Delay in Cleavage of Porcine Embryos after Intracytoplasmic Sperm Injection (ICSI) Shows Poorer Embryonic Development

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Abstract. In pigs, the embryonic developmental ability after intracytoplasmic sperm injection (ICSI) is inferior to that resulting from in vitro fertilization (IVF). We evaluated the timing of cell division up to blastocyst formation on embryonic development after ICSI using either whole sperm (w-ICSI) or the sperm head alone (h-ICSI) and IVF as a control. At 10 h after ICSI or IVF, we selected only zygotes, and each of the zygotes/embryos was evaluated for cleavage every 24 h until 168 h. We then observed a delay in the 1st and 2nd cleavages of h-ICSI embryos and also in blastocoele formation by w-ICSI embryos in comparison with IVF embryos. The rate of blastocyst formation and the quality of blastocysts in both ICSI groups were inferior to those in the IVF group. In conclusion, the delay in cleavage of porcine ICSI embryos shows poorer embryonic development.

Key words: Cleavage, Embryonic development, Intracytoplasmic sperm injection (ICSI), Pig
w-ICSI embryos were still at the 5-cell to morula stages, whereas a large proportion of IVF embryos had developed to the blastocyst stage (Fig. 1C and D). The proportion of embryos reaching the blastocyst stage and the mean number of cells per blastocyst at 144 h in the two ICSI groups were lower than those in the IVF group (Fig. 1D and E). The results at 168 h were similar to those at 144 h. This phenomenon may be caused by a delay in the timing of transition from the morula stage to the blastocyst stage (Fig. 1C and D). In other words, blastocoele formation was delayed in w-ICSI embryos compared with IVF embryos. In mice, it has been reported that intracellular calcium signaling and the phospholipase Cζ-mediated signaling pathway are required for blastocoele formation [12]. Induction of oocyte activation by whole-sperm injection and additional electrical stimulation in ICSI oocytes may not be the most suitable conditions for timely blastocoele formation. We have already confirmed that the sperm tail contains an extra sperm factor, phospholipase Cζ [13], which may be advantageous for the calcium signaling leading to normal oocyte activation resulting in the completion of embryo development. The whole pig sperm actually has higher competence for induction of oocyte activation than the sperm head alone [13]. In addition, electrical stimulation can promote development of ICSI oocytes into live offspring [14, 15]. However, w-ICSI embryos still showed a delay of embryonic development even after electrical stimulation. It has been suggested that no signaling mechanism leading to activation of the phosphoinositide pathway and generation of calcium signaling observed during natural fertilization is replicated by artificial activation such as electrical stimulation [16]. The pattern of calcium signaling is an alternative factor that affects the global pattern of gene expression [17]. Furthermore, it has been reported that the maternal to embryonic transition of genome activation may not proceed in an appropriate pattern in slow-cleaving embryos [5]. It remains clear to us that this issue requires further investigation in relation to the induction of physiological oocyte activation.

In conclusion, the delay in cleavage of porcine ICSI embryos shows poorer embryonic development. The reason for this seems to depend on the status of the injected sperm.
Materials and Methods

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

Oocyte collection and in vitro maturation (IVM)

Ovaries were obtained from prepubertal crossbred gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35 C. Cumulus-oocyte complexes (COCs) were collected from follicles 2 to 6 mm in diameter in TCM 199 (with Hanks’ salts) supplemented with 5% (v/v) fetal bovine serum (Gibco, Life Technologies, Grand Island, NY, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), 100 IU/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate. Maturation culture was performed as reported previously [14, 18]. Briefly, about 40 COCs were cultured in 500 µl of maturation medium for 20 to 22 h in four-well dishes (Nunc; Thermo Fisher Scientific, Waltham, MA, USA). The medium was modified North Carolina State University (NCSU)-37 solution [19] containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 µM β-mercaptoethanol, 1 mM dibutyl cAMP (dbcAMP), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml hCG (Puberogen 500 U; Novartis Animal Health, Tokyo, Japan). The COCs were subsequently cultured for 24 h in maturation medium without dbcAMP and hormones. Maturation culture was carried out at 39 C under conditions in which CO2, O2 and N2 were adjusted to 5%, 5% and 90%, respectively (5% O2). After culture, and served as matured oocytes.

Preparation of sperm

Epididymal spermatozoa from a Landrace boar were frozen [20]. They were thawed in TCM 199 (with Earle’s salts; Gibco) adjusted to pH 7.8 and centrifuged at 600 × g for 2 min. For ICSI, the sperm pellet was resuspended in Dulbecco’s phosphate-buffered saline (PBS; Nissui Pharmaceuticals, Tokyo, Japan) containing 5 mg/ml BSA (Fraction V). A portion of the spermatozoa were then subjected to sonication for 1 min at a power output of 700 W using ultrasonic cleaner (US-C ultrasonic cleaner; Dalton, Tokyo, Japan) to isolate the sperm heads. The heads were maintained at room temperature and used for ICSI (h-ICSI). Another portion of sperm, which had not been subjected to sonication but otherwise treated in the same manner, was also used as whole sperm for ICSI (w-ICSI). The remaining sperm were preincubated at 38 C for 15 min in TCM 199 (pH 7.8) and used for IVF.

