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SHORT COMMUNICATION

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Excess polyspermy reduces the ability of porcine oocytes to promote male pronuclear formation after in vitro fertilization

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Abstract

Male pronucleus (MPN) formation is a very important physiological event during fertilization, which affects in vitro production of transferrable embryos. The aim of this study was to find out the correlation between the number of penetrated sperm and the occurrence of failure of MPN formation in porcine oocytes. In vitro matured porcine oocytes were fertilized in vitro with frozen epididymal sperm. Two different frozen sperm lots were tested in this study, which were different in terms of polyspermy rates. The numbers and the status of penetrated sperm in oocytes were evaluated 10 h after insemination. Under high polyspermy condition, the polyspermy rate was 83.5% with an average mean of 3.5 sperms per penetrated oocyte, whereas the percentage of polyspermy was 65.5% with an average mean of 2.4 sperms per penetrated oocyte under moderate polyspermic condition. Correlation analysis revealed a negative correlation between the number of penetrated sperm and their MPN formation percentage both in the sperm lot of high polyspermy ($R = -0.560$, $p < 0.05$) and in the sperm lot of moderate polyspermy ($R = -0.405$, $p < 0.05$) which suggests that penetration of excessive spermatozoa disables the oocyte cytoplasm to promote MPN formation.

KEYWORDS
IVF, male pronucleus formation, oocyte, pig, polyspermy

1 | INTRODUCTION

In vitro fertilization (IVF) is an important technology for the utilization of sperm cryopreserved in livestock gene banks (Kikuchi et al., 2016). Besides this, IVF is a basic technology that enables the production of embryos in a large amount, which can be used for embryonic development research. However, polyspermy (penetration of the oocyte with more than one spermatozoon) is present in most mammalian IVF systems. Polyspermy occurs in relatively low frequencies in human (Kola et al., 1987; Rudak et al., 1984) and cattle (Iwasaki et al., 1989, 1992). However, in pigs, it is still a major problem causing abnormal chromosome numbers in embryos (Abeydeera & Day, 1997b; Grupen, 2014; Koo et al., 2005; Nagai et al., 2006). Although most embryos with of chromosomal abnormality fail to develop to term (Causio et al., 2002; Plachot, 1989; Yoshizawa, 2003), some polyspermic zygotes can develop to diploid embryos (Somfai et al., 2008) and even to live piglets (Han et al., 1999). Several different mechanisms to repair the chromosome status after polyspermic fertilization have been suggested (Funahashi, 2003; Han et al., 1999); however, experimental evidence

to prove these mechanisms is still lacking. In fact, we still have limited knowledge on the cytoplasmic mechanisms that occur in oocytes after polyspermic fertilization. The aim of the present study was to clarify if the number of penetrated spermatozoa affects the ability of the oocyte cytoplasm to promote male pronucleus (MPN) formation. To do so, we performed IVF of porcine oocytes in both highly and moderately polyspermic systems established earlier (Nguyen et al., 2020) and analyzed the correlation between the number of penetrated spermatozoa and the frequency of MPN formation in individual oocytes.

2 | MATERIALS AND METHODS

2.1 | Oocyte collection and in vitro maturation

The ovaries were collected from prepubertal crossbred gilts (Landrace × Large White × Duroc) at a local slaughterhouse and carried to the laboratory in Dulbecco's phosphate-buffered saline (PBS) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) at 35–37°C within 1 h. Cumulus–oocyte complexes (COCs) were collected from follicles 3–6 mm in diameter in collection medium consisting of Medium 199 (with Hanks' salts; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco; Invitrogen Corp., Carlsbad, CA, USA), 20-mM HEPES (Dojindo Laboratories, Kumamoto, Japan), and antibiotics (100-U/ml penicillin G potassium [Sigma] and 0.1-mg/ml streptomycin sulfate [Sigma]). In vitro maturation (IVM) of oocytes was carried out as reported previously (Kikuchi et al., 1999). In brief, about 50 COCs were cultured in each 500-μl aliquot of maturation medium, which was a modified North Carolina State University (NCSU)-37 solution (Petters & Wells, 1993) containing 10% (v/v) porcine follicular fluid, 0.6-mM cysteine (Sigma), 50-mM β-mercaptoethanol (Axon Medchem, Groningen, the Netherlands), 1-mM dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP; Sigma), 10-IU/ml equine chorionic gonadotropin (Setrotropin; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan), and 10-IU/ml human chorionic gonadotropin (Gonatropin; ASKA) in four-well dishes (Nunclon Multidishes; Thermo Scientific, Waltham, MA, USA) for 22 h in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. The COCs were subsequently cultured in the maturation medium without dbcAMP and hormones for an additional 24 h under the same atmosphere.

