Viable Piglets Generated from Porcine Oocytes Matured In Vitro and Fertilized by Intracytoplasmic Sperm Head Injection

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ABSTRACT

Intracytoplasmic sperm injection (ICSI) of a nonmotile cell into the ooplasm for assisted fertilization is a highly specialized procedure for producing the next generation. The production of piglets by ICSI has succeeded when in vivo-matured oocytes have been used as recipients. Our objective was to generate viable piglets by using porcine oocytes matured in vitro and fertilized by ICSI after evaluating the efficacy of using donor spermatozoa in which the acrosome had been artificially removed by treatment with calcium ionophore A23187 (Ca-I). The rate of acrosomal loss in spermatozoa was increased significantly as the duration of treatment with 10 μM Ca-I was prolonged for 30–120 min (Ca-I treated; 55.6–78.6%), whereas the rate was not different as the duration of incubation without Ca-I was prolonged for 30–120 min (control; 45.3–58.4%). On the sixth day of in vitro culture after injection of the sperm head and subsequent stimulation with an electrical pulse, the rates of blastocyst formation were not significantly different between the two groups: the rates for oocytes injected with Ca-I-treated sperm heads (incubated for 120 min) and for those injected with control sperm heads were 8.6% and 4.0%, respectively. The mean cell numbers of the blastocysts were not significantly different between the two groups (25.6 and 22.7, respectively). Within 2 h after the stimulation, the injected oocytes were transferred to estrous-synchronized recipients. The three recipients that received oocytes injected with Ca-I-treated sperm heads (77–150 oocytes per recipient) were not pregnant, whereas two of the four recipients given oocytes injected with control sperm heads (55–100 oocytes per recipient) were pregnant. One of these farrowed three (a male and two female) healthy piglets. The results demonstrate clearly that in vitro-matured oocytes injected with sperm heads are developmentally competent and can produce viable piglets. They also suggest that removal of the acrosome from the spermatozoon before injection does not affect the development of the blastocyst in vitro. This might not also improve the production of piglets in vivo.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) into the ooplasm is an established technique in many mammals for generating live offspring when their spermatozoa lack motility, causing infertility. Since the 1990s, there have been reports of successful production of live offspring after ICSI using both in vivo-matured (human [1, 2] and mouse [3–5]) and in vitro-matured (IVM) (cattle [6], ram [7] and rabbit [8]) oocytes. These reports confirm the cytoplasmic ability for fertilization and development to term after ICSI in both in vivo-matured or IVM oocytes in mammals. Boar spermatozoa are capable of being frozen and stored as genetic resources, but they sometimes show great loss of motility or immotility after thawing, depending on the individual from which the spermatozoa were collected [9]. For these motility-lost spermatozoa, ICSI is the optimum procedure for producing the next generation also in pigs. The successful production of piglets after ICSI has been reported only with the use of in vivo-matured oocytes [10–12]; the use of ICSI in porcine IVM oocytes resulted in the death of a piglet immediately after birth [13]. It is therefore important to seek clear evidence that IVM-ICSI oocytes have the ability to develop to normal, viable piglets.

ICSI is now considered to be a useful procedure, not only for producing live offspring from nonmotile sperm cells but also for generating transgenic animals through sperm-mediated gene transfer [14, 15]. However, the efficiency of ICSI is not good. A large number of matured oocytes are needed when DNA-conjugated sperm cells are used for the generation of live offspring. It is important to be able to use IVM oocytes supplied from slaughterhouse materials because of the cost and time required in the preparation of in vivo-matured oocytes. However, if IVM materials are to be used, then the success rate needs to be improved by modification of the procedure. In most ICSI studies, untreated spermatozoa have been injected. The acrosome is an outer membrane of the spermatozoon and appears to be a barrier to the integration of foreign DNA into the sperm genome. It has been reported in mice that acrosomal loss results in an increased pronuclear formation rate after ICSI [5]. Therefore, one would expect the presence of the acrosome during ICSI to have a negative effect on the success of sperm-mediated gene transfer.

