Veterinary and Animal Science 16 (2022) 100241

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas

Effects of the timing of electroporation during *in vitro* maturation on triple gene editing in porcine embryos using CRISPR/Cas9 system

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ARTICLE INFO

Keywords:
CRISPR/Cas9
Electroporation
Gene knockout
Permeability
Pig

ABSTRACT

Mosaicism, including alleles comprising both wild-type and mutant, is a serious problem for gene modification by gene editing using electroporation. One-step generation of F0 pigs with completely desired gene modifications saves cost and time, but the major obstacles have been mosaic mutations. We hypothesized that the timing of electroporation prior to *in vitro* fertilization (IVF) can increase the rates of biallelic mutation for multiple gene knockout as the permeability of mature oocytes is greater than that of zygotes. Hence, we determined whether the timing of electroporation during *in vitro* maturation (IVM) culture enhances triple gene editing in the resulting blastocysts. Three gRNAs targeting *KDR, PDX1*, and *SALL1* were simultaneously introduced into the oocytes that had been incubated for 40, 42, and 44 h from the start of the IVM culture. Electroporation with three gRNAs at 40 h and 42 h during IVM culture decreased the blastocyst formation rates and did not improve the mutation rates and target number of biallelic mutations in the resulting blastocysts. The blastocyst formation rate, mutation rates, and target numbers in the resulting blastocysts from oocytes treated by electroporation at 44 h of IVM culture were similar to those of control zygotes electroporated at 13 h after the initiation of IVF. In conclusion, multiple gene editing efficiency in the resulting blastocysts was comparable between oocytes electroporated before and after the fertilization, indicating that oocytes with completed maturation time may allow better functioning of materials accepting gene editing application.

Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (CRISPR/Cas) system has been successfully used for gene editing in several mammalian species (Liu, 2020). Pigs are one of the domesticated animals that have been widely used in the history of biomedical research, including gene therapy, because of their similarities to humans in terms of body conformation, anatomy, and physiology (Luo, Lin, Bolund, Jensen & Sorensen, 2012; Tao, Youfu & Yukun, 1996). We have previously published data that proved the efficacy of electroporation technique in the application of single genome mutation after introduction of the Cas9 protein in porcine embryos (Hirata et al., 2019b; Tanihara et al., 2021; Wittayarat et al., 2021). Research concerning one-step multiple gene targeting in pig models has recently attracted great interest in the recent years because some human diseases, such as type I diabetes, thrombosis, and liver cirrhosis, are caused due to mutations in multiple genes (Field, 2002; Liu et al., 2015; Tirado et al., 2004). This type of study would lead to a better understanding of the disease mechanism, and possibly to the future development of effective therapeutics for gene therapy in human medicine.

Such one-step multiple gene targeting system has faced some major obstacles, including mosaic mutations (Im, Moon & Kim, 2016). Mosaic mutations are characteristic of mutants with mixed alleles comprising both wild-type and mutant alleles (Foulkes & Real, 2013). Electroporation of Cas9 protein and sgRNA into zygotes within the time window between the fertilization and first DNA replication has been suggested to generate non-mosaic or biallelic mutants (Hashimoto, Yamashita &

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https://doi.org/10.1016/j.vas.2022.100241

Available online 28 February 2022

Takemoto, 2016). However, we still could not produce the desired biallelic mutation by one-step multiple gene targeting, in which the CRISPR/Cas9 system was electroporated into porcine putative zygotes during the gap time between the end of fertilization and onset of genome replication (Hirata et al., 2019a). The mechanism underlying this limitation is still obscure, and we hence decided to initiate a study to determine a better timing (oocyte stage or zygote stage) for the introduction of CRISPR/Cas9 system to increase the rates of biallelic mutation for triple gene knockout by electroporation.

Matured oocytes with intact zona pellucida are known to have permeability to molecules weighing up to 170 kDa, which is larger than that of zygotes with intact zona pellucida that has been observed to decrease permeability to molecules up to the weight of 110 kDa (Legge, 1995). However, our previous study demonstrated that the efficiency of single gene editing in the resulting blastocysts from matured oocytes electroporated with CRISPR/Cas9 system before IVF was comparable with that of zygotes electroporated at 13 h after the initiation of IVF (Hirata et al., 2019a). Therefore, in the present study, we investigated whether the timing of electroporation prior to fertilization (during *in vitro* maturation (IVM) culture) enhances triple gene editing in the resulting blastocysts. Three gRNAs targeting *KDR, PDX1*, and *SALL1* genes were simultaneously introduced into porcine oocytes that had been incubated for 40, 42, and 44 h in comparison to the control group, wherein the putative zygotes were electroporated with pooled gRNAs targeting all three genes at 13 h after the initiation of fertilization.

