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Effects of Sperm Membrane Disruption and Electrical Activation of Oocytes on *In vitro* Development and Transgenesis of Porcine Embryos Produced by Intracytoplasmic Sperm Injection*

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ABSTRACT : The intracytoplasmic sperm injection (ICSI) procedure has recently been utilized to produce transgenic animals and may serve as an alternative to the conventional pronuclear microinjection in species such as pigs whose ooplasm is opaque and pronuclei are often invisible. In this study, the effects of sperm membrane disruption and electrical activation of oocytes on *in vitro* development and expression of transgene green fluorescent protein (GFP) in ICSI embryos were tested to refine this recently developed procedure. Prior to ICSI, sperm heads were treated with Triton X-100+NaCl or Triton X-100+NaCl+NaOH, to disrupt membrane to be permeable to exogenous DNA, and incubated with linearized pEGFP-N1 vector. To induce activation of oocytes, a single DC pulse of 1.3 kV/cm was applied to oocytes for 30 μsec. After ICSI was performed with the aid of a micromanipulator, *in vitro* development of embryos and GFP expression were monitored. The chemical treatment to disrupt sperm membrane did not affect the developmental competence of embryos. 40 to 60% of oocytes were cleaved after injection of sperm heads with disrupted membrane, whereas 48.6% (34/70) were cleaved without chemical treatment. Regardless of electrical stimulation to induce activation, oocytes were cleaved after ICSI, reflecting that, despite sperm membrane disruption, the perinuclear soluble sperm factor known to mediate oocyte activation remained intact. After development to the 4-cell stage, 11.8 (2/17, Triton X-100+NaCl+NaOH) to 58.8% (10/17, Triton X-100+NaCl) of embryos expressed GFP. The expression of GFP beyond the stage of embryonic genome activation (4-cell stage in the pig) indicates that the exogenous DNA might have been integrated into the porcine genome. When sperm heads were co-incubated with exogenous DNA following the treatment of Triton X-100+NaCl, GFP expression was observed in high percentage (58.8%) of embryos, suggesting that transgenic pigs may efficiently be produced using ICSI. (**Key Words :** Intracytoplasmic Sperm Injection, GFP, Transgenic, Pigs)

INTRODUCTION

A number of methods have been developed to introduce genetic material into the animal genome. Direct microinjection of recombinant DNA into a pronucleus of a zygote (Gordon et al., 1980) has been commonly used to produce transgenic animal and, especially in mice, provided powerful tool for the study on the regulation of gene expression in mammalian development. Other procedures are also available, including the use of recombinant retrovirus to infect oocytes or preimplantation embryos (Jahner et al., 1985), replication-deficient adenovirus-mediated gene transfer (Kanegae et al., 1995) and spermatozoa as vehicles for DNA delivery during *in vitro* fertilization (Lavitrano et al., 1989). In the latter approach, live spermatozoa were used as a vector for introducing recombinant DNA into the oocyte (Lavitrano et al., 1989; Maione et al., 1998). However, the use of sperm-mediated gene transfer has been limited since the procedure is often unreliable with variable efficacy of transgenic animal production among researchers, and the underlying mechanism of transgene integration has not yet been clearly understood.

In an attempt to improve efficiency of transgenic animal production, the intracytoplasmic sperm injection (ICSI) technique has been combined with sperm-mediated gene transfer to produce transgenic mice with high efficiency

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(Perry et al., 1999). Since such procedure does not require zygote with visible pronuclei, it may be a practical alternative to the pronuclear microinjection in the pig because the pronucleus injection in this species is often difficult due to the opaque ooplasm.

Although transgenic pigs become important tools in biomedical research in the field of xenotransplantation and human genetic disease model, conventional pronucleus microinjection has not been to be the choice of procedure sufficient to produce transgenic pigs needed (Nottle et al., 1997). Generally the production efficiency of transgenic pigs is less than 1% of pronucleus-microinjected oocytes (Nagashima et al., 2003). Hence, attempts have been made to produce transgenic embryos and pigs using ICSI procedures, and recently transgenic pigs carrying transgene GFP have been reported (Kurome et al., 2006; Yong et al., 2006). However, the production efficiencies of transgenic pigs from ICSI embryos have been disappointingly low, for instance, 1 transgenic piglet from 390 embryos transferred (Kurome et al., 2006) and 1 transgenic piglet from 452 embryos transferred (Yong et al., 2006), suggesting that this new procedure still needs to be improved.

