with frozen-thawed spermatozoa at a concentration of 5×10^6/ml.*

IVF methods	No. of oocytes examined	No. of oocytes reaching MII (%)	No. of oocytes penetrated (%)	No. of polyspermic oocytes (%)	No. of normal fertilized oocytes† (%)
Microdrops	188	170 (90.4 ± 2.5)	124 (72.9 ± 1.0)[a]	65 (52.4 ± 2.1)[a]	30 (24.2 ± 2.0)[a]
Straws with air	180	159 (88.3 ± 3.1)	152 (96.5 ± 1.6)[b]	52 (34.2 ± 1.8)[b]	58 (38.2 ± 2.2)[b]
Straws without air	186	164 (88.2 ± 2.7)	134 (81.7 ± 1.5)[c]	49 (36.6 ± 1.9)[b]	43 (32.1 ± 1.7)[b]

* Values within parentheses are mean ± SD.
† Oocytes with one male and one female pronucleus were designated normal fertilized oocytes.
[abc] Values with different superscripts within the same column are significantly different ($P < 0.05$); experiments were repeated six times.

TABLE 3. Early development of porcine oocytes inseminated in microdrops and straws with frozen-thawed spermatozoa.*

IVF methods	No. of oocytes examined	No. of cleaving embryos (%)
Microdrops	395	82 (20.8 ± 3.3)[a]
Straws with air	345	123 (35.7 ± 2.0)[b]
Straws without air	346	85 (24.6 ± 2.5)[a]

* Values within parentheses are mean ± SD.
[ab] Values with different superscripts within the same column are significantly different ($P < 0.05$); experiments were repeated six times.

amined after IVF, it was found that oocytes started to released CGs 3 h after insemination in the straws (Fig. 3C), and most CGs had already been released from the oocytes. However, the oocytes inseminated in the microdrops started to release CGs 4 h after insemination, although the exact sperm penetration was not clear in the oocytes (Fig. 3D). Zona pellucida of the oocytes penetrated by spermatozoa was also labeled by FITC-PNA (Fig. 3, C and D).

DISCUSSION

The present study indicates that high proportions of porcine oocytes can be fertilized in the straw IVF system, and the polyspermic penetration can also be reduced by this new IVF system. The advantage of the straw IVF is that motile spermatozoa can reach and then fertilize the oocytes. When the straw IVF was compared with the traditional microdrop IVF, we found that more oocytes were fertilized normally in the straw IVF system.

It has been reported that coculture of spermatozoa with oviduct cells [11], follicle cells [13], oviductal fluid [14], follicular fluid [22], and other substances [31] can reduce the incidence of polyspermy in pig oocytes fertilized in vitro. All of these experiments were designed to mimic in vivo conditions in oviducts, so polyspermic penetration was reduced to some levels. However, because sperm number at the site of fertilization was not easily controlled in the microdrop insemination, polyspermic penetration in these approaches mentioned above was reduced, but overall sperm penetration rates were also reduced. In addition, extra works (such as culture of oviduct cells) are necessary and unstable factors (such as unstable components in follicular fluid and oviductal fluid during collection and treatment) are present in these methods. The results are unstable and not repeatable. In the present study, we used embryo cryopreservation straws instead of microdrops to mimic the process of spermatozoa moving, binding, and fertilizing of oocytes in the oviducts. Good results were obtained: reduced polyspermic penetration without reducing overall sperm penetration rate.

Air columns in the straws had no obvious effect on fertilization parameters, especially on the rates of normal fertilized oocytes when oocytes were inseminated in the straws. However, the rate of cleavage (2- to 4-cell embryos) was significantly higher in the straws with air columns than in straws without air columns. The embryos were not cultured for blastocyst development in the present study because all embryos were fixed for examination of fragmentation. In addition, blastocyst development was poor (<10%) in our culture conditions and poor air may have been the major problem. An experiment for improving blastocyst culture conditions is being conducted in our laboratory. The better early development of oocytes fertilized in the straws with an air column may be due to air columns in the straws being important for air equilibration when the