2.2 | IVF and in vitro culture

Oocytes were in vitro fertilized according to the two-step IVF method reported by Grupen and Nottle (2000) with some modifications (Nguyen et al., 2020). Excess layers of cumulus cells were removed from oocytes after a 30-s treatment with 0.1% (w/v) hyaluronidase in oocyte collection medium leaving only the corona radiata on the zona pellucida. The medium used for IVF was a modified Pig-FM medium (Suzuki et al., 2002) containing 10-mM HEPES, 2-mM caffeine, and 5-mg/ml BSA. The oocytes were washed three times in IVF medium. They were then transferred into 90-μl IVF droplets (approximately 20 oocytes in each droplet) covered by paraffin oil (Paraffin Liquid; Nacalai Tesque). Frozen–thawed epididymal spermatozoa from each of two Meishan boars were preincubated at 38.5°C in Medium 199 (with Earle's salts, Gibco, pH adjusted to 7.8) for 15 min (Kikuchi et al., 1998). To obtain the final sperm concentration (1×10^5 sperms/ml), 10 μl of the sperm suspension was introduced into the IVF medium containing oocytes and coincubated for 30 min at 38.5°C under 5% CO_2, 5% O_2, and 90% N_2. The oocytes with the attached spermatozoa were then transferred to a second droplet of the IVF medium containing no sperm and subsequently incubated for 3 h. At the end of IVF, spermatozoa were removed from the surface of zona pellucida by gentle pipetting with a fine glass pipette. Embryo culture was performed in 500-μl drops of NCSU-37 medium containing 4-mg/ml BSA and 50-mM β-mercaptoethanol supplemented with 0.17-mM sodium pyruvate and 2.73-mM sodium lactate (Kikuchi et al., 2002) in four-well dishes in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 39°C for 10 h.

2.3 | Evaluation of the number and status of fertilizing sperm in putative zygotes

The putative zygotes were fixed 10 h after IVF in a mixture of acetic acid ethanol 99.5% (v/v) (1:3) for at least 3 days, stained with 1% aceto-orcein (Sigma), and examined for sperm penetration and the status of penetrating sperm head under a phase-contrast microscope. Sperm head was classified as MPN, condensed sperm head (CSH), and enlarged sperm head (ESH) based on their morphological appearance (Figure 1).

2.4 | Experiment design

The oocytes after IVM for 46 h were fertilized with each of the two frozen sperm lots of different boars, which were earlier characterized by moderate and high polyspermy (Nguyen et al., 2020). The putative zygotes derived under moderate and high polyspermic conditions were fixed separately at 10 h after IVF for examining the number and status of fertilizing sperm in each oocyte. For each penetrated oocyte, the numbers of total fertilizing spermatozoa and the MPN formation were recorded.

2.5 | Statistical analysis

Percentages of polyspermic fertilization and mean numbers of sperms per penetrated oocyte were compared between sperm samples of high and moderate polyspermy by *t* test after arcsine transformation. Results are presented as mean ± SEM. The correlation between the total number of fertilizing sperm and the percentage of MPN