The present study was to refine the ICSI procedure to facilitate the transgenesis in porcine embryos and ultimately to improve the efficiency of producing transgenic pigs. During ICSI process the effects of sperm membrane disruption to permeabilize to exogenous DNA and electrical activation of oocytes on *in vitro* development and transgenesis in preimplantation ICSI embryos were tested. Porcine sperm heads pre-incubated with green fluorescent protein (GFP) gene construct were injected into oocytes, and without compromising viability of the cells an expression of GFP based on emission of fluorescence was used as an indicator of transgenesis.

MATERIALS AND METHODS

In vitro maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to laboratory in a warm box (25 to 30°C). Before use the ovaries were rinsed in 0.9% NaCl containing 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate and maintained at room temperature. Follicular fluid and cumulus-oocytes complexes (COC) from follicles of 5 to 6 mm in diameter were aspirated using an 18-gauge needle attached to 5-ml disposable syringe. Compact COC were selected and washed six times in HEPES-buffered tissue culture medium (TCM)-199 (Gibco BRL, Gaithersburg, MD). The *in vitro* maturation (IVM) medium was modified TCM-199 (Gibco BRL) supplemented with 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO), 10 IU/ml pregnant mare serum gonadotropin (PMSG; Intervet, Boxmeer, The Netherlands), 10 IU/ml human chorionic gonadotropin (hCG; Intervet) and 10% (v/v) porcine follicular fluid aspirated from antral follicles of 6 to 8 mm in diameter. After centrifuging at 1,600×g for 30 min, supernatants were collected, filtered sequentially through 1.2 μm and 0.45 μm syringe filters and stored at -20°C until use. A group of 50 COC was cultured in 500 μl of IVM medium at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. After culturing for 22 h, COC were transferred to PMSG- and hCG-free IVM medium and cultured for another 20 h. At the end of the maturation, oocytes were freed from cumulus cells by repeated pipetting in the IVM medium containing 0.5 mg/ml hyaluronidase (Sigma) for 1 min.

Preparation of sperm

Sperm pellet was recovered from porcine semen and washed twice by centrifugation at 800×g for 5 min in 0.9% NaCl (w/v) supplemented with 10 mg/ml BSA (fraction V; Sigma). Resulting spermatozoa were suspended in 1.5 ml of TL-HEPES. To obtain sperm heads, spermatozoa were resuspended in 5 ml of cold Tris-buffered saline (TBS), and the sonication was conducted in water bath for 1 min using 100% output from Branson 8510 ultrasonic sonicator (Branson Sonic Power Co., Danbury, CN). Then, the sonicated sperm suspension was centrifuged at 1,000×g for 10 min in two layer percoll gradient solution to isolate sperm heads. The sperm heads were washed twice in 1 ml of TBS by centrifugation at 800×g for 5 min.

Removal of sperm membrane

To remove acrosomal membrane, isolated sperm heads were suspended in 0.2% Triton X-100 and agitated for 1 h at 4°C. After agitation, sperm suspension was washed with TBS by centrifugation at 800×g for 5 min. Sperm heads were resuspended in 1 M NaCl and agitated for another 1 h at 4°C. For an additional removal of the perinuclear theca from spermatozoa, the sperm suspension was washed with TBS and resuspended 1M NaOH overnight at 4°C. Membrane-removed spermatozoa were placed in TBS until co-incubation with DNA.

Induction of oocyte activation

To induce the oocyte activation, BTX Electro Cell Manipulator (Genetronics, San Diego, CA) that has a chamber with two parallel platinum wire electrodes spaced 1-mm apart overlaid with electrofusion medium composed of 0.25 M mannitol, 0.01% polyvinyl alcohol, 0.5 mM HEPES, 100 μM $CaCl_2 \cdot 2H_2O$ and 25 μM $MgCl_2 \cdot 6H_2O$ was used. Thirty to 60 min before sperm cell injection, cumulus-free oocytes were stimulated by a single DC pulse of 1.3 kV/cm for 30 μs at room temperature as modified from Xu et al. (2007). The oocytes were then transferred to 500 μl of

Table 1. Effects of sperm membrane disruption and electrical stimulation on porcine oocyte activation following ICSI

Sperm treated with	Electrical stimulation	No. of oocytes injected	No. (%) of oocytes cleaved
Triton X-100+NaCl	Yes	70	33 (47.1)
Triton X-100+NaCl	No	70	40 (57.1)
Triton X-100+NaCl+NaOH	Yes	70	42 (60.0)
Triton X-100+NaCl+NaOH	No	70	28 (40.0)
No treatment	No	70	34 (48.6)