After evaluating the effect of calcium ionophore (Ca-I) as a tool for removal of the acrosome, we injected Ca-I-treated or untreated sperm heads into IVM oocytes and evaluated the ability of the oocytes to develop to the blastocyst stage in vitro and to viable piglets in vivo after transfer to recipient pigs.
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MATERIALS AND METHODS

Oocyte Collection and IVM

Ovaries were obtained from prepubertal cross-bred pigs (Landrace, Large White, and Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35°C. Cumulus cell oocyte complexes (COCs) were collected from follicles 3–5 mm in diameter in TCM 199 (with Hanks salts; Sigma Chemical, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (Gibco, Life Technologies, Grand Island, NY), 20 mM Hepes (Dojindo Laboratories, Kumamoto, Japan), 100 IU/ml penicillin G potassium (Sigma), and 0.1 mg/ml streptomyycin sulfate (Sigma). IVM of oocytes was performed by the method of Kikuchi et al. [16, 17]. In brief, about 40 COCs were cultured in each 500 μl of maturation medium, a modified North Carolina State University (NCSU)-37 solution [18] containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 μM β-mercaptoethanol, 1 mM dibutyl cAMP (dbcAMP; Sigma), 10 IU/ml eCG (PMS 1000 Tani NZ; Nihon Zenyaku Kogyo, Koriyama, Japan) and 10 IU/ml hCG (Puberogen 1500 U; Sankyo, Tokyo, Japan), which was equipped with Hoffman modulation contrast.

Preparation of Sperm

Epididymides from a boar of the Landrace breed were collected at a local slaughterhouse, and the epididymal spermatozoa were collected and frozen [19, 20]. Spermatozoa were thawed in TCM 199 (with Earls salts; Gibco) adjusted to pH 7.8 and preincubated in pig-FM [17, 21] consisting of 90 mM NaCl, 12 mM KCl, 25 mM NaHCO3, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 10 mM sodium lactate, 10 mM Hepes, 8 mM CaCl2, 2 mM sodium pyruvate, 2 mM caffeine, and 5 mg/ml BSA (Fraction V; Sigma) or in pig-FM supplemented with 10 μM Ca-I A23187 (C-7522; Sigma). The concentration of spermatozoa was diluted to 2.5 × 10^6/ml. The preincubated spermatozoa were then sonicated for 1 min for the isolation of sperm heads.

ICSI Procedure

ICSI was conducted with the aid of a pair of micromanipulators (MBU; Naritishige, Tokyo, Japan) on an inverted microscope (IX70; Olympus, Tokyo, Japan), which was equipped with Hoffman modulation contrast. About 20 oocytes were transferred into 20-μl drops of injection solution.

The injection solution consisted of NCSU-37 without glucose but supplemented with 4 mg/ml BSA, 50 μM β-mercaptoethanol, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate (IVC-PyrLac) [17], and also 20 mM Hepes, of which the osmolarity was adjusted to 285 osmol (IVC-PyrLac-Hepes). The solution containing oocytes was placed on the cover of a plastic dish (Falcon 35-1005; Becton Dickinson and Company, Franklin Lakes, NJ). A small volume (0.5 μl) of the sonicated sperm head suspension was transferred to 2-μl drops of injection solution with 4% (w/v) polyvinyl pyrrolidone (MW 360000; Sigma), which were prepared close to the drops for ICSI. All drops had been covered with paraffin oil (mineral oil; E.R. Squibb & Sons, Princeton, NJ). A single sperm head was aspirated into an injection pipette from the suspension and was moved to the drop containing oocytes. It was then injected into the ooplasm by using a Piezo-actuated micromanipulator (PMAS-CT150; Prime Tech Ltd., Tsuchiura, Japan).

Oocyte Activation

The sperm-injected oocytes were transferred to activation solution consisting of 0.28 M d-mannitol, 0.05 mM CaCl2, 0.1 mM MgSO4, and 0.01% (w/v) BSA and washed once. They were then stimulated with a direct current pulse of 1.5 kV/cm for a duration of 20 μsec by using a somatic hybridizer (SSH-10; Shimadzu, Kyoto, Japan).