Materials and methods

Ethical approval

The animal experiments were approved by the Institutional Animal Care and Use Committee of Tokushima University (approval number: T2019–11).

Oocyte collection, in vitro maturation (IVM), and fertilization

Pig ovaries were obtained from slaughtered prepubertal gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and were transported in physiological saline at 30 °C within 1 h to the laboratory. Ovaries were washed three times with prewarmed physiological saline solution supplemented with 100 IU/ml penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/ml streptomycin sulfate (Meiji). Follicles with diameters of 3–6 mm on the ovarian surface were sliced on a sterilized dish using a surgical blade, and cumulus-oocyte complexes (COCs) were visualized and collected under a stereomicroscope. Approximately 50 COCs were cultured in 500 μl of maturation medium consisting of tissue culture medium 199 with Earle's salts (TCM 199; Gibco/Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 50 μM β-mercaptoethanol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd.), 10 IU/ml equine chorionic gonadotropin (Kyoritsu Seiyaku, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Kyoritsu Seiyaku), and 50 μg/ml gentamicin (Sigma-Aldrich) for 22 h in 4-well dishes (Nunc A/S, Roskilde, Denmark). The COCs were then transferred to a maturation medium without hormones and were cultured for additional 22 h. COCs were incubated at 39 °C in a humidified incubator containing 5% CO_2.

The matured oocytes were subjected to *in vitro* fertilization (IVF) as described previously (Nguyen et al., 2017). Briefly, frozen-thawed ejaculated spermatozoa were transferred into 5 ml of fertilization medium (PFM; Research Institute for the Functional Peptides Co., Yamagata, Japan) and washed by centrifugation at 500 × *g* for 5 min. The pelleted spermatozoa were resuspended in fertilization medium and adjusted to 1×10^6 cells/ml. Subsequently, approximately 50 oocytes were transferred to 500 μl of sperm-containing fertilization medium, covered with mineral oil in 4-well dishes, and co-incubated for 5 h at 39 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. After the co-incubation, the presumptive zygotes were washed with pig zygote medium (PZM-5; Research Institute for Functional Peptides Co.) and cultured continuously *in vitro* at 39 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. Embryos cultured for 3 days were subsequently incubated in porcine blastocyst medium (PBM; Research Institute for Functional Peptides Co.) for 4 days.

Electroporation

Electroporation was performed as described previously (Tanihara et al., 2016). Briefly, an electrode (LF501PT1–20; BEX, Tokyo, Japan) was connected to a CUY21EDIT II electroporator (BEX) and was set under a stereoscopic microscope. The cumulus-free oocytes incubated for the designated times from the start of maturation culture were washed with Opti-MEM I solution (Thermo Fisher Scientific) and placed in a line between the gap in the electrode in a chamber slide filled with 10 μl of nuclease-free duplex buffer (Integrated DNA Technologies (IDT), Coralville, IA, USA) containing 100 ng/μl of three gRNAs (Alt-R CRISPR crRNAs and tracrRNA, chemically modified and length-optimized variants of the native guide RNAs purchased from IDT) and 100 ng/μl Cas9 protein (Guide-it Recombinant Cas9; Takara Bio, Shiga, Japan). The gRNAs were designed using the CRISPR direct webtool (https://crispr.dbcls.jp/) (Naito, Hino, Bono & Ui-Tei, 2015). To minimize off-target effects, 12 nucleotides at the 3′-end of the designed gRNAs had no sequence matches in the pig genome other than the target regions of *KDR, PDX1*, and *SALL1* (Table 1), as determined using the COSMID webtool (https://crispr.bme.gatech.edu/) (Cradick, Qiu, Lee, Fine & Bao, 2014).

