J Anim Reprod Biotechnol 2023;38:70-76
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.38.2.70

Original Article

Optimization of the *in vitro* fertilization system in pigs

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Received May 23, 2023
Revised June 8, 2023
Accepted June 9, 2023

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ABSTRACT

Background: Despite considerable technological advancements, polyspermy remains a significant challenge in *in vitro* fertilization (IVF) procedures in pigs, disrupting normal embryonic development. Here, we aimed to determine whether optimal fertilization conditions reduce the polyspermy incidence in pigs.

Methods: *In vitro*-matured oocytes were co-incubated with sperm according to a modified two-step culture system.

Results: In the first experiment, oocytes were briefly co-incubated with sperm, washed in IVF medium, and then moved to fresh IVF medium for 5 or 6 h. Although the 6 h sperm-free cultured group had a higher penetration rate than the 5 h cultured group, the polyspermy rate significantly increased in the 6 h sperm-free cultured group. The gamete co-incubation period was either 20 or 40 min. The 40 min cultured group had a higher rate of blastocyst formation and number of total cells in blastocysts than the 20 min cultured group. In experiment 2, oocytes were inseminated with sperm separated by Pecroll treatment. Percoll treatment increased the rate of oocyte penetration and blastocyst formation compared to the control. In experiment 3, fertilized oocytes were cultured in 25 μL microdroplets (10 gametes/drop) or 500 μL (100 gametes/well) of culture medium in 4-well plates. The large volume of medium significantly reduced the number of dead oocytes and increased the rate of blastocyst formation compared to the small volume.

Conclusions: Collectively, these results demonstrate that various fertilization conditions, including modified co-culture period, active sperm separation, and culture medium volume, enhance fertilization efficiency and subsequent embryonic development by decreasing polyspermy occurrence.

Keywords: co-culture time, embryonic development, *in vitro* fertilization, polyspermy

INTRODUCTION

In vitro fertilization (IVF) is a fundamental technique that promotes the generation of a substantial number of embryos and facilitates research on embryonic development (Romar et al., 2019). Despite recent advancements in *in vitro* maturation and IVF techniques for pig oocytes, porcine IVF programs continue to face significant challenges compared to other species, such as a notable prevalence of polyspermy and poor embryo quality (Macháty et al., 1998; Romar et al., 2019). Consequently, the majority of studies conducted thus far have focused on investigating the factors influencing the outcome of IVF. Various strategies have been adopted to address this issue, includ-

ing modifying gamete co-incubation times (Marchal et al., 2002; Gil et al., 2004), adjusting sperm concentration (Koo et al., 2005), considering the source of spermatozoa (Gil et al., 2005) and oocytes (Marchal et al., 2001), and examining the effect of the co-culture medium (Abeydeera and Day, 1997) on embryo production. However, a successful IVF system for pigs has yet to be established.

The occurrence of polyspermic penetration can be reduced by decreasing the number of sperms during IVF. However. This approach also leads to a decline in the overall sperm penetration rate (Coy et al., 1993) and compromises the fertilization efficiency. Along with polyspermy, the suboptimal quality of IVF-derived pig embryos poses another challenge that needs to be addressed for *in vitro* production systems. Specifically, compared to *in vivo*-derived blastocysts, IVF-derived blastocysts have a lower total cell number (Macháty et al., 1998). To resolve this problem, various sperm-selection methods are routinely applied to prepare semen for IVF in various species. These procedures aim to enhance sperm quality and eliminate seminal plasma/cryoprotectants (Henkel and Schill, 2003). Several recent studies have explored the potential benefits of reducing the co-incubation time of gametes in terms of pre-embryo development and implantation potential; however, the results have been inconsistent (Dirnfeld et al., 1999; Almiñana et al., 2005). Given the current state of research, comprehensive information on the impact of different conditions on IVF and embryo development would greatly contribute to our understanding of how processing methods influence IVF outcomes.

