Expression of GFP Gene in the Porcine Preimplantation Embryos after ICSI with DNA/Sperm Complex

Joo-Hee Han[1], Sungwoo Kim[1], Poongyeon Lee[1], Chun-Gyu Park[1], Hyun-Gi Lee[1], Boh-Suk Yang[1], Ki-Hyeong Rhee[2], Chang-Hyung Lee[3], Hoon-Taek Lee[4], Won-Kyong Chang[1] and Jin-Ki Park[1,†]

[1]*Animal Biotechnology Division, National Livestock Research Institute, RDA, Suwon 441-706, Korea*

[2]*College of Industrial Science, Kongju National University, Kongju 314-701, Korea*

[3]*Department of Animal Science, Korea National Agricultural College, RDA, Suwon 441-706, Korea,*

[4]*Animal Resources Research Center, Konkuk University, Seoul 143-701, Korea*

ABSTRACT

The possibility of producing transgenic embryos expressing the green fluorescence protein (GFP) gene have been evaluated after transfer of exogenous gene into the porcine zygote cytoplasm using the intracytoplasm sperm injection (ICSI) as gene delivery method. For DNA binding to sperm heads, 0.05% Triton X-100 or Lipofectin was used. After injection of the sperm bound to DNA by means of Lipofectin or Triton X-100 triturate, the blastocyst formation rates on day 6 were not significantly different from that of ICSI only group (18.8, 19.2 and 25.3%). In terms of GFP expression, more embryos were in GFP form in Triton X-100 group than in Lipofectin group (40.6 vs 36.4%), while percentage of non-mosaic embryos expressing the GFP gene in all blastomere was higher ($P<0.05$) in Lipofectin group than in Triton X-100 group (4.2 vs 0.9%). ICSI embryos derived from sperm treated with Lipofectin/DNA complex was transferred into 3 recipients and were collected by uterine flushing on days 5, 7 and 15 after embryo transfer, and then GFP expression was observed by a fluorescence microscopy. Over 26% of the collected embryos were normally expressed GFP gene. These results suggest that foreign gene transfer method with DNA bound sperm caused minimal damage to structure of oocytes that can result to full development of porcine embryos. This was confirmed in this study when the embryos that were transferred after ISCI of DNA bound sperm had a normal development and gene expression until preimplantation.

(Key words : Porcine, ICSI, GFP, Gene expression, Embryo transfer)

INTRODUCTION

Modification of animal genome is new an important field of research due to its wide application. Integration and stable germ line transmission of the foreign genes microinjected into mouse pronuclei was first reported (Gordon and Ruddle, 1981). At present, while most method can be achieved in few species such as mouse, efficiency of introducing a foreign gene is also limited. When other species were examined, these methods either were ineffective or with much lower efficiency. The first ICSI method for overcoming male infertility or for the study of fertilization has been employed in humans (Palermo *et al.*, 1992, 1993). Other workers (Lavitrano *et al.*, 1989; Perry *et al.*, 1999) suggested that sperm could be used as vectors for introducing foreign gene into mice zygote and sperm mediated gene transfer (SMGT) could be an easier method to achieve transgenesis. In porcine, a little information with regard to ICSI (Iritani, 1991; Lee *et al.*, 1998) and ICSI-mediated transgenic animal production has been reported (Lavitrano *et al.*, 1989, 2003). Moreover, all experiments have not been successful to produce transgenic pig by DNA bound sperm transfer method aside from low gene expression efficiency of transgenic porcine embryos. However successful productions of transgenic mice (Maione *et al.*, 1998; Perry *et al.*, 1999) or monkey (Chan *et al.*, 2000) by SMGT method have been reported. Thus, in the present study, we employed ICSI procedure to test the successful production of transgenic pigs by injecting the sperm conjugated with the foreign DNA through the treatment with Lipofectin or Triton X-100. Since liposomes could effectively transfer DNA into sperm (Bachiller *et al.*, 1991). DNA/liposome complex or Triton X-100 pretreatment was used to transfer the foreign DNA into oocytes (Lai *et al.*, 2001; Szczygiel and Ward,

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† Corresponding author : Phone: +82-31-290-1634, E-mail: parkjk@rda.go.kr

2002). Other experiment also involved gene transfer of silver sea bream (Lu *et al.*, 2002) using the liposome method by co-incubating the sperm with DNA pretreated with liposome (Lai *et al.*, 2001), however, production of the transgenic animal was not a success.

In the present study, the effects of activation and two treatments of sperms for DNA binding before ICSI into porcine oocytes were tested during the early embryo development. Aside from predicting the applicability of ICSI in transgenic pig, the expression pattern of the exogenous GFP gene was also investigated for long term.

