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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

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In pigs, intracytoplasmic sperm injection (ICSI) is considered to be a useful technique for producing live offspring from nonmotile sperm and for preventing polyspermy, which frequently occurs in porcine *in vitro* fertilization (IVF). However, the efficiency of *in vitro* embryo production by ICSI and the quality of the embryos are still inferior. It is therefore important to investigate the reasons for the poor developmental ability of ICSI embryos, not only for preservation of genetic resources but also for studies of the fundamental mechanisms of fertilization in pigs.

Failure of male pronucleus (PN) formation is one of the reasons for the poorer embryonic development observed after porcine ICSI [1]. The female PN is formed in sperm-injected porcine oocytes; however, in many cases, the sperm head does not decondense [2], resulting in failure of male PN formation and fertilization. Some previous studies have already evaluated the membrane [3, 4] and nucleus [5] of injected sperm at various time points after sperm injection until male PN formation. However, a higher frequency of normal fertilized oocytes showing two polar bodies and two PNs after ICSI does not guarantee embryonic development to the blastocyst stage [3]. Thus, even after normal fertilization, the proportion of ICSI embryos developing to the blastocyst stage is low.

The timing of cleavage or cell division during early embryogenesis (defined as "cleavage" hereafter in the present study) seems to be a critical parameter for predicting subsequent developmental ability. Previous studies have shown that a higher proportion of embryos with faster cleavage reach the blastocyst stage than those with slower cleavage [4, 5]. The timing of the first cleavage has an important effect on early embryonic development [6, 7]. However, to our knowledge, no previous study has indicated that porcine ICSI embryos showing poor developmental ability have a delayed cleavage timing in comparison with IVF embryos. Therefore, we examined the timing of cleavage in ICSI embryos after injection of whole sperm (w-ICSI) or sperm heads alone (h-ICSI) from the PN stage to the blastocyst stage in comparison with IVF embryos (IVF).

When sperm heads alone were injected into oocytes, a delay in the timing of the 1st and 2nd cleavages was observed (Fig. 1A and B). Furthermore, at 96 h after sperm injection, the proportion of embryos between the 5-cell to morula stages in the h-ICSI group was lower than in the other groups (Fig. 1C). For injection of sperm heads alone, in the present study, sperm were treated with sonication, which enables separation of the head from the neck and tail. The sperm centrosome, which is responsible for aster assembly during the first cell cycle, is located in the neck region and plays a role in progression of the oocyte cell cycle [8]. In domestic cats, any deficit of male centrosomal functions leads to delay of the first cleavage, a slower developmental rate and reduced formation of blastocysts [9]. In porcine oocytes, the maternal centrosomal material forms a microtubule network even in the absence of a paternal centrosome [10]. However, our present findings suggest that the paternal centrosome may hold the key to timely attainment of specific stages of porcine embryonic development. In IVF oocytes, cytoskeletal dynamics after sperm penetration are suggested to be very important for normal embryo development [11], and this also seems to be the case for ICSI oocytes.

On the other hand, the developmental kinetics of embryos in the w-ICSI group were almost the same as those of embryos in the IVF group until 96 h (Fig. 1A, B and C). However, at 120 h, most of the

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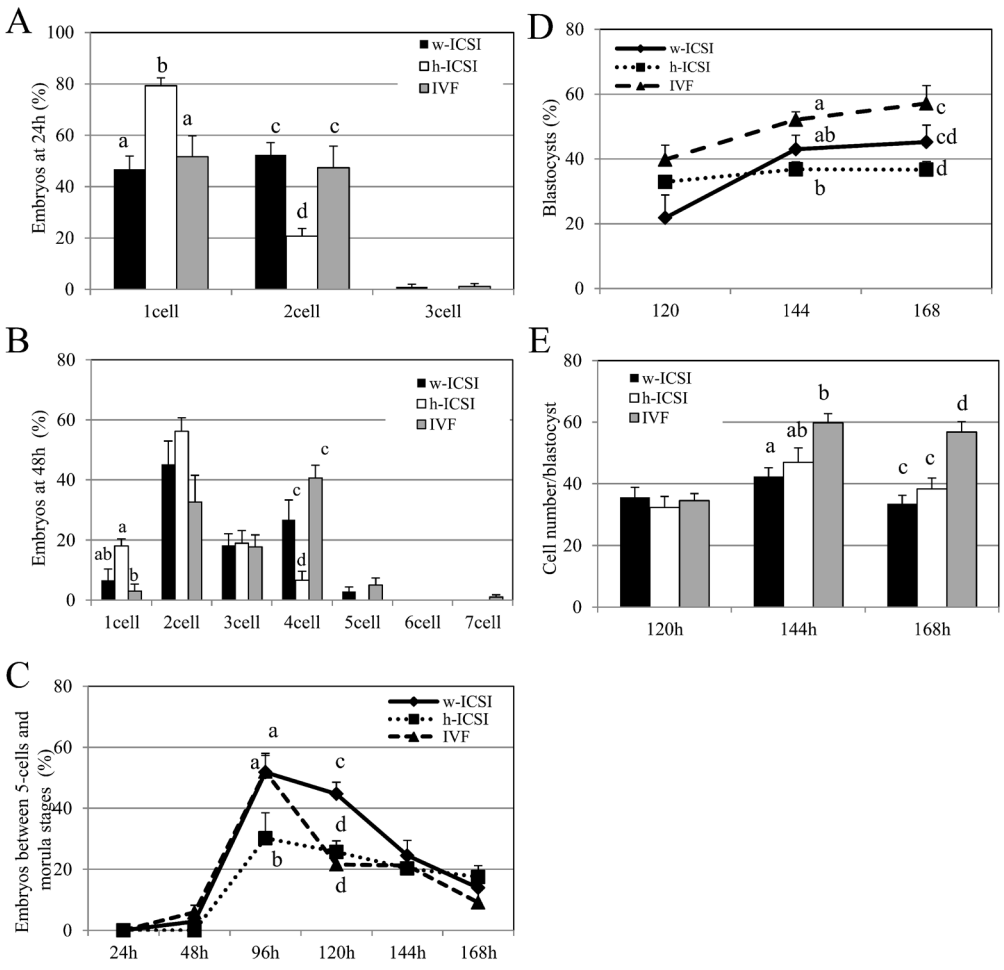