The aim of the present study was to assess the effect of different conditions during IVF on fertilization parameters and subsequent embryonic development and to improve fertilization conditions by reducing the high frequency of polyspermy in pigs.

MATERIALS AND METHODS

All chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Oocyte collection and *in vitro* maturation

Oocyte collection was performed as described previously (Kwon et al., 2022). Prepubertal porcine ovaries were obtained from a local slaughterhouse (Farm Story Hannang, Chungwon, Chungbuk, South Korea) and transported to the laboratory at 37℃ in saline supplemented with 75 mg/mL penicillin G and 50 mg/mL streptomycin sulfate. Follicles with a diameter of 3-6 mm were aspirated using an 18-gauge needle connected to a 10 mL disposable syringe. Cumulus-oocyte complexes (COCs) were chosen based on their morphological features, and those that exhibited a minimum of three layers of cumulus cells and an evenly granulated ooplasm were selected. After three washes with *in vitro* maturation medium TCM-199 (11150-059; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.1 g/L sodium pyruvate, 0.6 mJVI l-cysteine, 10 ng/mL epidermal growth factor, 10% (v/v) porcine follicular fluid, 10 IU/mL luteinizing hormone, and 10 IU/mL follicle-stimulating hormone, 100 COCs were transferred into 4-well dishes containing 500 μL of maturation medium covered with mineral oil. The plates were incubated at 38.5℃ in a humidified atmosphere of 5% CO_2 for 44 h.

Sperm preparation

Fresh liquid semen was supplied weekly from a Darby Pig Artificial Insemination Center (Darby Genetics, Anseong, Gyeonggi-do, South Korea) and kept at 18℃ prior to use. Sperm concentration was determined using a hemocytometer. Sperm motility was evaluated before fertilization, and > 80% motile sperm were used in each experiment. In experiment 3, sperms were chosen through a discontinuous Percoll gradient according to the treatment (90% and 45%). Percoll at each concentration was placed in a 1.5 mL tube at a volume of 200 μL, whereas the semen volume was 100 μL. After centrifugation at 5,000 × *g* for 5 min and subsequent washing in modified Tris-buffered medium (mTBM) medium, sperm concentration was evaluated prior to fertilization.

In vitro fertilization and *in vitro* culture

In vitro fertilization and culture were conducted as previously described (Jeon et al., 2015; Zhou et al., 2020), with some modifications. For IVF, cumulus cells were removed from the oocytes by repeated pipetting with 1 mg/mL hyaluronidase. Then, groups of 15 denuded oocytes were randomly placed into 40 μL droplets of mTBM. Next, the semen sample was washed twice with Dulbecco's phosphate-buffered saline supplemented with 0.1% bovine serum albumin (BSA) by centrifugation at 2,000 × *g* for

2 min. After washing, the sperm pellet was resuspended in mTBM, which had been pre-equilibrated for 18 h at 38.5℃ in the presence of 5% CO_2. After proper dilution, 5 μL of the sperm suspension was added to a 40 μL drop of mTBM to achieve a final sperm concentration of 5.0 × 10^5 sperm/mL. A modified two-step culture system was used to utilize stored liquid semen. The oocytes were co-incubated with sperm for 20 or 40 min at 38.5℃ in a humidified atmosphere of 5% CO_2. After the co-incubation period, any loosely attached sperm on the zona pellucida was gently removed by pipetting. Subsequently, the oocytes underwent three washes in mTBM and were incubated in mTBM without sperm for 5 or 6 h at 38.5℃. Next, the gametes were thoroughly washed and cultured in porcine zygote medium-5 supplemented 4 mg/mL BSA in 25 μL microdroplets or 500 μL in 4-well plates for 7 days at 38.5℃ and 5% CO_2.

Assessment of fertilization parameters

To assess sperm penetration and pronucleus formation, presumptive zygotes from each group were stained with DAPI for 5 min 12 h after the initiation of IVF. After embryos were washed in embryo culture medium, they were placed in a droplet of HEPES and immediately imaged using a digital camera (DP72; Olympus, Tokyo, Japan) connected to a fluorescence microscope (IX70; Olympus). The number of penetrated oocytes, presence of monospermy and polyspermy, and efficiency of fertilization (number of monospermic oocytes/total number of inseminated oocytes) were assessed as the number of pronuclei under a fluorescence microscope.