MATERIALS AND METHODS

Oocytes Collection and *In Vitro* Maturation

All animals that were maintained and used conformed to the protocol of the Institutional Animal Care and Use Committee of the National Livestock Research Institute based on the Guide for Care and Use of Domestic Animal. Porcine ovaries were obtained from an abattoir and transported to the laboratory in 0.9% (w/v) saline solution and physiologically maintained at 30 to 35°C. Cumulus oocyte complexes (COCs) were aspirated from follicles of 3 to 6 mm in diameter with an 18-gauge needle attached to a disposable 10 ml syringe. The COCs were washed three times with TL-HEPES supplemented with 0.1% polyvinyl alcohol (PVA) (Abeydeera *et al.*, 2000). Approximately 70 ~100 COCs were put into individual 4-well dish containing TCM-199 (Gibco, Grand island, NY) supplemented with PVA (0.1%), D-glucose (3.05 mM), sodium pyruvate (0.91 mM), penicillin (75 μg/ml), streptomycin (50 μg/ml), cysteine (0.57 mM), luteinising hormone (LH; 0.5 μg/ml), follicle stimulating hormone (FSH; 0.5 μg/ml) and epidermal growth factor (EGF; 10 μg/ml) for 42~44 hr at 38.5°C in 5% CO_2.

Preparation of Lipofectin/DNA Complex

Fresh boar semen was collected and washed twice by centrifugation at 800 ×g for 5 min in D-PBS (Gibco BRL, USA) supplemented with 0.1% BSA (Sigma, St. Louis, MO.). Spermatozoa were suspended in 1.5 ml of D-PBS, and 0.5 μl of Lipofectin (Boehringer Mannheneim, Germany) and 10 ng/μl DNA (pCX-EGFP/Neo) mixture was incubated at 17°C (Francolini *et al.*, 1993) for 1 hr. The DNA-Lipofectin mixture was co-incubated with a suspended spermatozoa (with a concentration of $1\sim2\times10^6$ sperm cells/ml) for 6 hr before ISCI.

Sperm Pretreament by Triton X-100

Boar semen was prepared by the same procedure used for Lipofectin (Lipofectin®Reagent, Gibco BRL, MD) treatment. Spermtozoa were washed twice in nuclear isolation medium (NIM) containing 125 mM KCl, 2.6 mM NaCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4 and 3.0 mM EDTA (pH 7.2) (Perry *et al.*, 1999). Triton X-100 to a final concentration of 0.05% (v/v) was added in the sperm suspension and triturated for 60 sec. The triturated spermatozoa with Triton X-100 were centrifuged twice for 1 min at 20,000 ×g at 2°C and resuspended in ice-cold NIM. The 200~400 μl NIM including spermatozoa was mixed with 10 ng/μl DNA.

Preparation of DNA

The pCX-EGFP/Neo (Ikawa *et al.*, 1995; Okabe *et al.*, 1997; Miyoshi *et al.*, 2000) gene containing a neomycin resistant gene and enhanced green fluorescent protein (EGFP) gene regulated by a phosphoglycerate kinase promoter was linearized by Sal I restriction enzyme for sperm/DNA complex. Size of the used DNA was 7.4 kb, which contained the gene encoding GFP and neo^r regulated by a separate promoter.

ICSI

The same volume of fresh boar semen used for Lipofectin or Trition X-100 was employed for ICSI. Oocytes were denuded from cumulus cell by repeated pipetting in 0.1% hyaluronidase. The oocytes with visible first polar body and excellent morphology were used for the experiment. A 60-mm sterile dish containing 5~7 μl injection medium (TL-HEPES-PVA: 10% polyvinyl pyrrolidone solution 1:1) overlaid with mineral oil was placed on Nikon inverted microscope equipped with Narishige micromanipulators. The injection needle which was made of glass pipette has an inner and outer diameter of 6~7 μm and 8~9 μm, respectively. The oocyte was captured by a holding pipette and immobilized with its polar body at 6 or 12 o'clock, and the site of injection was at 3 o'clock. Development of injected oocytes and expression of GFP during the experimental period was monitored by a fluorescent microscope (Olympus, Tokyo, Japan).