In Vitro Culture of Sperm-Injected Oocytes

The sperm-injected oocytes before or after stimulation were cultured in vitro. Two types of in vitro culture (IVC) medium were prepared [17]. The first was IVC-PyrLac. The second contained 5.55 mM glucose, as originally reported, and also 4 mg/ml BSA and 50 μM β-mercaptoethanol (IVC-Glu). IVC-PyrLac was used from Day 0 (the day of ICSI and electric stimulation was defined as Day 0) up to Day 2. For the subsequent IVC for evaluation of the ability of the injected oocytes to develop to the blastocyst stage, the medium was changed once to IVC-Glu at Day 2 and the embryos were fixed at Day 6. IVC was carried out at 38.5°C under 5% O2.

Assessment of Embryonic Development

The cultured embryos were mounted on glass slides and fixed in 25% (v/v) acetic acid in ethanol, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined under a phase-contrast microscope.

Statistical Analysis

The percentage acrosome status, oocyte activation and blastocyst formation, and the mean number of cells per blastocyst were subjected to analysis of variance (ANOVA) using the GLM procedure of the Statistical Analysis System (SAS Institute, Cary, NC) and were then analyzed by the Duncan multiple range test. Percentage data were transformed by arcsine transformation [22] before SAS analysis.

Experiment I: Assessment of Acrosome Status after Treatment with Ca-I

To produce acrosome-free sperm heads, thawed spermatozoa were treated with Ca-I. The effective duration of the treatment with Ca-I was determined. The spermatozoa were preincubated in pig-FM containing 0 or 10 μM Ca-I at 37°C for 30, 60, or 120 min. Then they were placed on to glass slides and air dried. The specimens were stained by a triple-stain technique [19, 20, 23]. The stained samples were examined under a phase-contrast microscope with a 100× objective lens. Four types of spermatozoa were identified: 1) live acrosome-intact cells; 2) dead acrosome-intact cells; 3) live acrosome-free cells; and 4) dead acrosome-free cells. The percentages of acrosome-intact (1 and 2) or -free spermatozoa (3 and 4) were calculated. The triple stain was also carried out in some specimens before and after the sonication. Three preparations were made for each sperm sample and about 100 spermatozoa were observed in each preparation under the light microscope.

Experiment II: IVC of Sperm-Injected Oocytes

We evaluated the in vitro developmental ability of oocytes injected with sperm heads that were either untreated (control) or treated with Ca-I (Ca-I treated). At 6 h after ICSI, the oocytes were electrically stimulated and were cultured in vitro as described above. On Day 6, the cultured
Experiment III: Transfer of Sperm-Injected Oocytes to Recipients

Estrus synchronization of the recipient gilts was carried out basically as reported previously [16, 24]. In brief, an i.m. injection of 1000 IU of eCG (Nihon Zenyaku Kogyo) and, 72 h later, an injection of 500 IU of hCG (Sankyo) were given to nonpregnant gilts (5–6 mo old, 100–110 kg). Ovulation was expected at 40–45 h after the hCG injection. Sperm-injected and stimulated oocytes were transported to the farm at 37 °C in IVCPyrLac-Hepes. At 2 h after stimulation, the oocytes were transferred to both oviducts of estrous-synchronized recipient gilts, in which ovulation was confirmed. Pregnancy was diagnosed in the recipients by using an ultrasound pregnancy detector (Medeta Systems Ltd., Arundel, West Sussex, UK) at least twice between Days 66 and 86 after the embryo transfer, and the pregnancies were allowed to continue to term.

RESULTS

Experiment I: Acrosomal Loss after Treatment with Ca-I

The percentage acrosome losses in the frozen-thawed boar epididymal spermatozoa after preincubation without Ca-I (control) or with 10 μM Ca-I (Ca-I treated) are shown in Figure 1. When spermatozoa were not treated with Ca-I and were preincubated for 30–120 min, the rate of acrosomal loss did not differ significantly with the duration of preincubation (45.3–58.4%). Ca-I treatment accelerated significantly (P < 0.01) the acrosome loss as the duration of treatment was prolonged (55.6–78.6%). Because a significantly higher rate of loss (P < 0.05) was detected in the Ca-I group treated for 120 min (78.6%) than in the control group preincubated for 120 min (58.4%), in subsequent experiments, spermatozoa preincubated for 120 min with or without Ca-I were used as controls and as Ca-I-treated spermatozoa, respectively. The acrosome integrity in the spermatozoa before and after the sonication are shown in Figure 2, where the percentages of acrosome-intact spermatozoa (sperm heads) before and after the sonication did not differ significantly (P = 0.15; 64.0% and 54.3%, respectively).