Fig. 1. Cleavage statuses at 24 h (A) and 48 h (B) after whole sperm ICSI (w-ICSI), sperm head ICSI (h-ICSI) or IVF. Time-dependent changes in the rates of embryos from the 5-cell to morula stage (C) and in the rates of blastocyst stage embryos (D). Mean numbers of cells per blastocyst are also shown (E). Different superscripts (a, b, c and d) within the same culture period indicate that values are significantly different ($P < 0.05$).

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 CO_2, O_2 and N_2 were adjusted to 5%, 5% and 90%, respectively (5% O_2). After culture, cumulus cells were removed from the oocytes by treatment with 150 IU/ml hyaluronidase and gentle pipetting. Denuded oocytes with the first polar body were harvested under a stereomicroscope 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.

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 $CaCl_2$ (Katayama Chemical Industries, Osaka, Japan), 0.1 mM $MgSO_4$ (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 (SSH-10; Shimadzu, Kyoto, Japan).

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 1×10^5 sperm/ml and then co-incubated for 2.5 h at 39 C under 5% O_2.

In vitro culture (IVC)

Two types of IVC medium were prepared [18]. The first was IVC-PyrLac. The second contained 5.55 mM glucose, as used in the originally reported NCSU-37 medium, and was also supplemented with 4 mg/ml BSA and 50 µM β-mercaptoethanol (IVC-Glu). IVC-PyrLac was used from day 0 (the day of ICSI or IVF) up to day 2. The medium was changed once, to IVC-Glu, on day 2, and this medium was used for subsequent culture. IVC was carried out at 38.5 C under 5% O_2.

Selection of normal zygotes

At 10 h after ICSI or insemination, oocytes were placed in 700 µl of IVC-PyrLac-Hepes and centrifuged at 10,000 × *g* at 37 C for 20 min in a microcentrifuge [24]. We defined "0 h" as the time point of electrical stimulation in the ICSI groups and as the time point for completion of gamete co-incubation in the IVF group (Fig. 2). The centrifuged oocytes were examined for their content of pronuclei and polar bodies under an inverted microscope (IX70; Olympus, Tokyo, Japan). Normal zygotes that had two polar bodies and two PNs were then cultured. The rates of normal fertilization in IVF, w-ICSI and h-ICSI are 25.8, 48.6 and 37.7% respectively. We then prepared around 100 normal fertilized embryos for each observation timing (24, 48, 96, 120, 144 and 168 h).

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.

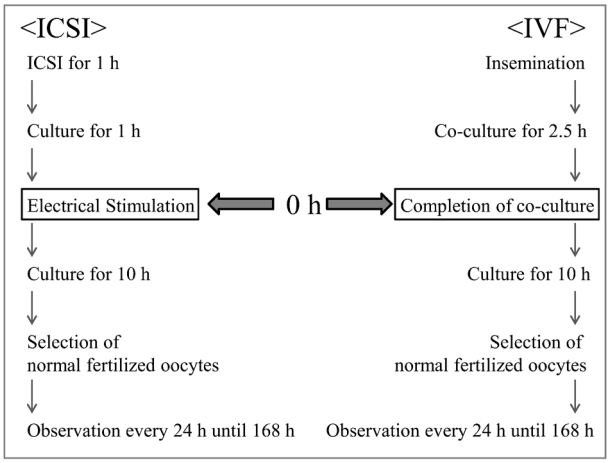

Fig. 2. The time table for the ICSI and IVF procedures. During the IVF procedure, sperm began to penetrate into oocytes from 2.5 to 3.5 h after insemination. Activation of the oocytes was considered to be triggered immediately after penetration. In the ICSI groups, 1 h is required for completion of the 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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