Embryo Transfer and Collection

Sexually mature gilt and sows were used as recipients. Estrus cycles were synchronized with altrenogest (Regumate, Hoechst Roussel Pharmaceuticals Inc., Somerville, NJ), whose active ingredient is altrenogest, a synthetic progesterone. The recipients were fed altrenogest (20 mg/day) from day 1 to 9 of estrus cycle (Day 0 being the day of onset of the estrus), and intramuscularly injected with 750 IU hCG 5 days after the final altrenogest treatment to induce ovulation. Two days after hCG administration, embryos were transferred into the isthmus of the recipient's oviduct. The surgical procedure for embryo transfer into recipient was performed as follows. Animal was premedicated with hypnodil and anesthesia was maintained with halothane (Sinsung Pharmacy Company, Korea). Genital tract was probed through ventral midline incision in the caudal abdominal region and embryos were transferred in the isthmus region of the oviduct using a fine

capillary tube with a small volume of NCSU23 medium including 0.4% BSA. On days 5, 7 or 15 after embryo transfer, recipients were sacrificed and embryos were flushed from uterine horns using PBS with 0.1% BSA. Development of transferred embryos and expression of GFP was monitored under a fluorescence microscope (Olympus, Tokyo, Japan).

Statical Analysis

The statistical significance among treatment groups in each experiment was determined by using General Linear Models Procedure and *t*-test of SAS.

RESULTS

In Vitro Development of Porcine Embryos after ICSI of Lipofectin/DNA Complex and Triton X-100 Treated Sperm

In vitro development of porcine embryos in a SMGT is shown in Table 1. After ICSI, the cleavage rates of ICSI only, Lipofectin and Triton X-100 group were 62.7, 59.4, and 62.1%, respectively. Developmental rates by *in vitro* of blastocysts were 18.8, 19.2 and 25.3%, respectively with no significant difference among the treatment groups. To evaluate the *in vitro* developmental ability of porcine oocytes and the influence of sperm microinjection, ICSI embryos were cultured for 6 days *in vitro*.

Analysis of GFP Expression Pattern in Porcine Embryos after ICSI

As shown in Table 2, after DNA bound sperm injection, the green fluorescent signal detected an embryo prevalence rate of 36.4% (34/96) and 40.6% (48/116) in Lipofectin and Triton X-100 groups, respectively. The fluorescent embryos at the blastocyst stage on day 7 after *in vitro* culture had detection rates of 9.4% and 4.3% using the Lipofectin and Triton X-100 DNA-sperm head binders, respectively. Among the injected embryos and whole embryos, the number of embryos expressing the GFP gene from all blastomeres were significantly higher ($P<0.05$) in the Lipofectin group (4.2%) than in Triton X-100 group (0.9%). At day 7 after ICSI, control blastocysts did not expressed of GFP reaction (Fig. 1B), however the Lipofectin and Triton X-100 treated blastocysts manifested strong expression of GFP reaction(Fig. 1D and F).

Table 1. *In vitro* development of porcine embryos after ICSI the DNA binding sperm by Lipofectin/DNA complex or Triton X-100

Treatment	No. of ICSI	No. (%) of ≥ 2 cell embryos	No. (%) of blastocyst
ICSI only	75	47(62.7)	19(25.3)
Lipofectin/DNA complex + ICSI	96	57(59.4)	18(18.8)
Triton X-100 + ICSI	116	72(62.1)	23(19.2)

Analysis of GFP Expression by Embryos Collected at Different Days after Transfering ICSI Embryos

To know the potential of transgenesis via SMGT, a total 295 ICSI embryos were transferred to surrogate dams that were fed with Regumate for 9 days to induce estrus or to maintain natural estrus. As shown in Table 3, 107 embryos were recovered from the uterus of three different surrogate gilts by surgical laparotomy on day 5, 7 and 15 after embryo transfer or *in vivo* development. The rates of GFP expression in respective groups were 26.9, 34.4 and 43.4%. On day 15, the elongated embryos collected from porcine uterine was cultured in DMEM medium supplemented with 20% FBS for 7 days.