In vitro fertilization (IVF)

IVF was carried out according to the method described by Kikuchi et al. (2002) [18]. The oocytes were washed three times in pig-fertilization medium (Pig-FM) [23] and then placed in individual 80-µl drops of the same medium that had been covered with warm paraffin oil (Paraffin Liquid, Nakarai Tesque, Kyoto, Japan). Generally, 10 µl of preincubation medium containing sperm was added to each fertilization drop to give a final concentration of 1 × 105 sperm/ml and then co-incubated for 2.5 h at 39 C under 5% O2.

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Injection procedure

ICSI was carried out as described previously [14, 15, 21, 22]. Two solutions were prepared for ICSI: (1) modified NCSU-37 for oocytes, which did not contain glucose but was supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate (Wako Pure Chemical Industries, Osaka, Japan), 4 mg/ml BSA, 50 µM β-mercaptoethanol (IVC-PyrLac; [15]) and 20 mM HEPES (IVC-PyrLac-Hepes; [14]), and (2) IVC-PyrLac-Hepes for sperm, which was supplemented with 4% (w/v) polyvinylpyrrolidone (MW 360,000) (IVC-PyrLac-Hepes-PVP). Sperm were injected into oocytes using a Piezo-actuated micromanipulator (PMAS-CT150; Prime Tech, Tsuchiura, Japan).

Oocyte stimulation

One hour after ICSI, the sperm-injected oocytes were transferred to an activation solution consisting of 0.28 M D-mannitol, 0.05 mM CaCl2 (Katayama Chemical Industries, Osaka, Japan), 0.1 mM MgSO4 (Wako) and 0.1 mg/ml BSA and washed once. They were then stimulated with a direct current pulse of 1.5 kV/cm for 20 µs using a somatic hybridizer (SSIP-10; Shimadzu, Kyoto, Japan).

Assessment of embryonic development

We mounted a proportion of ICSI or IVF embryos on glass slides every 24 h until 168 h, and the gametes/embryos were fixed in 25% (v/v) acetic acid in ethanol, stained with 1% aceto-orcein and examined under a phase-contrast microscope to evaluate the stage of cleavage and the mean number of cells per blastocyst. We defined the blastocyst stage as an embryo with more than 10 cells and a clear blastocoele. Embryos with more than 10 cells and without any distinguishable blastocoele were considered to be at the morula stage.
Fig. 2. The time table for the ICSI and IVF procedures. During the IVF procedure, sperm began to penetrate into oocytes from 2.5 to 3.5 h after insemination. Activation of the oocytes was considered to be triggered immediately after penetration. In the ICSI group, 1 h is required for completion of the injection procedure, and the oocytes were stimulated by an electrical pulse at 1 h after the ICSI procedure. In our system, almost all ICSI oocytes do not resume meiosis until electrical stimulation. Therefore, the time point of electrical stimulation in the ICSI groups was defined “0 h” in the present study, and this was the time point for completion of co-culture of sperm and oocytes in the IVF group. At 10 h later, normal fertilized oocytes in both the ICSI and IVF groups were selected and cultured. The cleavage stages were then observed every 24 h until 168 h.

Statistical analysis
The cleavage stage evaluated according to the number of cells was scored at various time points during culture after ICSI or IVF. The mean number of cells per blastocyst was also counted. Percentage data were arcsine transformed [25]. All the data were then subjected to analysis of variance (ANOVA) and Tukey’s multiple range test using the Statcel 2 software (OMS Publishing, Saitama, Japan).

