In Vitro Production of Pig Embryos using Intracytoplasmic Injection of Flow Cytometry Sorted Boar Spermatozoa

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ABSTRACT

The ability to preselect the sex of piglets is advantageous in the pig industry. The objective of this study was to examine the feasibility of using intracytoplasmic sperm injection (ICSI) with sorted spermatozoa to produce piglets with a preselected sex. Pig embryos were produced by ICSI of frozen X- and Y-sperm that had been separated by flow cytometry. The developmental competence of the embryos was investigated *in vitro* and *in vivo*. The populations of X- and Y-spermatozoa were 52.7% and 47.3%, respectively in our samples. The *in vitro* development of ICSI embryos was enhanced by longer of *in vitro* maturation of oocytes (44~48 h vs. 40~43 h). Their cleavage (65~70%) and blastocyst formation (9~12%) rates were not significantly different between male and female ICSI embryos, or between sorted and unsorted sperm-derived embryos. One pregnancy was established in a recipient that was transferred with 110 female ICSI embryos, but the pregnancy was terminated on Day 89 of gestation. Our results suggest that the separation X- and Y-spermatozoa by flow cytometric sorting can be a useful tool in combination with ICSI for the production of pig embryos and piglets of preselected sex.

(Key words : flow cytometry, ICSI, spermatozoa, embryo development, pig)

INTRODUCTION

The theoretical ratio of male and female offspring is 1:1 in naturally breeding animal populations. The livestock industries place different values on the sexes of cattle and hogs and therefore would benefit from tools designed to allow the preselection of offspring sex. In the dairy industry, female calves produced by the dairy cattle are raised for milk production while the male calves are shunted to beef production. Likewise, male piglets and beef calves are frequently castrated to improve their meat quality, which creates additional costs for management of these undesired animals. Therefore, developing a technique enabling the preselection of animals with the desired sex would be advantageous to the livestock industry by reducing the costs of managing animals with unwanted sexes. Especially for multiparous livestock animals like pigs, the production of offspring with preselected sex allows for standardization of nutrition and breeding, thereby increasing the efficiency of farm management.

Since the development of sperm-sexing technology, numerous improvements have lead to increased efficiency of sperm separation. Consequently, it became possible to produce offspring of cattle, pigs, and sheep with preselected sexes (Grossfeld *et al.*, 2005; Garner, 2006). Sex-sorting technology is currently being applied to other species including horses, cats, gorillas, and dolphins with success rates of 90~95% for shifting the sex ratio of spermatozoa or offspring (reviewed by Johnson *et al.*, 2005). Sorted spermatozoa can be used for artificial insemination (AI) and *in vitro* fertilization (IVF) with subsequent embryo transfer (Johnson *et al.*, 1989; Abeydeera *et al.*, 1998; Rath *et al.*, 1999; Probst and Rath, 2003; Vazquez *et al.*, 2003). However, for practical and commercial applications of this technology, it is necessary to secure large numbers of sorted spermatozoa with normal motility and fertilization ability. Spermatozoa experience mechanical and chemical stresses during sorting steps due to exposure to laser light, emitted fluorescence, and various media with different compositions. Boar spermatozoa are more susceptible than bull spermatozoa to the stresses of flow cytometric sorting and to freezing-thawing preservation processes (Woelders *et al.*, 2005; Holt *et al.*, 2005). Damage to boar spermatozoa during flow cytometric sorting reduces viability, storage capability, and fertilization ability of the spermatozoa (Maxwell and Johnson, 1999; Garcia *et al.*, 2007).

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These limitations prevent the production of large numbers of spermatozoa with normal fertilizing capability, and the application of sex sorting by flow cytometry is therefore not currently practiced in the swine AI industry.