Table 2. Effects of sperm membrane disruption and electrical stimulation on *in vitro* development and transgene GFP expression in porcine ICSI embryos

Sperm treated with	Electrical stimulation	No. of oocytes injected	No. (%) of > 4-cell embryos	No. (%) of GFP+embryos
Triton X-100+NaCl	Yes	65	13 (20.0)	3 (23.1)
Triton X-100+NaCl	No	65	17 (26.2)	10 (58.8)**
Triton X-100+NaCl+NaOH	Yes	65	15 (23.1)	4 (26.7)
Triton X-100+NaCl+NaOH	No	65	17 (26.2)	2 (11.8)
No treatment*	No	65	26 (40.0)**	0 (0.0)

* Sperm without any treatment, but co-incubated with DNA before ICSI.
** $p<0.05$ compared to the other treatments in the same column.

TCM-199 medium and cultured at 39°C in an atmosphere of 5% CO_2 in air until injection.

Preparation of DNA

pEGFP-N1 Vector (Clonetech, Palo Alto, CA) was amplified in DH5α competent cells. DNA was isolated using Maxiprep kit (Promega, Madison, WI) according to the manufacturer's protocol and linearized by EcoO 109 I restriction. The resulting size of the DNA used was 4.7 kb, containing the genes encoding GFP and neo^r selection cassette under the regulation of separate promoters.

Preparation of sperm-DNA complex

Nine microliters of membrane-disrupted sperm suspension containing 5×10^5 sperm heads in TBS were mixed with 1 μl of linearized pEGFP-N1 to give a final DNA concentration of 7 ng/μl. The DNA-sperm mixture was incubated at room temperature for 1 min.

Intracytoplasmic sperm injection

Isolated sperm heads or sperm-DNA complex was centrifuged at 400×g for 5 min and resuspended in 1:1 mixture of TBS and 7% polyvinylpyrrolidone (PVP) solution. A few microdrops (5 to 9 μl) of this suspension were placed on a lid of falcon 1007 petri dish (Becton Dickinson, Franklin Lakes, NJ). The dish was placed on Nikon inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with Narishige micromanipulator (Narishige Scientific Instrument Lab, Tokyo, Japan). After freed from cumulus cells, the oocytes with visible first polar body were selected and centrifuged for 1 to 2 min at 12,000×g in 100 μl of TBS. The injection of isolated sperm heads into the oocytes cytoplasm was performed by the method of Lee et al. (1998).

Statistical analysis

The data were pooled at least from three replicates, and the results were analyzed by analysis of variance (ANOVA).

RESULTS

Effects of sperm membrane disruption and electrical stimulation on oocyte activation following ICSI

To test whether isolated sperm head retains a competence of fertilization, sperm heads with chemical treatments were injected into porcine oocytes. Based on normal cleavage of oocytes referred as successful oocyte activation at 48 h following ICSI, the incidences of activation were not significantly different among Triton X-100+NaCl, Triton X-100+NaCl+NaOH and no treatment group (Table 1). This result suggests that neither Triton X-100+NaCl nor additional NaOH treatment affected the fertilizing ability of the sperm regardless of electrical activation.

Effect of sperm membrane disruption and electrical stimulation on *in vitro* development and transgene expression in ICSI embryos

Table 2 summarizes *in vitro* development and transgene expression in porcine embryos at 96 to 120 h after sperm head injection. The rates of embryonic development beyond 4-cell stage, which considered as post-embryonic genome activation, in all treatment groups were significantly lower ($p<0.05$) than the rate in the group without any treatments. The highest rate (58.8%) of transgene GFP expression was achieved in the group that Triton X-100+NaCl-treated sperm head were injected into oocytes without electrical activation. In contrast, the injection of Triton X-100+NaCl-treated sperm into electrically activated oocytes and injection of Triton X-100+NaCl+NaOH-treated sperm head