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References
1. Lee JW, Tian XC, Yang X. Failure of male pronucleus formation is the major cause of lack of fertilization and embryonic development in pig oocytes subjected to intracytoplasmic sperm injection. Biol Reprod 2003; 68: 1341–1347. [Medline] [CrossRef]
2. Kren R, Kikuchi K, Nakai M, Miyano T, Ogushi S, Nagai T, Suzuki S, Fukia J, Fulka J Jr. Intracytoplasmic sperm injection in the pig: where is the problem?. J Reprod Dev 2003; 49: 271–273. [Medline] [CrossRef]
3. Yong HY, Hong JY, Kang SK, Lee BC, Lee ES, Hwang WS. Sperm movement in the ooplasm, dithiothreitol pretreatment and sperm freezing are not required for the development of porcine embryos derived from injection of head membrane-damaged sperm. Theriogenology 2005; 63: 783–794. [Medline] [CrossRef]
4. Mateusen B, Van Soom A, Maes DGD, Donnay I, Duchateau I, Lequarre AS. Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal laser scanning microscopic study. Reproduction 2005; 129: 443–452. [Medline] [CrossRef]
5. Isom SC, Li RF, Whitworth KM, Prather RS. Timing of first embryonic cleavage is a positive indicator of the in vitro developmental potential of porcine embryos derived from in vitro fertilization, somatic cell nuclear transfer and parthenogenesis. Mol Reprod Dev 2012; 79: 197–207. [Medline] [CrossRef]
6. Comizzoli P, Marquant-Le Guenne B, Heyman Y, Renard JP. Onset of the first S-phase is determined by a paternal effect during the G1-phase in bovine zygotes. Biol Reprod 2000; 62: 1677–1684. [Medline] [CrossRef]
7. Leoni GG, Succu S, Berlinger F, Rosati L, B Ebbede B; Bogliolo L, Ledda S, Naitana S. Delay on the in vitro kinetic development of prepubertal ovine embryos. Anim Reprod Sci 2006; 92: 373–383. [Medline] [CrossRef]
8. Schatten H, Sun QY. The functional significance of centrosomes in mammalian meiosis, fertilization, development, nuclear transfer, and stem cell differentiation. Environ Mol Mutagen 2009; 50: 620–636. [Medline] [CrossRef]
9. Comizzoli P, Wildt DE, Pukazhenthi BS. Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biol Reprod 2006; 75: 252–260. [Medline] [CrossRef]
10. Kim NH, Smirioiu G, Funahashi H, Schatten G, Day BN. Microtubule organization in porcine oocytes during fertilization and parthenogenesis. Biol Reprod 1996; 54: 1397–1404. [Medline] [CrossRef]
11. Sun QY, Lai L, Park KW, Kühnholzer B, Prather RS, Schatten H. Dynamic events are differently mediated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilization in vitro. Biol Reprod 2001; 64: 879–889. [Medline] [CrossRef]
12. Stachekli JJ, Arment DR. Regulation of blastocoele formation by intracellular calcium release is mediated through a phospholipase C-dependent pathway in mice. Biol Reprod 1996; 55: 1292–1298. [Medline] [CrossRef]
13. Nakai M, Ito T, Sato K, Noguchi J, Kaneko H, Kashiwazaki N, Kikuchi K. Pre-treatment of sperm reduces success of ICSI in the pig. Reproduction 2011; 142: 285–293. [Medline] [CrossRef]
14. Nakai M, Kashiwazaki N, Takizawa A, Hayashi Y, Nakatsuka E, Fuchimoto D, Noguchi J, Kaneko H, Shino M, Kikuchi K. Viable piglets generated from porcine oocytes matured in vitro and fertilized by intracytoplasmic sperm head injection. Biol Reprod 2003; 68: 1003–1008. [Medline] [CrossRef]
15. Nakai M, Kaneko H, Somfai T, Maedolami N, Ozawa M, Noguchi J, Ito T, Kashiwazaki N, Kikuchi K. Production of viable piglets for the first time using sperm derived from eutestic testicular xenografts. Reproduction 2010; 139: 331–335. [Medline] [CrossRef]
16. Sun FZ, Hylund J, Huang X, Mason W, Moor RM. A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. Development 1992; 115: 947–956. [Medline]
17. Ozil JP, Banrezes B, Töth S, Pan H, Schultz RM. Ca2+ oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. Dev Biol 2006; 300: 534–544. [Medline] [CrossRef]
18. Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. Biol Reprod 2002; 66: 1033–1041. [Medline] [CrossRef]
19. Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil Suppl 1993; 48: 61–73. [Medline]
20. Kikuchi K, Nagai T, Kashiwazaki N, Ikeda H, Noguchi J, Shimada A, Soley E, Kaneko H. Cryopreservation and ensuing in vitro fertilization ability of boar spermatozoa from epididymides stored at 4°C. Theriogenology 1998; 50: 615–623. [Medline] [CrossRef]
21. Nakai M, Kashiwazaki N, Takizawa A, Maedolami N, Ozawa M, Noguchi J, Kaneko H, Shino M, Kikuchi K. Morphologic changes in boar sperm nuclei with reduced disulfide bonds in electrostimulated porcine oocytes. Reproduction 2006; 131: 663–671. [Medline] [CrossRef]
22. Nakai M, Kaneko H, Somfai T, Maedolami N, Ozawa M, Noguchi J, Kashiwazaki N, Kikuchi K. Generation of porcine diploid blastocysts after injection of spermatozoa grown in nude mice. Theriogenology 2009; 72: 2–9. [Medline] [CrossRef]
23. Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodriguez-Martinez H. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. Int J Androl 2002; 25: 84–93. [Medline] [CrossRef]
24. Somfai T, Ozawa M, Noguchi J, Kaneko H, Karja NWK, Fahrudin M, Nakai M, Maedolami N, Dinnyés A, Nagai T, Kikuchi K. In vitro development of polyspermic porcine oocytes: Relationship between early fragmentation and excessive number of penetrating spermatozoa. Anim Reprod Sci 2008; 107: 131–147. [Medline] [CrossRef]
25. Snedecor GW, Cochran WG. In: Statistical Methods, 8th ed. Ames, IA: Iowa State University Press; 1989: 273–296.