ICSI is an alternative to AI or IVF for the production of sexed piglets with sorted spermatozoa and embryo transfer. ICSI requires only small numbers of spermatozoa for the production of viable embryos. In addition, ICSI makes it possible to produce normal pig embryos from the use of immotile or membrane-damaged spermatozoa that cannot be used for AI or IVF. Although several litters of piglets were produced after transfer of ICSI embryos (Martin, 2000; Probst and Rath, 2003), the efficiency of piglet production was low. Therefore, the ICSI procedure using sorted spermatozoa requires further refinement to increase the efficiency of the production of piglets with preselected sexes. The objective of this study was to examine the feasibility of ICSI using sorted spermatozoa in the production of sexed piglets. We produced pig embryos by intracytoplasmic injection of frozen X- and Y-spermatozoa that were separated by flow cytometry, and then investigated their *in vitro* and *in vivo* developmental competence after *in vitro* culture or embryo transfer.

MATERIALS AND METHODS

1. Flow Cytometric Sperm Sorting and Freezing of Sorted Spermatozoa

Ejaculated Duroc semen was obtained from Darby Genetics Inc. (Anseong, Korea). Sperm concentration and motility were determined using a routine semen evaluation technique. Semen was washed three times in Dulbecco's phosphate-buffered saline (DPBS, Invitrogen, Grand Island, NY, USA) at 300 × g for 3 min. After washing, the concentration of the sperm was adjusted to 9.5×10^8 sperm/ml in DPBS. Spermatozoa were stained in DPBS containing 20.8 μM Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Spermatozoa were sorted with a FACS Vantage SE (Becton Dickinson, Franklin Lakes, NJ, USA) using DPBS as sheath fluid. The parameter was FL5-H and data were analyzed with Cellquest software (Becton Dickinson, San Jose, CA, USA). After sorting, X- and Y-spermatozoa were collected in conical tubes and for transport. The sorted X- and Y-spermatozoa were concentrated by centrifugation and suspended in a 3 : 1 : 1 mixture of Dulbecco's Modified Eagle Medium (DMEM): fetal bovine serum (FBS): dimethyl sulfoxide (DMSO) solution. Sperm suspensions were aliquotted to 1.5-ml microtubes, refrigerated at 4℃ for 2 h, and frozen at −70℃ overnight. Frozen spermatozoa were stored in liquid nitrogen until use.

2. Oocyte Collection and *In Vitro* Maturation (IVM)

Ovaries of prepubertal gilts were obtained from a local slaughterhouse. Follicular contents were aspirated from superficial follicles 3～8 mm in diameter and pooled into 15-ml conical tubes. The supernatant was gently removed and the sediment was placed in HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA) (Bavister *et al.*, 1983). Only cumulus-oocyte complexes (COCs) with multilayers of compact cumulus cells were selected and cultured for IVM. After washing in IVM medium, a group of 50～70 COCs was placed into each well of a four- well multi-dish (Nunc, Roskilde, Denmark) containing 500 μl of IVM medium with 10 IU/ml equine chorionic gonadotropin (eCG) (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/ml human chorionic gonadotropin (hCG) (Intervet International BV). COCs were cultured at 39℃ in a humidified atmosphere of 5% CO_2. After 22 h in the maturation culture, the COCs were washed three times in fresh hormone-free IVM medium and then cultured in fresh hormone-free IVM medium for an additional 18～26 h.

3. ICSI Using Sorted Spermatozoa and *In Vitro* Culture (IVC) of Embryos

Sorted spermatozoa frozen in a microtube were thawed in warm water at 39℃ and diluted in 1 ml of DPBS containing 0.01% polyvinyl alcohol (PVA) (PBS-PVA). The sperm suspension was washed twice by centrifugation at 350 × g for 3 min, and the final sperm pellet was resuspended in PBS-PVA. The ICSI procedure was essentially the same as previously described (Yong *et al.*, 2003). Briefly, an oocyte with the polar body was immobilized with a holding pipette such that its polar body was at either the 6 or 12 o'clock position. A sorted sperm was aspirated tail first into an injection pipette (3～4 μm in inner diameter) connected to open-ended tubing, injected, and mixed with a small quantity of cytoplasm.