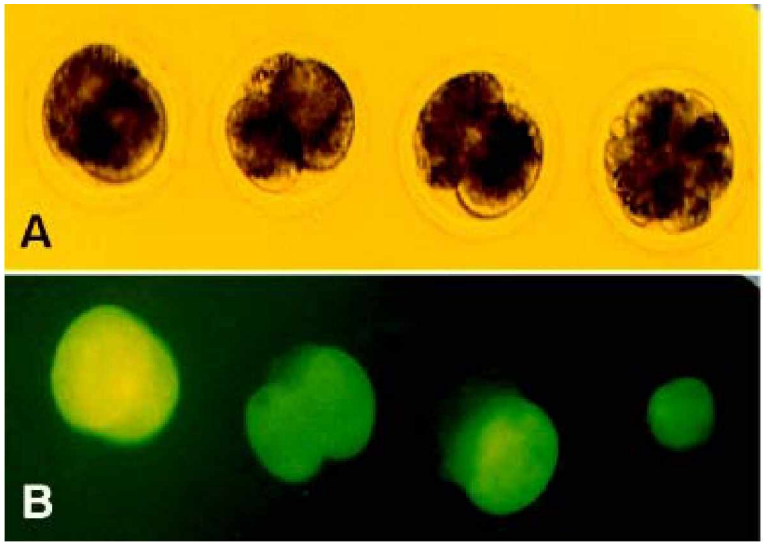

Figure 1. Expression of GFP in porcine ICSI embryos. (A) Embryos developed from the oocytes injected with sperm head after incubation with DNA encoding GFP (phase-contrast microscopy, 200×). (B) The same embryos as in panel A expressing GFP. Note that 8-cell embryo far right shows a mosaicism that only one blastomere expresses GFP (fluorescent microscopy, 200×).

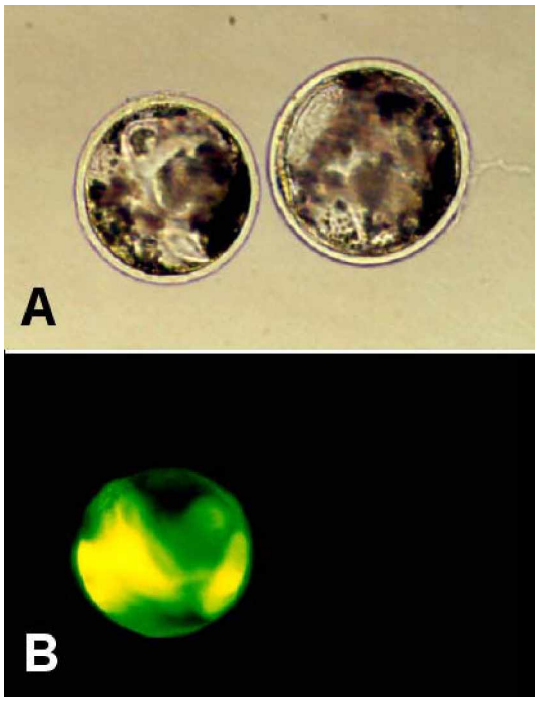

Figure 2. Expression of GFP in porcine blastocyst. (A) The blastocyst on the left developed from the oocyte injected with a sperm head after incubation with DNA encoding GFP. In contrast, the blastocyst on the right was developed from the oocyte injected with a sperm head without DNA co-incubation (phase-contrast microscopy, 200×). (B) The same embryos as in panel A. The blastocyst on the left expresses GFP in all cells contained, as opposed to no GFP expression from the blastocyst on the right (fluorescent microscopy, 200×).

into both electrically activated or non-activated oocytes showed significantly lower ($p<0.05$) GFP expression rate than those from Triton X-100+NaCl-treated sperm into non-activated oocytes. In all groups, most of the embryos expressing GFP were mosaic. As an example shown in Figure 1, approximately 95% of the total GFP-positive embryos represented mosaicism. However, as shown in Figure 2, the embryos expressed GFP from all blastomeres comprising the embryos were also observed.

DISCUSSION

Sperm-mediated gene transfer has been studied in mice (Lavitrano et al., 1989; Maione et al., 1998) and pigs (Lavitrano et al., 2002), but efficacy of transgenesis achieved in these studies has been controversial. Combining the ICSI procedure with sperm-mediated gene transfer that improved an efficiency of producing transgenic mice has been reported (Perry et al., 1999). This study demonstrated that membrane-disrupted mouse spermatozoa support the genomic integration and subsequent expression of exogenous DNA in developing embryos after ICSI. In the present study the effects of sperm membrane disruption and electrical activation of oocytes on *in vitro* development and transgenesis were assessed in preimplantation ICSI embryos.