After electroporation (five 1-ms square pulses at 25 V), the oocytes were incubated in the same maturation medium until 44 h of the total culture period. The matured oocytes were subjected to *in vitro* fertilization, as described above. Subsequently, the embryos were cultured for 7 days to evaluate their ability to develop to the blastocyst stage as well as the genotypes of the resulting blastocysts.

Analysis of the targeted genes after electroporation

We analyzed the frequencies of base insertions or deletions (indels) in the target regions of individual blastocysts to compare the efficiency of the introduction of target mutations in the embryos. Genomic DNA was extracted from embryos by heat treatment in 50 mM NaOH. After neutralization, the DNA samples were subjected to polymerase chain reaction (PCR) using KOD One PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The primers used for the amplification are listed in Table 1. After purification of PCR products using Fast Gene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), we analyzed the target region sequences using Sanger sequencing with a BigDye Terminator Cycle Sequencing Kit version 3.1 (Thermo Fisher Scientific K.K., Tokyo, Japan) in an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The Tracking of Indels by Decomposition (TIDE; https://tide.deskgen.com/) bioinformatics package was used to quantify the frequency of indel mutation events in the resulting blastocysts derived from oocytes electroporated with three gRNAs (Brinkman, Chen, Amendola & van Steensel, 2014). According to the target region sequences, blastocysts were classified as having biallelic mutations (carrying no wild-type (WT) sequences), mosaics (carrying more than one type of mutation and WT sequence), or WT (carrying only the WT sequence).

Experimental design

To determine whether the timing of electroporation prior to IVF enhances triple gene editing in the resulting blastocysts, three gRNAs targeting *KDR, PDX1*, and *SALL1* were simultaneously introduced into

Table 1
gRNA and primer sequences used for sequencing analysis.

Target gene (Chromosome localization *)	gRNA Target sequence	PAM	Target	Strand	Primer Forward primer	Reverse primer
KDR (Chromosome 8, NC_010450.3.)	AAAAGACATACTTACCATTA	TGG	Exon 2	Sense	CATCTGCCCATTCTTCCTGT	GTGTGTGCTGGCAGAGGATA
PDX1 (Chromosome 11, NC_010453.4.)	GGGGTCCTTGTAGAGCTGCG	TGG	Exon 1	Antisense	ATAGAAGTCCAAATATTTTCCCCGC	ACCTCGTACGGGGAGATGTC
SALL1** (Chromosome 6, NC_010448.3.)	TTTGCCCAACATCGGAACGA	CGG	Exon 2	Sense	CCCAATCCAGCTACCTCAGA	GGTACTGGTGGGGATGTTGT

* Based on NCBI: Sus scrofa isolate TJ Tabasco breed Duroc, whole genome shotgun sequence, Sscrofa11.1 (GCF_000003025.5).
** SALL1#3 shown in supplementary Fig. 1.

oocytes that had been incubated for 40, 42, and 44 h after the start of IVM culture. Gene editing efficiencies of gRNA targeting *SALL1* had been evaluated in advance (Supplementary Table S1, S2, and Fig. S1). The gRNAs targeting *KDR* and *PDX1* were evaluated in our previous study (Hirata et al., 2021; Tanihara et al., 2020). The oocytes incubated for each time point were mechanically freed from cumulus cells in Dulbecco's PBS (DPBS; Invitrogen Co.) supplemented with 1 mg/ml hyaluronidase (Sigma-Aldrich). The three types of gRNAs targeting each gene were mixed at a concentration of 100 ng/μl and simultaneously introduced into the cumulus-free oocytes by electroporation with 100 ng/μl of Cas9 protein. After electroporation, the oocytes were incubated in the same maturation medium until 44 h of the total culture period and then subjected to IVF. As a control, three gRNAs were simultaneously introduced into zygotes by electroporation at 12 h after the initiation of IVF. The rate of formation of blastocysts from oocytes upon the introduction of three gRNAs as well as the target mutations in the resulting blastocysts were evaluated.

Statistical analysis

The percentages of embryos that developed to the blastocyst stage were subjected to arcsine transformation. The transformed data were evaluated using analysis of variance, followed by protected Fisher's least significant difference tests. The analysis was performed using StatView software (Abacus Concepts, Berkeley, CA, USA). The percentages of mutated blastocysts were analyzed using chi-squared tests with Yates' correction. Differences with probability values (p) < 0.05 were considered statistically significant.