Embryo evaluation and total cell count

To determine the total cell number, blastocysts were randomly selected on day 7 and fixed in 3.7% paraformaldehyde for 30 min at room temperature. Subsequently, the blastocysts were stained with 10 mg/mL Hoechst 33342 (bisbenzimide) for 5 min, washed thrice with PBS-PVA, and mounted onto glass slides. Images were obtained using a digital camera (DP72; Olympus) connected to a fluorescence microscope (IX70; Olympus).

Experimental design

In experiment 1, the effects of the sperm-oocyte co-culture period on polyspermy and blastocyst formation in a two-step culture system of IVF embryos were investigated. *In vitro*-matured oocytes were briefly co-incubated with sperm for 20 or 40 min, washed in IVF medium by gentle pipetting to remove spermatozoa loosely attached to the zona pellucida, and transferred to fresh IVF medium (sperm-free) for 5 or 6 h. The sperm-free incubation period was 5 or 6 h, and fertilization parameters and rate of polyspermy were investigated. The first co-incubation period was 20 or 40 min to evaluate its effect on embryonic development. For each group, the rate of blastocyst formation and total number of cells were determined.

In experiment 2, we investigated the effects of Percoll treatment on sperm penetration and subsequent embryonic development. Fresh semen was treated with different concentrations of Percoll (90% and 45%), and active sperms were separated in 90% Percoll. The oocytes were inseminated with the separated sperm. The number of penetrating oocytes and the blastocyst formation rates were recorded for each group.

In experiment 3, the effects of culture medium volume on embryonic survival and development were investigated. The oocytes were divided into two groups for *in vitro* culture; 25 μL microdroplets (10 gametes/drop) or 500 μL in 4-well plates (100 gametes/well). Twenty-five-microliter microdroplets were renewed at 48 h (day 2) and 96 h (day 4) following IVF. The number of dead embryos and blastocyst formation rates were examined for each group.

Statistical analysis

Each experiment was repeated at least three times, and data were analyzed using Student's *t*-test. All percentage data were subjected to arcsine transformation before conducting statistical analysis and are presented as the means ± SEM. Statistical significance was set at $p < 0.05$. All calculations were performed using the GraphPad Prism 6 software (GraphPad, San Diego, CA, USA).

RESULTS

Effects of sperm-free culture time on polyspermy in porcine embryos

To investigate the effect of sperm-free culture time on polyspermy occurrence during IVF, oocytes bound to spermatozoa were incubated for 5 or 6 h in sperm-free medium according to a two-step culture system. As shown in Fig. 1A, the rate of penetrated oocytes in the 6 h cultured group was higher than that in the 5 h cultured group ($p <$

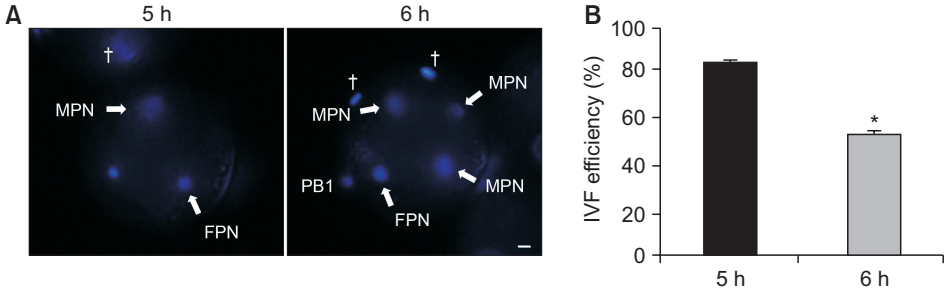

Fig. 1. Effects of sperm-free culture time on polyspermy and fertilization efficiency in porcine embryos. (A) Representative images of the polyspermy and monospermy in the 5 or 6 h sperm-free cultured group, respectively. †Spermatozoa attached to the zona pellucida. Bar = 20 μm. (B) The rate of efficiency of fertilization (number of monospermic oocytes/total inseminated) in porcine embryos. *$p < 0.05$. MPN, male pronuclear; FPN, female pronuclear; PB1, first polar body.