In the first experiment, whether the treatment of sperm head affects fertilizing ability of sperm was tested. All groups with chemically-treated sperm could activate oocytes regardless of electrical stimulation of oocytes (Table 1). This result was not as expected, considering that the initial treatment (Triton X-100+NaCl) solubilizes the acrosome, sperm head membranes, and hydrophobic and ionically bound proteins, leaving essentially a shell of

insoluble perinuclear theca surrounding the condensed nucleus, and subsequent treatment with NaOH solubilizes the perinuclear theca but leaves the nucleus in its condensed form (Oko and Maravei, 1994). Perhaps the treatments of sperm membrane in the present study were mild enough for sperm to be capable of fertilization. The perinuclear membrane may remain intact and soluble sperm factor covered by the membrane may be preserved and later directed oocyte activation after ICSI was performed. In addition, an electrical stimulation has minimal or no effect on activation of oocytes if once a sperm was injected (Table 1). Yong et al. (2005) reported a similar result that electrical stimulation was not necessary in porcine ICSI although the stimulation was given to oocytes post ICSI as opposed to prior to ICSI as demonstrated in the present study.

The results of the second experiment demonstrate developmental capability and transgenesis in ICSI embryos. As represented in Table 2, the rates of *in vitro* development to the 4-cell stage in all treatment groups were significantly lower than the rate in no treatment group, suggesting that either the chemical treatment of sperm or electrical stimulation of oocytes or both might have adverse effect to the development of ICSI embryos. The highest expression of transgene GFP (58.8%) was achieved after injecting Triton X-100+NaCl-treated sperm heads into non-activated oocytes. Only a report from Nagashima et al. (2003) demonstrated approximately 50% of ICSI embryos were transgenic, which was a few times higher than transgenic efficiency generally obtained from pronucleus microinjection. Effective transgenesis in the present study may be on account of appropriate timing of oocytes activation induced not by electrical stimulation but by sperm soluble factor and proper occurrence of sperm chromatin decondensation, which might have affected an integration of transgene.

Mechanisms involving correlation between sperm's capability of fertilization and DNA uptake have been postulated to be complex. Fifteen to 22% of sperm-bound DNA was spontaneously internalized into the nuclei within 30 to 60 min of incubation (Francolini et al., 1993). The interaction between sperm and exogenous DNA takes place in subacrosomal segment (Lavitrano et al., 1992; Sperandio et al., 1996). This can be mediated by a class of DNA-binding proteins of 30-35 kDa (Balhorn et al., 1977). Foreign DNA molecules internalized into sperm nuclei become heavily rearranged and undergo recombination events with the genomic DNA (Zoraqi and Spadafora, 1997). This suggests that the exogenous DNA is exposed to the action of sperm endonucleases early after nuclear internalization. The activation of nucleases in mature sperm cells may, on one hand, be responsible for the rearrangement of foreign DNA sequences that are found to be integrated into the host genomes (Lavitrano et al., 1989; Sperandio et al., 1996), and on the other hand, may cause the degradation of the chromosomal DNA as well as the loss of fertility and viability of spermatozoa.

In the present study, attempt to detect incorporation of exogenous DNA into the sperm using rhodamine-tagged DNA encoding GFP as described by Chan et al. (2000) was unsuccessful (data not shown). One-minute co-incubation of sperm with DNA in the present experiment adapted from previous report by Perry et al. (1999) might not be sufficient for exogenous DNA to be incorporated into the sperm. Exogenous DNA may rather adhere to the sperm, and integration of the DNA into host genome may occur after the injection of sperm into oocytes. Moreover, the integration of transgene taking place even after the cleavage of injected oocytes might have caused mosaicism of transgene expression that frequently observed in the present study (Figure 1). Such mosaicism due to integration of transgene at multicellular embryonic stages has also been reported in transgenic founder animals from pronucleus-microinjection (Whitelaw et al., 1993) and ICSI (Moreira et al., 2007).

The method of mammalian transgenesis described in this study affords advantages over conventional pronuclear microinjection. Transgenesis by ICSI may circumvent certain difficulties to the conventional pronuclear microinjection, especially in species such as cattle or pigs whose pronuclei are often difficult to observe due to dark cytoplasm of oocytes. Moreover, ICSI does not require additional *in vitro* fertilization step to obtain zygotes for pronuclear microinjection. However, a mosaicism possibly due to an asymmetric integration of transgene after cleavage was observed in most of embryos produced by ICSI in this study. Such drawback needs to be further solved for this new technique to be widely accepted for the production of transgenic pigs.

The results of the present study demonstrated that sperm heads with disrupted membrane can interact with exogenous DNA, and subsequent injection of sperm head-DNA complex into oocytes gave rise to transgenic embryos with high percentage (58.8%), suggesting that the refinement of the ICSI-based gene transfer procedure may enable efficient production of transgenic pigs.

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