The medium for embryo development was North Carolina State University-23 (NCUS-23) medium (Petters and Wells, 1993), which was modified by replacing 5.5 mM glucose with 5.0 mM sodium lactate and 0.5 mM sodium pyruvate (Park *et al.*, 2005). After ICSI, oocytes were washed, transferred to 30 μl droplets (10～15 zygotes/droplet) of culture medium co-

vered with warm mineral oil, and cultured for 156 h in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. After 48 and 156 h of culture, embryo cleavage and blastocyst formation, respectively, were assessed using a stereomicroscope. Total blastocyst cell number was assessed using Hoechst 33342 staining and an epifluorescence microscope.

4. PCR for Sexing of ICSI Embryos

A single ICSI embryo in a 0.6 ml PCR tube was digested by adding 100 or 200 μl of PCR buffer (Qiagen, Chatsworth, CA, USA), 5 μl of proteinase K solution (20 mg/ml, Qiagen), and 0.45% Tween-20 (Sigma-Aldrich) and incubating for 1 h in a water bath at 55℃. Proteinase K was inactivated by placing samples in > 95℃ water for 10 min. After this procedure, 50 μl of the reaction mixture (1 U Taq DNA polymerase, nucleotides 250 μM each, tris-HCl pH 9.0, 40 mM KCl, and 1.5 mM $MgCl_2$, Qiagen) was added to the material to be amplified. For amplification, pairs of primers for chromosomes 1 and Y were used (Table 1).

5. Embryo Transfer

Embryo transfers were performed at the research farm of the Gyeonggi Veterinary Service, Korea. ICSI embryos at the one-cell stage were transferred into two naturally cycling gilts on the first day of standing estrus. A mid-ventral laparotomy was performed under general anesthesia using isoflurane. The reproductive tract was exposed, and the ICSI embryos were transferred into an oviduct at the ampullary isthmic junction. The pregnancy was diagnosed on Day 30 (Day 0 was the day of embryo transfer), and then was checked regularly at 4-week intervals using ultrasonography.

6. Experimental Design and Statistical Analysis

In Experiment 1, spermatozoa were separated by flow cytometric sorting and the ratio of X- to Y-bearing spermatozoa was determined. The effect of culture duration (40~43 h vs. 44~48 h) for IVM of oocytes on the *in vitro* development of ICSI embryos was investigated in Experiment 2. In Experiment 3, pig embryos were produced from ICSI of sorted X-, Y-spermatozoa, and unsorted spermatozoa. Unsorted spermatozoa were immobilized by dipping into liquid nitrogen for 1 min and then thawing in a water bath at 39℃. Some ICSI embryos were sexed by PCR using primers for porcine chromosomes 1 and Y. In Experiment 4, ICSI embryos derived from X- and Y-spermatozoa were transferred surgically to recipient gilts to examine the *in vivo* viability.

All statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Data were analyzed using a general linear model, followed by the least significant difference mean separation procedure when treatments differed at $p<0.05$. Percentage data were arcsine transformed prior to analysis to maintain homogeneity of variances.

RESULTS

Experiment 1: Flow Cytometric Sorting of Boar Spermatozoa

Fig. 1 displays a histogram and dot plot illustrating the separation of X- and Y-spermatozoa by flow cytometry. The subpopulations of X- and Y-spermatozoa were 52.7% and 47.3%, respectively (Table 2). After freezing and thawing, the sorted spermatozoa had no motility.

Experiment 2: Effect of IVM Duration on the In Vitro Development of ICSI Embryos

Development of ICSI embryos to the blastocyst stage (14%) was significantly higher when IVM oocytes were cultured for longer than 44 h (Table 3). However, variances in the *in vitro* maturation duration did not alter the embryo cleavage rates or the mean number of cells in per blastocyst.