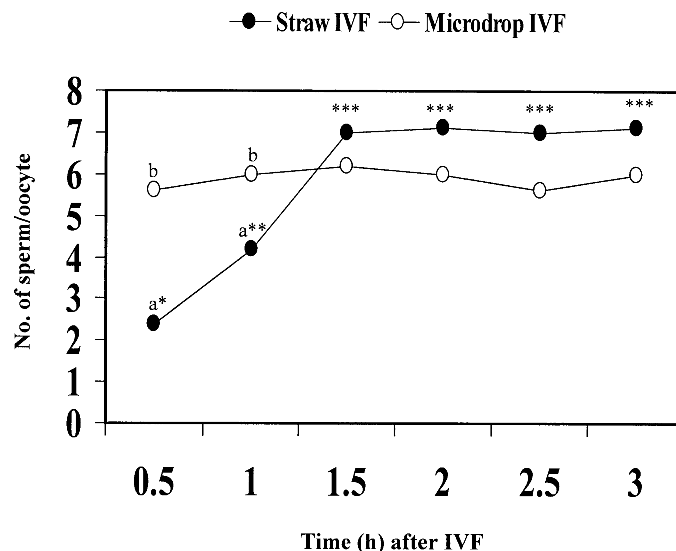

FIG. 2. Sperm binding in porcine oocytes inseminated in straws and in microdrops. Sperm binding was examined at various times after insemination. The [ab]values within the same time of examination between straw and microdrop were significantly different ($p < 0.05$). *, Values within the same IVF system were significantly different ($p < 0.05$).

straws were placed in the CO_2 incubator. It would appear that such a small difference affected the oocyte quality, which in turn influenced subsequent embryonic developmental competence.

An optimal number of fully capacitated spermatozoa at the site of fertilization is extremely important for reducing the incidence of polyspermy in pigs [24–26] because it not only maintains a high proportion of oocytes being fertilized but also avoids a high rate of polyspermic penetration. As in conventional microdrop IVF [5], in the present study, we found that increasing sperm concentrations also increased polyspermic penetration in the straw IVF. However, as the actual sperm concentration was very low in the present study, polyspermic penetration was not increased too much. For example, when sperm concentration was increased from 1×10^6 sperm cells/ml to 5×10^6 sperm cells/ml, polyspermic penetration was increased by less than 10%. These results indicate that oocytes were not directly exposed to so many spermatozoa in the straw IVF, and thus simultaneous sperm penetration was reduced. Recently, Gil et al. [32] found that medium volume and oocyte number also influenced the fertilization parameters and the embryo development in porcine IVF. A low volume of IVF medium and 30–50 oocytes inseminated with 2000 sperm cells per oocyte were beneficial to subsequent embryo development [32]. These results indicate that a high number of spermatozoa during IVF not only induces high polyspermic penetration, but dead sperm may also be detrimental to oocytes, thus affecting subsequent embryo development. In the present study, when oocytes were inseminated in the straw IVF system as compared with the microdrop IVF, sperm concentration in the straws was diluted further (1:7 in straws with air columns), the sperm densities in the straws at different segments were different, and the number around the oocytes was significantly reduced. Furthermore, only motile spermatozoa were at the site of fertilization in the straw IVF, so the effect of dead spermatozoa on the oocytes was minimized. However, if sperm concentration was increased, polyspermic penetration was also increased, indicating that too many motile spermatozoa swam to the oocytes and thus