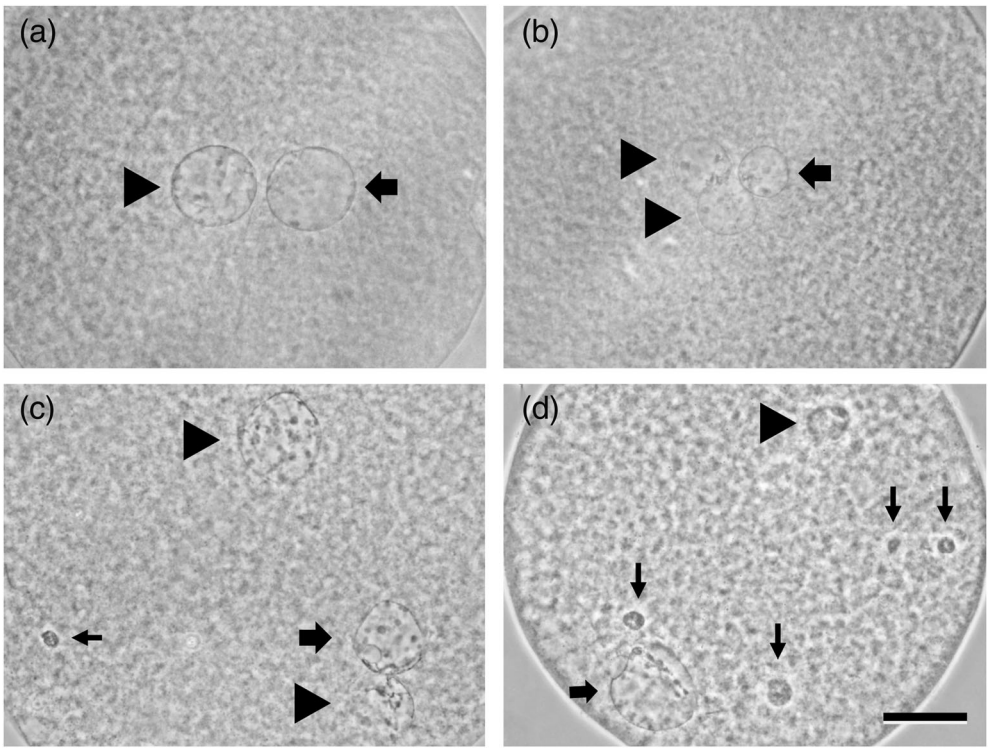

FIGURE 1 Penetrated oocytes at 10 h after in vitro fertilization stained by orcein, under a phase-contrast microscope. The oocyte was penetrated by (a) one, (b) two, (c) three, and (d) five sperm(s). When the oocytes were penetrated by more than four sperms, the ability of male pronucleus formation decreased. The arrow heads show MPN, which is associated with a tail (out of focus). The bold arrows show female pronucleus, and thin arrows show condense/enlarged sperm head. The area of ooplasm varies depending on the oocyte (figure plate) depending on the state of specimen preparation. The scale bar represents 50 μm

TABLE 1 Polyspermy rates with two different sperm samples

Sperm type	No. of fertilized oocytes examined	Polyspermy (%)	No. of sperms per penetrated oocyte
High polyspermy	383	325 (83.5 ± 3.6%)$_a$	3.5 ± 0.1$_a$
Moderate polyspermy	73	48 (65.5 ± 4.3%)$_b$	2.4 ± 0.2$_b$

Note: Fifteen replications for high polyspermy condition and three replications for moderate polyspermy condition were carried out. Results with different subscripts (a and b) are significantly different ($p < 0.05$). Data are presented as mean ± SEM.

formation from total penetrating sperm was analyzed in a total of 383 oocytes in 15 replications under high polyspermic condition and in a total of 73 oocytes in three replications under moderate polyspermic condition by linear correlation analysis using the KyPlot 5.0 software (KyensLab Inc., Tokyo, Japan). A value of $p < 0.05$ was considered statistically significant.

3 | RESULTS

Representative figures of monospermic and polyspermic fertilization are shown in Figure 1. Overall polyspermy rates and sperm numbers per oocyte in moderate and high polyspermic conditions are shown in Table 1. The polyspermy rate and the average mean of sperm penetrated into an oocyte in high polyspermy condition (83.5 ± 3.6% and 3.5 ± 0.1, respectively) were significantly higher than those of moderate polyspermy ($p < 0.05$, 65.5 ± 4.3% and 2.4 ± 0.2, respectively). Linear correlation analysis revealed a statistically significant negative correlation between the total number of penetrating spermatozoa and their percentage to form an MPN under both high and moderate polyspermy conditions (Figure 2).

4 | DISCUSSION

The results of this study revealed that the more sperm penetrated the oocytes, the less frequently they formed a normal pronucleus in porcine IVF, and this result was confirmed in two frozen/thawed sperm samples, which were different in terms of the frequencies of polyspermic penetration.

Irrespective of the sperm used for IVF, in nearly all penetrated oocytes, MPN formation was observed by 10 h after IVF; however, in cases of excessive sperm penetration, some spermatozoa failed to develop to MPN and remain at the enlarged or condensed state (Figure 1c,d). The results revealed that the incidences of MPN formation failure increased dramatically in oocytes penetrated excess number (under moderate polyspermy more than three) of spermatozoa (Figure 2).

One possible speculation for the cause of MPN formation failure is simply the lack of time for late-penetrating spermatozoa because late-fertilizing sperm might have less time to form pronucleus. However, it cannot be the case here because in this study, status of fertilizing sperm in oocyte cytoplasm was evaluated at 10 h after fertilization whereas sperm and oocytes were coincubated for only