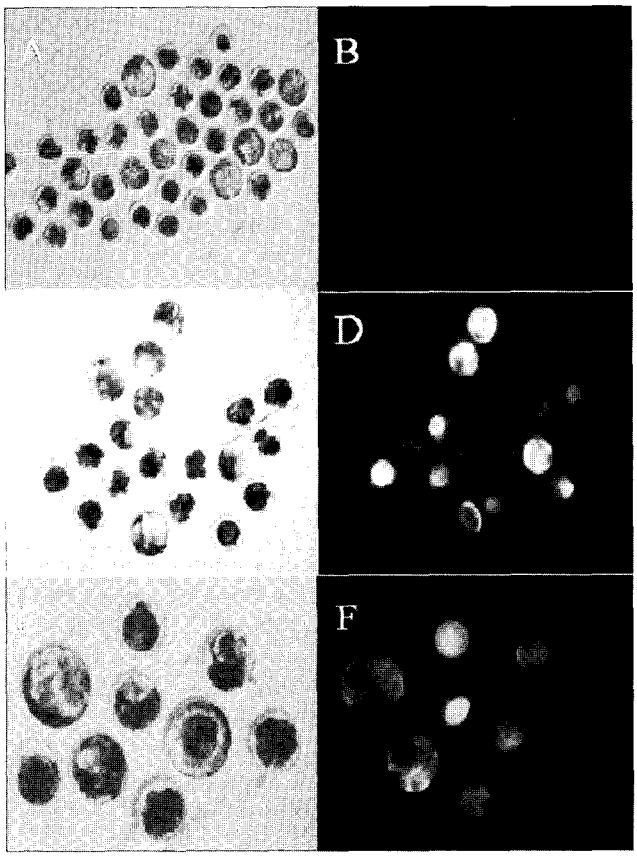

Fig. 1. GFP gene expression of *in vitro* developed embryos on day 7 after ICSI with GFP gene binding sperm. Light and green fluorescence image in porcine embryos derived from sperm treated with ICSI only (A, B), Triton X-100 (C, D) and Lipofectin/DNA complex (E, F) were observed on a fluorescence microscopy. Magnifications (A, B, C, D ×100 and E, F ×200).

Table 2. Analysis of GFP expression pattern in *in vitro* cultured porcine embryos after ICSI*

Treatment	No. of oocytes	Gene expression	No.(%) of fluorescence embryos			
			1-Cell	≥ 2-Cell	Blastocyst	Total
Lipofectin/ DNA complex	96	Whole	3(3.1)	4(4.2)	4(4.2)[a]	11(13.5)
		Mosaic	0(0.0)	17(17.7)	5(5.2)	22(22.9)
Triton X-100	116	Whole	7(6.0)	9(7.8)	1(0.9)[b]	18(14.7)
		Mosaic	5(4.3)	21(18.1)	4(3.4)	30(25.9)

* GFP gene expression at each developmental stage was observed on day 6 after ICSI. All experiments were replicated three and four times, respectively.

[a,b] Values with different superscripts are significantly different ($P<0.05$).

Table 3. Analysis of GFP gene expression at different days after transfer the embryos derived from sperm mediated gene transfer*

Parameter	The day of embryo collection after embryo transfer		
	Day 5	Day 7	Day 15
No. of oocytes transferred	129	80	86
No. of recipient	1	1	1
No. of embryos collected	52	32	23
Embryos developed (n) to	Degenerated(22) ≥ 2-Cell(24) Blastocyst(6)	Degenerated(18) ≥ 2-Cell(9) Blastocyst(5)	Elongated blstocysts(23)
No. (%) of GFP expression	14/52(26.9)	11/32(34.4)	10/23(43.4)

* Embryos were collected from gilts by uterine flushing on Day 5, 7 and 15 after embryo transfer. Development stage and gene expression of embryos were observed on each collected day.

DISCUSSION

Sperm mediated gene transfer has been studied in mice and rats (Lavitrano *et al.*, 1989; Huguet *et al.*, 1998; Chan *et al.*, 2000), but the efficacy of transgenesis achieved in these studies have been still argumentative. However, Perry and co-workers (Perry *et al.*, 1999) reported high efficiency of producing transgenic mice using ICSI procedures. Showing that membrane-disrupted mouse spermatozoa support the genomic integration and subsequent expression of exogenous DNA in developing embryos after ICSI. In the present study, technical disruption of spermatozoa membrane by chemicals and liposome complex, supported the genomic integration of sperm bound gene and subsequent expression of GFP in developing porcine embryos. As Bachiller *et al.* (1991) reported that liposomes could be used effectively for transfection of DNA into sperm head the possibility of sperm transfection with liposome. On the other hand, ICSI of Triton X-100 treated sperm has been reported that Triton X-100 treatment before ICSI in mice (Ahmadi *et al.*, 1997; Kasai *et al.*, 1999) was not affected to *in vitro* development and fertilization, sperm head condensation by ICSI with a membrane-damaged sperm was significantly improved in human (Ahmadi *et al.*, 1999). These results suggested that sperm membrane-damage would be effectively enhanced the success rate of ICSI in mice and human.

The results in porcine embryos that also membrane-damaged with Triton X-100 was no difference on *in vitro* development of porcine embryos, it is indicated that pig sperm are more similar to those of human's membrane properties (Lee *et al.*, 2004). *In vitro* development ability in blastocyst stage was investigated after ICSI with Triton X-100 treated or Lipofectin/DNA complex sperm for DNA binding. In the study, after ICSI of membrane-damaged sperm by Triton X-100, *in vitro* development rate of porcine embryos was not different from liposome and ICSI only groups (see Table 1).