Table 1. PCR primers for sex determination of ICSI embryos at the cleavage stages derived from X- and Y-spermatozoa sorted by flow cytometry

Markers	Primer sequence	Product size (bp)	Chromosome
Chromosome 1 (F)	5'-GTTGCACTTTCACGGACGCAGC-3'	244	X and Y
Chromosome 1 (R)	5'-CTAGCCCATTGCTCGCCATAGC-3'		
Chromosome Y (F)	5'-AATCCACCATACCTCATGGACC-3'	377	Y
Chromosome Y (R)	5'-TTTCTCCTGTATCCTCCTGC-3'		

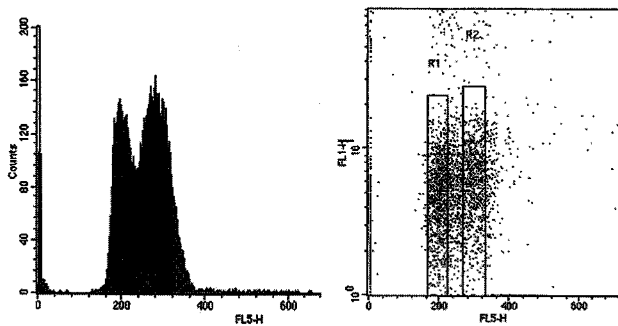

Fig. 1. Flowcytometric histogram (left) and dotplot (right). R1 and R2 channels in the dotplot indicate the first and second peaks, respectively, in the histogram. The first peak and the second peak were verified as Y- and X-bearing spermatozoa by PCR using primers for porcine chromosome 1 and chromosome Y.

Table 2. Population of X- and Y-spermatozoa sorted by flow cytometry

Peak	Sex	Number of spermatozoa	%
R1	Y	798,000	47.3
R2	X	890,000	52.7

Experiment 3: *In Vitro* Development of ICSI Embryos Derived from Sorted or Unsorted Spermatozoa

The *in vitro* development of ICSI embryos derived from sorted X- or Y-spermatozoa was examined and compared to embryos derived from unsorted spermatozoa. There were no significant differences in the rates of cleavage (65~70%) or blastocyst formation (9~12%) between male and female ICSI embryos (Table 4, Fig. 2). ICSI embryos derived from unsorted spermatozoa also showed similar embryo cleavage rates (61%) and blastocyst formation (10%). When the sex of the ICSI embryos was determined by PCR amplification of porcine chromosomes 1 and Y, the sex of the ICSI embryos was consistent with the sex of sorted spermatozoa used for ICSI (Fig. 3).

Table 4. *In vitro* development of pig embryos derived from ICSI using sorted X- and Y-spermatozoa or unsorted spermatozoa

Source of spermatozoa	No. of ICSI embryos cultured*	% of embryos developed to		No. of cells in blastocyst
		≥ 2-cell	Blastocyst	
Sorted X	194	70	9	23
Sorted Y	171	65	12	29
Unsorted	225	61	10	28

* Nine replicates. Oocytes matured for 44~48 h were used for ICSI.

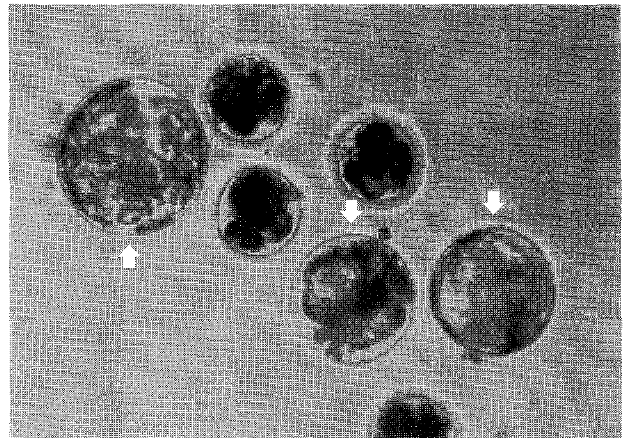

Fig. 2. Pig blastocysts (arrows) developed on Day 7 from the *in vitro* culture of ICSI embryos that were derived from sorted X-spermatozoa.