Table 2
Effects of the timing of electroporation (EP) during *in vitro* maturation (IVM) on the blastocyst formation of oocytes after *in vitro* fertilization (IVF).

EP time during IVM (h)	No. of embryos examined	No. (%) of embryos Cleaved**	Developed to blastocysts
Control	306	270 (88.3 ± 0.5)^{a}	41 (14.6 ± 4.2)^{a,c}
40 h	269	168 (62.1 ± 9.0)^{b}	20 (7.4 ± 1.5)^{a,b}
42 h	278	161 (57.3 ± 9.2)^{b}	15 (5.4 ± 1.0)^{b}
44 h	270	242 (89.9 ± 2.6)^{a}	44 (16.5 ± 1.8)^{c}

*All experiments were repeated five times. Data are expressed as the mean ± SEM.
**Electroporation with three gRNAs targeting *KDR, PDX1*, and *SALL1* was performed at the designated times from the start of maturation culture, and then the oocytes were incubated in the same maturation medium until 44 h of the total culture period. As a control, putative zygotes that were collected 13 h after the start of IVF were electroporated with three gRNAs.
$^{a\text{-}c}$Values with different superscripts in the same column are significantly different ($p < 0.05$).

Results

We examined the effects of the timing of electroporation with three types of gRNAs prior to IVF on the embryonic development (Table 2). The blastocyst formation rates of oocytes treated by electroporation before the end of the total IVM culture period were significantly lower ($p < 0.05$) than those of oocytes cultured for the total culture period. However, the blastocyst formation rate of oocytes treated by electroporation at the end of the total culture period was similar to that of control zygotes electroporated at 13 h after the initiation of IVF.

When evaluating the mutation rates by sequencing each target site in the resulting blastocysts (Fig. 1), the timing of electroporation prior to IVF did not improve the total mutation rates and total biallelic mutation rates in the resulting blastocysts. Moreover, there were no significant differences in the total mutation rates between oocytes and control zygotes electroporated simultaneously with the three types of gRNAs. The rate of triple mutations in the resulting blastocysts from oocytes electroporated at 44 h was significantly higher ($p < 0.05$) than that of oocytes electroporated at 40 h (Fig. 1A). However, the timing of electroporation did not affect the target numbers of biallelic mutations in the resulting blastocysts (Fig. 1B).

The simultaneous introduction of triple mutations was only observed in the resulting blastocysts from oocytes electroporated at 44 h and from control zygotes electroporated at 13 h after the initiation of IVF. Next, we compared mutation types introduced into each targeting gene in both groups (Fig. 2). There were no significant differences between the two groups in the total mutation rates and total biallelic mutation rates of each targeting gene in the resulting blastocysts.

Discussion

To drive the success of genetic modification approaches, it is critical to consider the timing at which the CRISPR/Cas9 system is delivered into fertilized oocytes or mature oocytes arrested at metaphase II (Hirata et al., 2019a). Therefore, we conducted the present study to test whether the timing of electroporation during IVM culture can increase the rates of total mutations and biallelic mutations during triple gene knockout. Although no significant differences in the total mutation rates between oocytes and control zygotes electroporated simultaneously with three types of gRNAs were observed in the present study, the percentage of blastocysts carrying mutations in all three genes was higher in the group derived from oocytes that had been incubated for 44 h than that in those incubated for 40 h. These results indicate that oocytes that have completed their maturation time may function as gene editing material rather than oocytes in the middle of maturation culture. During IVM, immature oocytes normally undergo chromatin changes by increased condensation to prepare the genome to enter meiotic phases and accomplish the metaphase II stage (Belli et al., 2014). It is evident that editing machinery preferentially targets decondensed chromatin rather than condensed chromatin due to protein depletion during chromatin decondensation, which makes it a better substrate for gene editing (Suzuki, Asami & Perry, 2014; Yoshida, Brahmajosyula, Shoji, Amanai & Perry, 2007). In this study, it may help to explain why mature oocytes that complete the maturation process and proceed to the