Table 1. Effect of sperm-free culture time on polyspermy in porcine IVF embryos

Treatment	Total (n)	Penetrated oocytes	Polyspermic oocytes
		% (No.)	% (No.)
5 h	84	48.01 ± 1.87 (39)	15.17 ± 1.40 (6)
6 h	80	69.44 ± 1.31 (56)*	47.01 ± 1.68 (26)**

Polyspermy rate was calculated as the no. of polyspemic oocytes divided by the no. of penetrated oocytes × 100. Data are indicated as the mean ± SEM. No, number. *$p < 0.05$; **$p < 0.01$.

0.05). Although the rate of sperm penetration increased, the rate of polyspermy significantly increased in the 6 h cultured group (Table 1, $p < 0.01$). Finally, the 6 h cultured group showed significantly reduced fertilization efficiency in IVF embryos compared to the 5 h cultured group (Fig. 1B, $p < 0.05$). These results indicate that a short sperm-free culture time reduces polyspermy frequency in pig IVF embryos.

Effect of sperm–oocyte co-culture time on development and quality of embryos

To determine the optimal IVF co-culture period for embryo quality, oocytes were co-cultured with sperm for 20 or 40 min during the first step of the culture system, and the rates of blastocyst formation and quality were investigated. Given the observed effect of a sperm-free culture time of 5 h, oocytes were incubated for 5 h in sperm-free medium after the first step of culturing for 20 or 40 min. In the 40 min cultured group, the rate of blastocyst formation was significantly higher than that in the 20 min culture (Fig. 2A, $p < 0.05$). Moreover, the 40 min cultured group showed significantly increased total cell numbers in blastocysts compared to the 20 min culture, resulting in good-quality embryos (Fig. 2B and 2C, $p < 0.05$). Collectively, these results suggest that a long co-culture time in the first step of the culture system enhances the developmental competence and quality of IVF embryos.

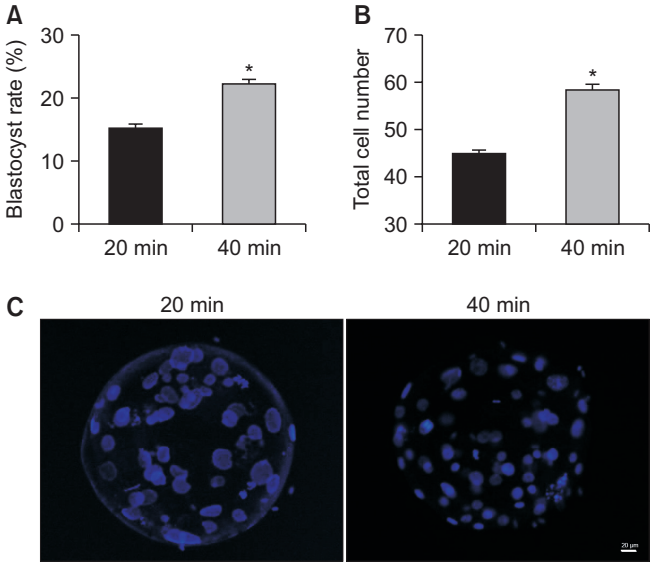

Fig. 2. Effects of sperm-oocyte co-culture time on development and quality of IVF-derived embryos. (A) The rate of blastocyst formation in the 20 or 40 min co-cultured group. *$p < 0.05$. (B) Total cell number in porcine blastocyst in the 20 or 40 min co-culture group. (C) Representative images of the total cells according to different co-culture time in IVF-derived embryos. Blue, DNA.