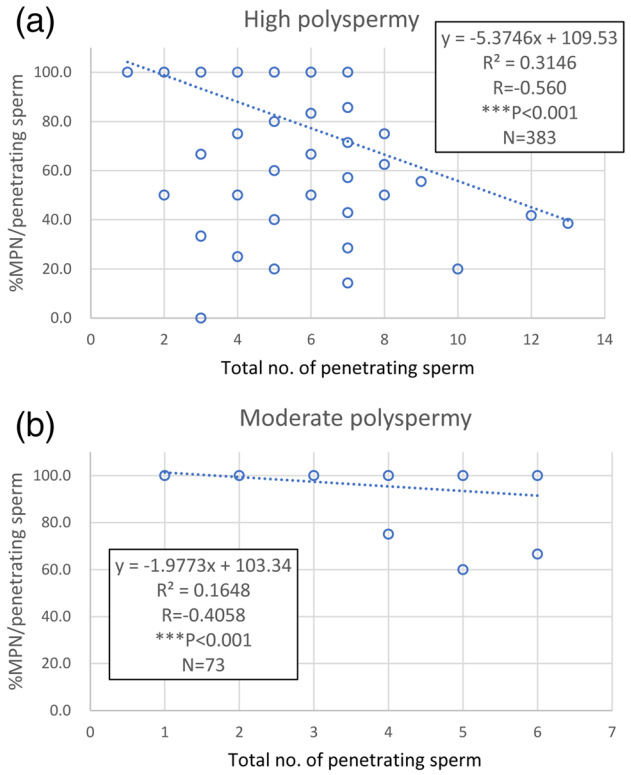

FIGURE 2 The correlation between the total number fertilizing sperm and the success of male pronucleus (MPN) formation in an in vitro fertilization system utilized under high and moderate polyspermy conditions

30 min (thus, all fertilizing sperm had to finish attachment to oocytes within this time frame), and in the present system, sperm penetration into oocytes occurs within 3 h (Kikuchi et al., 2002, 2006). Furthermore, pronuclear formation in pigs is observed about 6 h after sperm penetration (Abeydeera & Day, 1997a). This suggests that failure of MPN formation might have occurred due to the lack of the ability of oocyte cytoplasm to facilitate MPN formation for the excessive fertilizing sperm.

Unlike the chromatin of somatic cells, the DNA in mammalian spermatozoa is associated with a sperm-specific protein, protamine, in a crystalline-like structure (McLay & Clarke, 2003). It makes the chromatin the most highly compacted in eukaryotes (McLay & Clarke, 2003) and therefore "packageable" into the relatively small head of the sperm (Jenkins & Carrell, 2012; Nakazawa et al., 2002). After penetration into oocytes, the chromatin of porcine sperm undergoes structural and morphological changes (chromatin remodeling) during which the compact (condensed) chromatin first enlarges (decondenses) and then recondenses and forms a large membrane-bound MPN (Jenkins & Carrell, 2012; Kikuchi et al., 2006; Yanagimachi, 1994). This process is associated with the replacement of protamine to histone (McLay & Clarke, 2003). These processes require glutathione (GSH) and histone from the oocyte cytoplasm (Jenkins & Carrell, 2012; Nakazawa et al., 2002; Shimada et al., 2000). In porcine oocytes, GSH is accumulated in the cytoplasm during IVM (Nagai, 2001) and insufficient cytoplasmic GSH levels were proven to be associated with the failure of MPN formation (Yoshida, 1993; Yoshida et al., 1993). During IVM, GSH is synthesized from cysteine (Yoshida et al., 1993) and GSH accumulation in oocytes requires cumulus cells attached to oocytes by gap junctions (Maedomari et al., 2007). The cases for insufficient MPN formation in the present study are likely to be caused by the failure of the oocyte cytoplasm to facilitate MPN formation from excessively penetrating sperm. Nevertheless, it must be noted that in the present study, nearly all (382/383) fertilized oocytes had at least one MPN. This was no surprise because both cysteine and cumulus cells were given in the current IVM system, which has been proven previously to be efficient to promote MPN formation after IVF and even development to term (Kikuchi et al., 2002). Based on the mentioned above, our results suggest that to some extent, all oocytes could facilitate the formation of at least one MPN; however, as the number of penetrating spermatozoa increased, some oocytes may have lost their ability to promote MPN formation for the excessive penetrating sperm. It is possible that excessive spermatozoa employ too much of the oocyte GSH and/or histone for chromatin remodeling and the oocytes cannot provide such material to promote MPN formation for some sperm heads. Further studies will be needed to clarify this theory. Furthermore, the use of the highly polyspermic condition revealed a great variation of oocytes in terms of their ability to promote MPN formation when penetrated by the same numbers of sperm. This suggests a variation in the oocyte population in their ability to develop further after polyspermic penetration.

In conclusion, our study suggests that the insufficient MPN formation in polyspermic oocytes may be caused by the failure of the oocyte to facilitate MPN formation from excess spermatozoa in cytoplasm. The negative correlation between the number of fertilizing sperm and MPN formation ability of oocyte cytoplasm is revealed in this study, which could be useful information to understand the development of polyspermic embryos in future studies.

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CONFLICT OF INTERESTS

All authors declare no conflicts of interest.

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