Experiment II: In Vitro Development of IVM-ICSI Oocytes

The percentages of whole and part blastocysts (plus the percentage of total blastocysts) after ICSI with the control or Ca-I-treated sperm heads are shown in Figure 2. The percentages of whole blastocysts were not significantly different in the two treatment groups, but the Ca-I treatment group (3.7%) had a tendency toward a higher percentage (P = 0.057) than the control group (0.7%). The percentages of part blastocysts were not significantly different between

FIG. 2. Acrosome status before (A, B) and after (C, D) sonication. Most of the sonicated sperm heads were detached from the tail; however, acrosome was maintained on the heads. A, C) Categorized as acrosome-intact spermatozoa (heads), with acrosome stained as pink. B, D) Categorized as sperm without acrosome. All the sperm heads were considered to be dead because of dark brown staining on the postacrosomal region. Bar = 3 μm.

FIG. 3. Mean percentages (A) and mean cell numbers (B) SEM of blastocysts categorized as whole or part blastocysts (plus their total rates of occurrence). A sperm head after treatment without (control) or with calcium ionophore (Ca-I treated) was injected into each in vitro-matured oocyte. The oocytes were cultured in vitro for 6 days. No significant difference was detected in each blastocyst category.
Experiment III: Pregnancy and Farrowing after Transfer of IVM-ICSI Oocytes

The results of IVM-ICSI oocyte transfer to recipients and their successful pregnancies and farrowing are shown in Table 1. Only two recipients, both of which received oocytes injected with control sperm heads, were found to be pregnant, and none of the recipients that received oocytes injected with Ca-I-treated sperm heads were found to be pregnant. One of the pregnant recipients completed her pregnancy, but the other pregnancy was not maintained. Because the pregnant recipient that went to term showed no signs of farrowing (e.g., development of udder, swelling of vulva) until the 118th day after embryo transfer, a Cesarean operation was carried out under full anesthesia. Three healthy piglets (one male and two female) were obtained. Their birth weights ranged from 1.1 to 1.3 kg.

Table 1. Pregnancy and farrow after transfer of IVM-ICSI oocytes.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Treatment of spermatozoa with Ca-I</th>
<th>No. of oocytes transferred&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pregnancy&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of piglets farrowed (male + female)</th>
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<td>80</td>
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<td>2</td>
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<sup>a</sup> All the sperm-injected oocytes were transferred to recipients.

<sup>b</sup> Pregnancy was evaluated at the 66–86th day after embryo transfer.

<sup>c</sup> Healthy piglets were delivered by a Cesarean operation on the 118th day after oocyte transfer.

DISCUSSION

We demonstrated that IVM-ICSI oocytes transferred to recipients could develop into viable piglets. Lai et al. [13] demonstrated that porcine IVM-ICSI oocytes could develop to term, but the resulting piglet died immediately after birth. They observed normal development in all the organs of the cadaver, but there remained the possibility of poor function of the organs originated from IVM oocytes, which had been suggested to show poor developmental ability [25]. It was important to confirm the viability after the birth of piglets generated from IVM-ICSI oocytes. To our knowledge, our study is the first to obtain multiple viable piglets after transfer of IVM-ICSI oocytes, although a Cesarean operation was carried out because of the recipient’s condition.