Effect of Percoll treatment of sperm on penetration and blastocyst formation in embryos

To evaluate the effect of Percoll treatment on the penetration rate and developmental competence of IVF embryos, oocytes were fertilized with selected sperms using a discontinuous Percoll gradient (90% and 45%). The

Table 2. Effect of Percoll treatment of sperm on penetration and development in IVF embryos

Treatment	Total (n)	Penetrated oocytes	Blastocyst formation
		% (No.)	% (No.)
Control	365	63.66 ± 1.63 (230)	13.03 ± 1.03 (48)
Percoll	420	79.51 ± 1.03 (327)*	18.72 ± 0.88 (77)*

Fertilization rate was calculated as the no. penetrated oocytes (2-cell embryos) divided by the total no. of inseminated oocytes × 100. Data are indicated as the mean ± SEM. No, number. *$p < 0.05$.

number of penetrating oocytes increased significantly after Percoll treatment. In addition, Percoll treatment significantly increased the rate of blastocyst formation compared to the untreated control, suggesting that it improves developmental competence (Table 2). Therefore, these results suggest that the separation of active sperm enhances the fertilization rate and development of IVF embryos.

Effect of volume of culture medium on survivability and development of embryos

To set IVF and culture condition, embryos were cultured in two volumes of culture medium: 25 or 500 μL (10 oocytes/drop or 100 oocytes/well, respectively). As shown in Table 3, 500 μL volume of medium significantly reduced the dead embryos compared to the 25 μL volume group, suggesting that it improves the survivability of embryos. Moreover, the rate of blastocyst formation in the 500 μL volume group was significantly higher than that in the 25 μL volume group. These results indicate that an increase in medium volume enhances the stability and developmental competence of IVF embryos.

DISCUSSION

Polyspermic fertilization is observed more frequently in pigs than in other species, even when *in vivo* fertilization is conducted under diverse experimental conditions (Hunter, 1990; Hunter, 1991). In the present study, we demonstrated that several IVF and culture conditions induced a decrease in polyspermy and an increase in fertilization efficiency, resulting in an increase in developmental competence in pigs. Our results showed that a sperm-free culture period of 6 h significantly increased the polyspermy rate compared to the 5 h culture period. On the other hand, a long oocyte-sperm co-culture time of 40 min increased the rate of embryonic development and its quality by increasing the total cell number of blastocysts compared to the 20 min cultured group. In the second experiment, fertilization with sperm-treated Percoll resulted in a higher rate of oocyte penetration and blastocyst formation than in the control with no treatment. In the third experiment, a high culture volume significantly increased the survivability of embryos and developmental rate by reducing the number of dead embryos compared to a low culture volume.

Table 3. Effect of volume of culture medium on survivability and development in IVF embryos

Treatment	Total (n)	Dead oocytes	Blastocyst formation
		% (No.)	% (No.)
25 μL	401	63.72 ± 1.57 (269)	12.55 ± 0.8 (49)
500 μL	543	12.27 ± 1.30 (70)*	21.38 ± 0.84 (125) *

Data are indicated as the mean ± SEM. No, number. *$p < 0.05$.

In general, polyspermy arises more frequently *in vitro* than *in vivo* (Mahé et al., 2021), and greater sperm concentrations and longer periods of sperm-oocyte co-incubation result in enhanced frequencies of polyspermic penetration (Marchal et al., 2002; Matás et al., 2003). Sperm-free culture time for 6 or 5 h had been applied for porcine IVF system, but comparative effects have not been studied yet. In this study, the degree of polyspermy and the rate of oocyte penetration significantly increased when the sperm-free culture time was used for 6 h compared to the 5 h during IVF. The efficiency of fertilization was significantly decreased in the 6 h cultured group. Some studies have reported that co-incubation for 2 h and 4 h leads to a higher rate of monospermy compared to the group cultured for 6 h, even when the penetration and number of spermatozoa per oocyte increase over time (Matás et al., 2003). These results suggest that a short sperm-free culture period can reduce the rate of polyspermy in porcine IVF embryos. However, these results indicated that short sperm-free incubation period still has a problem that reduced the penetration rate during IVF. Spermatozoa that bind to the zona pellucida within the initial 10 min can fertilize a considerable number of oocytes (Gil et al., 2004), suggesting that this is a crucial period during IVF. Several studies focused on gamete co-incubation time have been showed different effects under the various conditions in human and pig (Almiñana et al., 2005; Le Bras et al., 2017), gamete co-incubation for 20 or 40 min was investigated to improve embryonic development and