Experiment 4: *In Vivo* Development of Male and Female ICSI Embryos

When 72 and 110 ICSI embryos derived from X- and Y-spermatozoa, respectively, were transferred to recipient gilts, a pregnancy was established in a recipient transferred with female ICSI embryos. The pregnancy was terminated on Day 89 after embryo transfer (Table 5). A deformity was found in the

Table 3. Effect of duration of maturation culture on oocyte maturation and development of ICSI embryos derived from X- and Y-spermatozoa

Maturation duration (h)	No. of oocytes matured	No. (%) of oocytes reached metaphase II	No. of ICSI embryos cultured*	% of embryos developed to		No. of cells in blastocyst
				≥ 2-cell	Blastocyst	
40~43	411	386 (94)	271	61	6[a]	26
44~48	446	424 (95)	271	67	14[b]	26

* Eight replicates.

[ab] Values with different superscripts within each column are significantly different ($p<0.05$).

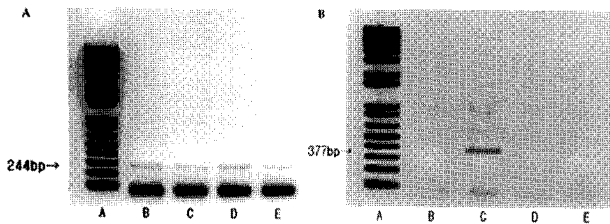

Fig. 3. PCR for sex determination in pig embryos at 4- to 8-cell stages that were derived from using ICSI of X- and Y-spermatozoa. DNAs from presumptive male and female ICSI embryos were amplified by the porcine chromosome 1 (244 bp)-specific (A) and chromosome Y (377 bp)-specific primers (B).
Lane A: 1 Kb ladder
Lane B: ICSI embryo from X-sperm (PCR buffer: 100 μl)
Lane C: ICSI embryo from Y-sperm (PCR buffer: 100 μl)
Lane D: ICSI embryo from X-sperm (PCR buffer: 200 μl)
Lane E: ICSI embryo from Y-sperm (PCR buffer: 200 μl)

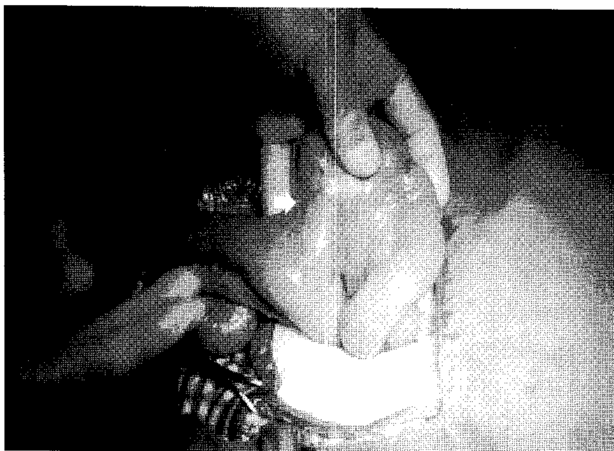

Fig. 4. A deformity found in the uterus of Recipient B in Table 4. A missing part of the uterine horn was found in the left uterus (arrow) just after the embryo transfer. This recipient was transferred with 110 ICSI embryos derived from Y-spermatozoa. Pregnancy was established but terminated on Day 89 of transfer.

left uterine horn ipsilateral to the oviduct into which the embryos were transferred (Fig. 4).

DISCUSSION

In this study, flow cytometrically sorted boar spermatozoa were successfully used to produce pig embryos *in vitro* using ICSI. Although the sorted spermatozoa showed no motility after freezing and thawing, they could support the development of embryos to the blastocyst stage after ICSI. The ratio of X- and Y-spermatozoa was determined to be approximately 1:1, which is similar to the sex ratio of offspring that are born after natural breeding or artificial insemination. In this study, sorted X- and Y-spermatozoa were frozen separately for IVF or ICSI. After thawing, the spermatozoa showed no motility and therefore it was not possible to use sorted spermatozoa for IVF. As an alternative, we employed the ICSI technique to produce pig embryos using sorted X- or Y-sperm. The ability of sorted sperm-derived ICSI embryos to develop to the blastocyst stage has previously demonstrated that ICSI can be used to produce pig embryos even when motile spermatozoa are not available (Yong *et al.*, 2005; Tian *et al.*, 2006). However, for industrial application, further studies are required to improve the viability of spermatozoa after flow cytometric sorting.