We have already confirmed the developmental ability of porcine IVM oocytes fertilized in vitro [16, 17, 24]. When oocytes were matured under 5% oxygen tension and fertilized in vitro, blastocysts after 6 days have significantly more cells (43.5) than those matured under 5% CO₂ in air (37.8) [17]. However, IVM-ICSI blastocysts generated from oocytes through the same IVM system seem to be of poor quality, with small cell numbers (22.7 for the control group). Lai et al. [13] reported that, when oocytes matured under 5% CO₂ in air and fertilized by ICSI were cultured for 7 days, their cell number was 23.5, whereas their IVM-IVF blastocysts had an average of 26.0 cells. These numbers are quite similar to those of our IVM-ICSI blastocysts. Although their culture system was different from ours, it may be suggested that the IVM-ICSI oocytes are damaged mechanically or by other nonphysiological factors during micromanipulation for sperm injection and that this damage results in poor developmental ability, even after maturation under 5% O₂ tension. One of the possible nonphysiological factors is the carrying of the acrosome, which is attached to the anterior part of the injected sperm membrane, into the cytoplasm. At the early stage of fertilization, protamine is replaced by histone just after sperm penetration [26, 27], and then decondensation of sperm nuclei and DNA synthesis occur. The presence of the acrosome on the injected sperm head may prevent these physiological changes oc-
curing in ooplasm. To improve the success rate of fertilization by ICSI, we examined the possibility of injecting acrosome-free sperm heads. Although incubation with Ca-I had a significant effect on acroson removal (78.6% after incubation for 120 min) compared with the control (58.4%), we did not observe any significant effects on blastocyst development in vitro. Whole blastocysts from Ca-I-treated sperm heads tended to have an advantage, but there was no difference in rates or quality (cell number) of blastocysts between the two categories (control and Ca-I-treated groups). Lacham-Kaplan and Trounson [5] injected mouse spermatozoon treated with Ca-I into mouse ooplasm. They reported that the rate of normal fertilization (formation of two pronuclei with the second polar body) of the acrosome-free group was significantly better than that of the control group but that there was no difference in blastocyst rates between the two groups. Furthermore, in our study, Ca-I treatment did not improve the rate of successful pregnancy to term after transfer to recipients. The results suggested that, in pigs, acrosomal removal before ICSI does not affect either blastocyst formation in vitro or development to term in vivo after transfer to recipients.

It should be noted that, after sonication, we injected only the sperm head into the ooplasm. This procedure is different from that in the previous reports of piglet production after ICSI [10–13], wherein the spermatozoon was immobilized individually by scoring the tail, and the whole spermatozoon, with the tail, was injected into the ooplasm. In most animals, except mice and hamsters, the penetrating spermatozoon provides a centrosome from its neck, which is the source of the sperm aster and plays an important role for completion of normal fertilization [28, 29]. A study of microtubules in porcine parthenogenesis suggests that maternal centrosomal material is present and can form a microtubule network even in the absence of a paternal centrosome [30]. This seems to be explained by the meiotic spindle formation by the nuclear mitotic apparatus protein without the centrosome in oocytes [31], and fertilization processes can occur by maternal-assembled microtubules in the absence of a sperm centrosome [32]. Our results suggest clearly that, in pigs, normal development to term can be possible even in the absence of a sperm centrosome during fertilization, as has been reported in mice [33–35] and cows [36].

Successful piglet production by ICSI paves the way for the utilization of boar genetic resources, including not only nonmotile spermatozoon before or after freezing but also spermatozoon preserved by methods other than freezing. The most likely application is a freeze-drying method in combination with ICSI [37]. This procedure enables long-time preservation at room temperature without the need for a freezing system and appears less costly and time-consuming than the ordinal cryopreservation procedure. However, because only one report is available and this is of a study in mice, the freeze-drying protocol will need to be investigated further for application in other species. Establishment of the porcine IVM-ICSI procedure may help to generate transgenic pigs by sperm-mediated gene transfer. The possibility of mammalian spermatozoon being able to act as vectors for foreign DNA has been reported by Brackett et al. [38], and the successful fertilization by gene-mediated spermatozoon has been reported in mice [39], sea urchins [40], and pigs [41–43]. This method, combined with ICSI, has succeeded in mice [34] and is now expected to be used in other mammalian species, including pigs. Although these reports confirm the efficacy of the sperm-mediated gene transfer method compared with microinjection method into the pronuclei, in the production of transgenic animals [14, 15], many mature oocytes are still needed for the generation of transgenic embryos. It is not difficult to supply as many porcine IVM oocytes as are needed. The efficacy of transgenic pig production may be increased by using a combination of IVM oocytes and sperm-mediated gene transfer by ICSI. These studies describing the freeze-drying method or sperm-mediated gene transfer in combination with ICSI have been carried out using acrosome-intact spermatozoa. The discussion about efficacy of removal of the acrosome before these procedures seems to be necessary for the establishment of these techniques.

In conclusion, we demonstrated that IVM oocytes injected with sperm heads are capable of developing into viable piglets. Removal of the acrosome from the sperm head before injection did not affect development to the blastocyst stage in vitro. This might not also improve the production of piglets in vivo.

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