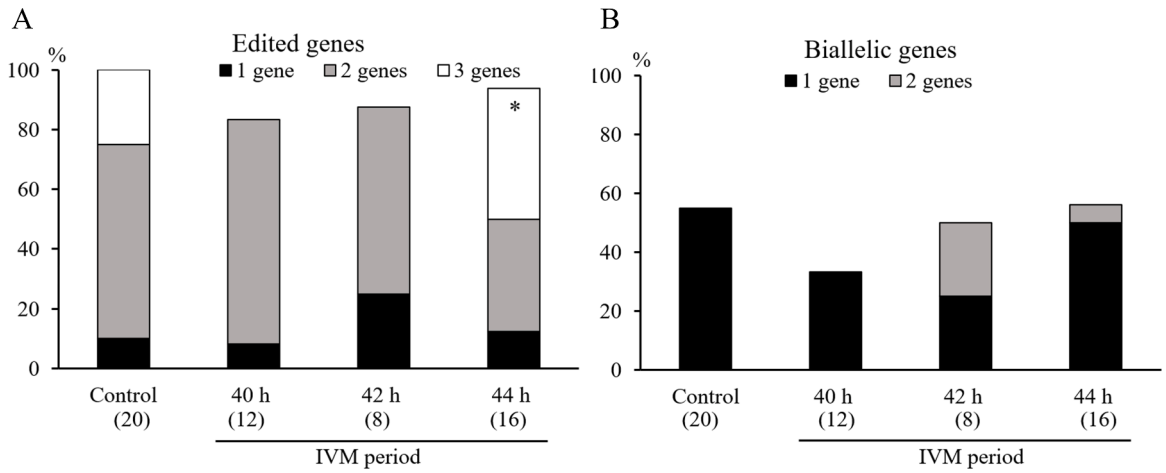

Fig. 1. Mutation (A) and biallelic mutation (B) derived from the resulting blastocysts following electroporation treatment with gRNAs targeting 3 genes during *in vitro* maturation (IVM). Electroporation with three gRNAs targeting *KDR, PDX1*, and *SALL1* was performed at the designated times from the start of the IVM culture, and then the oocytes were incubated in the same maturation medium until 44 h of the total IVM culture period. As a control, putative zygotes that were collected 12 h after the start of *in vitro* fertilization were electroporated with three gRNAs targeting *KDR, PDX1*, and *SALL1*. Genotypes of blastocysts were determined using TIDE. Numbers within the parentheses indicate the total number of examined blastocysts. $*p < 0.05$ compared with the rate of triple mutations in the resulting blastocysts from oocytes electroporated at 40 h.

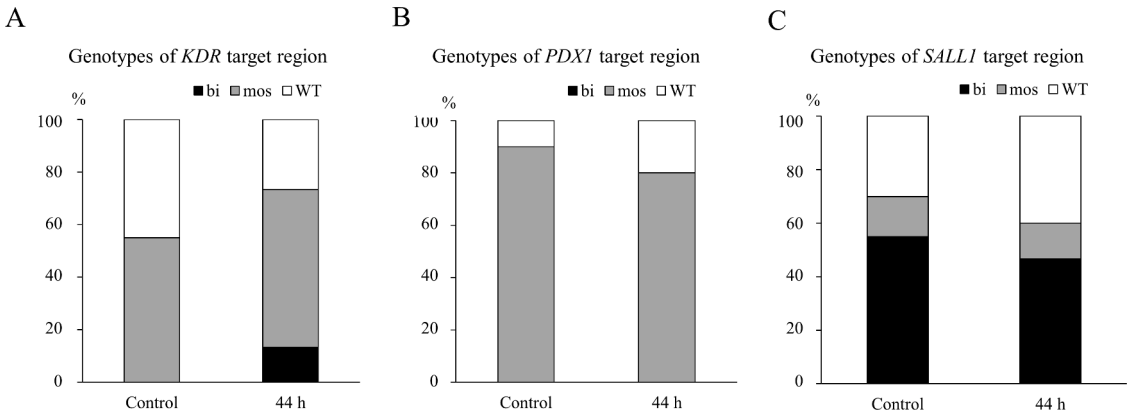

Fig. 2. Genotypes of blastocysts after individual sequencing the target sites of (A) *KDR*, (B) *PDX1*, and (C) *SALL1* genes. Blastocysts derived from oocytes electroporated at 44 h after the initiation of IVM culture (44 h), and that from putative zygotes electroporated at 12 h after the initiation of *in vitro* fertilization (Control) were analyzed. Genotypes of blastocysts were determined using TIDE. Numbers within parentheses indicate the total numbers of examined blastocysts. bi: blastocysts having biallelic mutations; mos: blastocysts having mosaic mutation; WT: wild-type.

fertilization step support a more efficient CRISPR/Cas9 system.