There are many factors influencing the efficiency of the production of pig embryos. IVM of immature oocytes is one of the critical factors which determines the developmental competence of embryos. It has been reported that IVM duration influences the development of embryos after IVF, parthenogenetic activation (PA), and somatic cell nuclear transfer (SCNT) (Ikeda and Takahashi, 2001; Miyoshi *et al.*, 2001; Hölker *et al.*, 2005). In this study, IVM oocytes matured for longer than 44 h had higher developmental potential following ICSI than oocytes matured for less than 44 h. This result is consistent with the observation that SCNT embryo development improved

Table 5. *In vivo* developmental potential of ICSI pig embryos derived from X- and Y-spermatozoa

Recipient ID	Spermatozoa used for ICSI	No. of ICSI embryos transferred*	Pregnancy		Remarks
			On Day 30	To term	
A	X	72	No	No	–
B	Y	110	Yes	No	Aborted on Day 89 of gestation

* Oocytes matured for 44~48 h were used for ICSI.

when oocytes matured for 42 h were used as recipient cytoplasts in comparison to oocytes matured for 36 or 39 h. In contrast, Ikeda and Takahashi (2001) reported that pig oocytes matured for 33 h showed higher cleavage and blastocyst formation rates after SCNT than those matured for 44 h (Ikeda and Takahashi, 2001). In addition, Alfonso *et al.* (2008) reported that there was no difference in the maturation and development rates of PA and ICSI embryos using oocytes matured *in vitro* for 45 or 50 h. MPF activity and cytoskeletal configuration are known to be affected by oocyte aging and may affect the developmental competence of embryos matured *in vitro* for different amounts of time (Kim *et al.*, 1996; Kikuchi *et al.*, 2000). In this study, it was not clear how embryo development was influenced by the extended IVM duration. Further study is needed to determine the optimal IVM duration for the production of ICSI embryos in pigs.

The fluorescent dye, Hoechst 33342, has been reported to be toxic and have mutagenic effects on certain types of cells (Durand and Olive, 1982). Ultraviolet light can also cause chrosomosomal abnormalities in mouse spermatozoa (Matsuda and Tobari, 1988). Sperm sorted by flow cytometry are exposed to both Hoescht 33342 and ultraviolet light, and therefore, may have lowered developmental competence. We compared the developmental capacity of sorted sperm-derived ICSI embryos to that of ICSI embryos derived from unsorted spermatozoa. No difference was found in the blastocyst formation rates of the sorted and unsorted-sperm derived ICSI embryos, which indicates that sorted spermatozoa can be used for ICSI to produce pig embryos even though their viability is decreased by the sorting process. We analyzed the sex of ICSI embryos by PCR co-amplification of the Y-chromosomal sequence and an autosomal sequence (chromosome 1). The sex of ICSI embryos corresponded to the sex of sperm used for ICSI (data not shown).

The *in vivo* developmental viability of the sorted sperm-derived ICSI embryos was demonstrated by the pregnancy established in one of two recipients transferred with sorted sperm-derived ICSI embryos. However, the pregnancy was terminated at 89 days of gestation, probably due to a uterine horn malformation discovered after embryo transfer. The pregnancy rate after embryo transfer is affected by many factors including the estrous stage of the recipient animal at the time of transfer, embryo quality, and the number of embryos transferred (Pope *et al.*, 1972; Polge, 1982). Due to small replications of embryo transfers in this study, it is not possible to determine if the termination of pregnancy before term was due to embryonic factors, the deformity present in the uterus, or to other environmental conditions relating to embryo transfer. Extensive embryo transfer studies would be needed to verify the ability of flow cytometry sorted sperm-derived ICSI embryos to develop to term *in vivo*. In summary, the results of our study suggest that separation of X- and Y-spermatozoa by flow cytometric sorting combining with ICSI can be a useful tool to produce pig embryos and piglets with preselected sex.

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