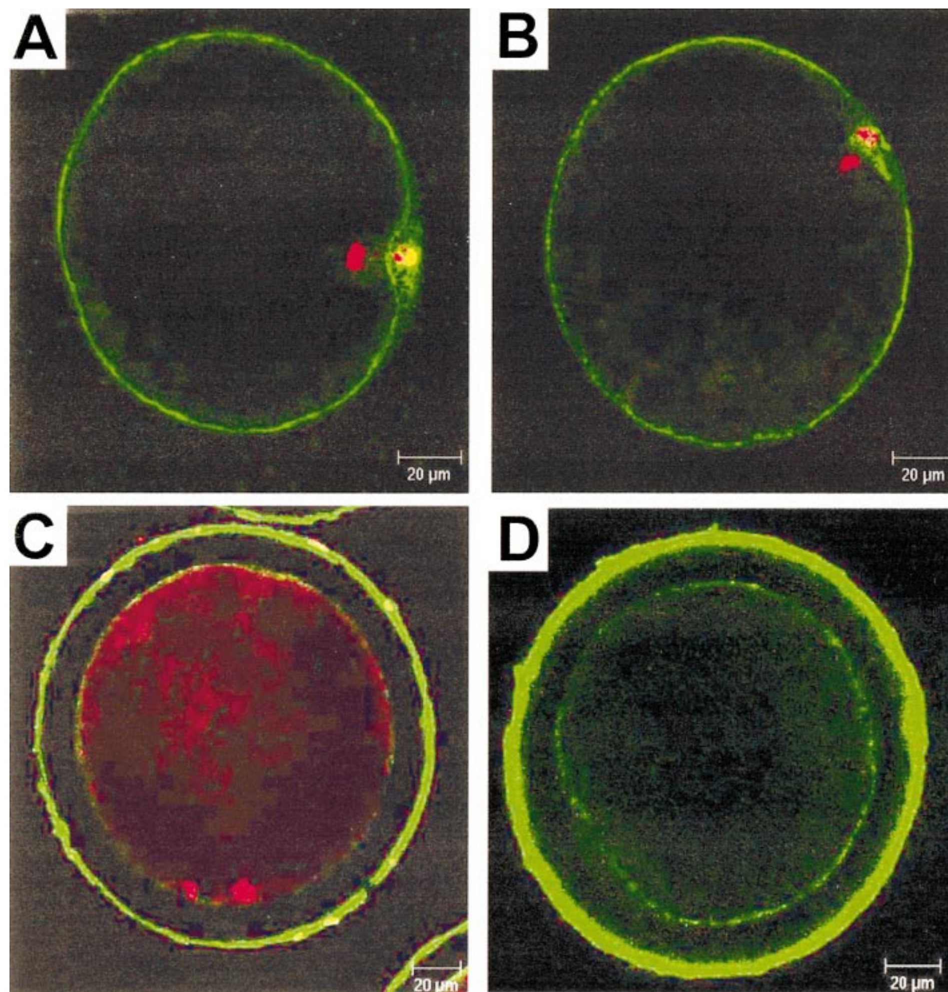

FIG. 3. Confocal microscopic images of CGs and zona pellucida in pig oocytes stained with FITC-PNA. **A**) A monolayer CG distribution under plasma membrane was observed in an oocyte after 44 h of culture. Zona pelluicda was not stained. **B**) CGs were still observed as a monolayer under plasma membrane in an oocyte cultured for 44 h and then 6 h in the insemination medium, showing no CG exocytosis and no zona pellucida staining. **C**) Most CGs had been released from the oocyte inseminated in the straws and examined 3 h after insemination. The zona pellucida was also strongly stained. **D**) Partial CG exocytosis was observed in an oocyte inseminated in the microdrop and examined 4 h after insemination. The zona pellucida was also strongly stained. The images were taken at the equatorial section of oocytes. Red images represent DNA staining, and green images represent CG and zona staining.

induced simultaneous sperm penetration. In this case, it is possible that a longer straw may be more effective than the straws used in the present study.

Sperm concentration used for the microdrop IVF was diluted two times (50 μl semen into 50 μl mBO medium), which means that the actual sperm concentration in the microdrop IVF was 3.5 times more than that in the straw IVF. However, sperm penetration rate was lower in the microdrop IVF than in the straw IVF. These results indicate that in porcine oocytes, straw IVF is more effective than microdrop IVF; high sperm penetrations accompanied by low polyspermic penetration can be obtained with a low sperm concentration.

It is clear that improved fertilization was obtained in the present straw IVF system. To further investigate the mechanism(s) by which polyspermic penetration was reduced without reducing fertilization rate, we examined sperm-egg binding and CG exocytosis. It was found that very motile spermatozoa could swim toward the oocytes and then fertilize the oocytes in the straws, and the number of spermatozoa exposed to the oocytes was increased gradually; thus, the time of sperm binding to oocytes and undergoing the acrosome reaction was different among the spermatozoa. As a result, simultaneous sperm penetration was reduced. However, in microdrops, spermatozoa bound the oocytes almost at the same time during the first 30 min after insemination. Therefore, spermatozoa may penetrate the oocytes simultaneously before the oocytes can establish a block to polyspermic penetration.

CG exocytosis and zona modification are two important events in blocking polyspermy in most mammalian oocytes [15]. In the present study, we examined CG exocytosis in zona-intact oocytes by FITC-PNA because PNA labels only CGs, not zona pellucida of in vitro-matured porcine oocytes [9]. As in the previous study [9], CGs were distributed under plasma membrane of oocytes and formed a monolayer, and the zona pellucida was not stained by FITC-PNA. However, when fertilized oocytes were examined, it was found that CG exocytosis was faster in the oocyte inseminated in the straws than in the microdrops. It is probable that more active spermatozoa fertilized the oocytes in the straws than in the microdrops, and thus induced a more complete CG exocytosis. Furthermore, it would appear that once CGs were released from the oocytes, irrespective of straw or microdrop IVF, it would establish a block to polyspermic penetration as zona pellucida would have been modified. The zona pellucida was not labeled by FITC-PNA in control oocytes but was labeled in the fertilized oocytes. Two possibilities can be used to explain such an interesting finding. First, CG contents released from oocytes may be diffused to the zona pellucida; thus, the zona would also be stained by FITC-PNA. Second, zona pellucida may be modified by the enzymes released from CGs; thus, the modified compositions in the zona would be labeled by FITC-PNA because zona protein modifications occur after zona reaction. Although the exact reason for zona staining after CG exocytosis was not clear in the present study, our results indicate that functional CG and zona re-

actions occurred in the oocytes penetrated by spermatozoa in the present study.

In conclusion, the straw IVF system developed in the present study is an easy and effective new IVF system for porcine oocytes. Improved fertilization and reduced polyspermic penetration were obtained by the straw IVF system. Furthermore, the zygotes produced by the straw IVF had a better developmental competence than those produced by microdrop IVF; thus, this method can be used to produce viable porcine embryos under in vitro conditions.

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