Following the delivery of CRISPR/Cas9 into fertilized oocytes, gene editing events may still occur after genome replication in multi-cell stage embryos, leading to mosaicism (Wu, Shen, Zhang & Meng, 2018). Given that the CRISPR/Cas9 system during oocyte maturation would allow for a better control of gene mutations as the editing can occur during the fertilization stage and can also continuously edit the paternal genome, leads to the generation of multiple mutated alleles of both maternal and paternal copies (Wu et al., 2018). However, contrary to our expectations, the timing of electroporation prior to fertilization did not improve the total biallelic mutation rates in the resulting blastocysts. This might be explained based on the study of Suzuki et al. (2014), who reported that oocyte chromatin organization during meiotic exit may be refractory to the editing machinery and leads to a short period of tolerance for the CRISPR/Cas9 system. Most cases of mixed alleles are believed to occur when only one allele is available at the time of editing, suggesting the possibility that maternal gene editing in this study occurred after the first round of DNA replication, although the CRISPR/Cas9 system was electroporated prior to fertilization (Suzuki et al., 2014). It should also be noted that since Cas9 protein may have a short half-life due to proteolysis (Yang, Li & Li, 2018), the existing activity of Cas9 protein at the time of the activity of editing machinery may not be sufficient to drive the gene-editing reaction. However, further studies are required to elucidate the underlying mechanisms.

The developmental competence of oocytes treated with electroporation before the end of the IVM culture (40 h and 42 h) was lower than that of oocytes treated with electroporation at the end of the IVM culture (44 h) and control zygotes. Our previous study has shown that oocytes are more sensitive to electrical pulses than zygotes (Hirata et al., 2019a). The electric field largely affects the membrane structure in response to creating membrane pores and facilitating the penetration of not only the editing machinery but also reactive oxygen species (ROS) across the membrane to the interior of the cell (Yadav, Kumar, Choi & Kim, 2021). Parthenogenesis of matured oocytes by electroporation simulation, which will increase the blastocyst formation rate of each experimental group, is also a major concern to consider (Nanassy, Lee, Javor & Machaty, 2008). In this study, the blastocyst formation rate from oocytes stimulated by electroporation during maturation culture was decreased compared with the rate from control zygotes electroporated after IVF. During oocyte maturation, the negative effect of electroporation on the developmental competence of oocytes appears to be greater than the

increase in blastocyst formation rate due to parthenogenesis. However, parthenogenesis caused by electroporation stimulation should be considered in the experimental design. Moreover, removal of cumulus cells during IVM culture impairs oocyte nuclear maturation, fertilization, and subsequent embryo development (Wongsrikeao et al., 2005). Therefore, our results indicate that electroporation of cumulus-free oocytes during IVM may not only induce a stress response from membrane damage but also cause subsequent embryonic developmental arrest, possibly through ROS production. Polyspermic penetration is a persistent obstacle to porcine IVF systems (Funahashi, 2003). Using our IVF system, the normal and polyspermic fertilization rates were approximately 50 to 60%, and < 15%, respectively (Nguyen et al., 2017). Removal of cumulus cells before the IVF reduces sperm penetration (Kikuchi, Nagai, Motlik, Shioya & Izaike, 1993). Therefore, the risk of polyspermic penetration is presumably not high. However, it does complicate the interpretation of the results. The use of intracytoplasmic sperm injection may be a solution to consider.

In conclusion, the results of this study suggested that oocytes with completed maturation time may allow better functioning of gene-editing materials and indicated no difference in the total mutation rates and total biallelic mutation rates in the resulting blastocysts between oocytes electroporated before and after the fertilization. Although there are still some issues to be evaluated in future studies, such as the effect of electroporation before IVF on implantation, their widened time window without waiting for the completion of IVF enables flexible research design for embryonic gene modification in pigs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Nippon Food Packer, K. K. Shikoku (Tokushima, Japan), for supplying pig ovaries. We acknowledge Tokushima University for their financial support of the Research Clusters program of Tokushima University.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2022.100241.

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