quality. The results showed that the rate of blastocyst formation and the number of total cells in blastocysts significantly increased in the 40 min cultured group, suggesting that it enhanced the quality of IVF-derived embryos in pigs. Polyspermy triggers the abnormal development and death of early embryos (Hunter, 1991). However, even polyspermic porcine IVF embryos exhibiting multiple pronuclei can develop to the blastocyst stage at a rate similar to that of monospermic IVF embryos. Blastocysts from polypronuclear oocytes have a lower number of inner cell mass nuclei than blastocysts derived from oocytes with two pronuclei (Han et al., 1999). Therefore, these results demonstrate that proper two periods of porcine IVF system have an effect on the rate of monospermy and penetration resulting in high developmental competence and quality.

Sperm selection methods such as swim-up, Percoll gradient, and centrifugation-based washing are commonly used in IVF systems for diverse species (Rodriguez-Martinez et al., 1997; Palomo et al., 1999). Compared with the swim-up method, the Percoll gradient technique yields a much higher recovery of motile spermatozoa (Parrish et al., 1995) and more successful IVF results. In experiment 2, the results indicated that fertilization with sperm separated by a 90% Percoll gradient greatly increased the rate of oocyte penetration and subsequent embryonic development. Moreover, the behavior pattern showed that the number of reacted live spermatozoa consistently improves throughout incubation. Spermatozoa subjected to Percoll treatment exhibit the fastest oocyte penetration and the highest penetrability in pigs (Matás et al., 2003). Percoll gradient treatment of frozen-thawed boar semen significantly enhances cleavage and blastocyst development in pigs (Jeong and Yang, 2001). Thus, these results suggest that active sperms separated from fresh semen have a higher capacity for penetration, resulting in an increase in embryonic development.

Embryo density refers to the ratio of embryos to the volume achieved during *in vitro* culture; in other words, it quantifies the number of embryos within a defined volume of the culture medium (Reed, 2012). To evaluate the appropriate culture volume for embryonic development, the culture density of 10 or 100 embryos cultured in different volumes (25-μL droplets or 500 μL/well, respectively) was investigated. The results showed that low-density culture greatly reduced the number of dead embryos, which resulted in an increase in the rate of blastocyst formation in pigs compared to high-density culture. Some studies have suggested that embryos cultured together produce better quality than cultured embryos, and increasing embryo density might enhance the *in vitro* culture of mammalian embryos (Reed, 2012). However, high culture density where 8-10 embryos were cultured in 20 μL droplets negatively affected embryo development in cats (Sananmuang et al., 2011). Similarly, these results indicate that a low culture density increases the developmental rate by enhancing embryonic survivability in porcine IVF embryos.

CONCLUSION

In conclusion, the modified culture time in the two-step culture system reduced the rate of polyspermy, increased efficiency, and improved embryonic development in pigs. Percoll treatment of sperm and low culture density enhances embryonic development in IVF embryos. Finally, this could contribute to effective production and improved quality of IVF embryos in pigs.

Author Contributions: Conceptualization, X-S.C., S-H.L.; methodology and data curation, S-H.L.; writing-original draft and review and editing, S-H.L., X-S.C.; supervision, X-S.C.

Funding: This work was supported by the National Research Foundation (NRF) of Korea grant funded by the Korea government (MSIT) (No. 2022R1A2C300769), Republic of Korea.

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

Acknowledgements: None.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

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