We observed that the optimal concentration of DNA that can be transferred via sperm was 10 ng/μl for high

transgene expression without activation of oocytes, we used 10 ng/μl of DNA concentration in our subsequent experiments (data not shown).

In the case of Lipofectin/DNA complex, transfection of sperm head with DNA could be completed at 2 hr after mixing DNA and liposome as reported by Francolini *et al.*, (1993) who tested the transfection time by time lapse from 2 to 6 hr with intervals of 2 hr. In the present study, however, we exposed sperm to DNA for 6 hr to compensate incubation temperature (17°C) of sperm (Maione *et al.*, 1998).

The GFP expression patterns of Lipofectin/DNA complex and Triton X-100 groups was studied and presented in Table 2. Recent report (Perry *et al.*, 1999) demonstrated that co-injection of mouse oocytes with sperm heads and exogenous DNA produced high percent transgene expressing embryos and generated about 20% offspring expressing the integrated transgene. The full expression, as evidenced by healthy blastocysts was regarded as marker of stable integration of exogenous DNA while the mosaic expression in cleaved embryos or blastocysts was regarded as indicator of incomplete integration of transferred DNA. The whole expression of GFP in the blastocyst form, in Lipofectin/DNA complex treated group was higher ($P<0.05$) than that in Triton X-100 group. These results showing the abnormal development and high frequency of mosaicisms in these sperm injected oocytes and sperm binding DNA injection to cytoplasm can only be an efficient method in transgenic pig production it the process of DNA integration does not inhibit the development of embryos. The transfer of porcine *in vitro* produced embryos introducing GFP gene into the porcine oviduct may provide a relatively new model for the development of porcine embryos. In the present study, we collected 23 elongated embryos after transfer of embryos derived from ICSI with GFP binding sperm and ten of 23 embryos showed GFP gene expression paternally. When the collected embryos were cultured *in vitro* for seven days, green florescence image did not disappear. This indicated that the DNA imported by sperm-mediated gene transfer is normally expressed without gene silence in the cell (see Table 3).

In conclusion, membrane manipulation by sperm injection with Triton-X 100 or liposome pretreatment binding DNA to the porcine sperm head did not affect *in vitro* developing oocytes. We suggest that ICSI embryos harboring GFP gene could be developed with no silences of the foreign gene for long term until just before implantation. With these results, the ICSI using sperm manipulation techniques could be used as a simple and reliable procedure to produce transgenic pig.

REFERENCES

1. Abeydeera LR, Wang WH, Cantley TC, Rieke A, Murphy CN, Prather RS, Day BN (2000): Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. Theriogenology 54:787-797.
2. Ahmadi A, Ng SC (1997): Fertilization and development of mouse oocytes injected with membrane-damaged spermatozoa. Hum Reprod 12:2797-2801.
3. Ahmadi A, Ng SC (1999): Influence of sperm plasma membrane destruction on human head decondensation and pronuclear formation. Arch Androl 42:1-7.
4. Bachiller D, Schellander K, Peli J, Ruther U (1991): Liposome-mediated DNA uptake by sperm cells. Mol Reprod Dev 30:194-200.
5. Chan AW, Luetjens CM, Dominko T, Ramalho-Santos J, Simerly CR, Hewitson L, Schatten G (2000): Foreign DNA transmission by ICSI: injection of spermatozoa bound with exogenous DNA results in embryonic GFP expression and live rhesus monkey births. Mol Hum Reprod 6:26-33.
6. Francolini M, Lavitrano M, Lamia CL, French D, Frati L, Cotelli F, Spadafora C (1993): Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. Mol Reprod Dev 34:133-139.
7. Gordon JW, Ruddle FH (1981): Integration and stable germ line transmission of genes injected into mouse pronuclei. Science 214:1244-1246.
8. Huguet E, Esponda P (1998): Foreign DNA introduced into the vas deferens is gained by mammalian spermatozoa. Mol Reprod Dev 51:42-52.
9. Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M (1995): Green fluroescent protein as a marker in transgenic mice. Develop Growth Differ 37:455-459.
10. Iritani A (1991): Micromanipulation of gametes for *in vitro* assisted fertilization. Mol Repro Dev 28:199-207.
11. Kasai T, Hoshi K, Yanagimachi R (1999): Effect of sperm immobilizationand demembranation on the oocyte activation rate in the mouse. Zygote 7:187-193.
12. Lai L, Sun Q, Wu G, Murphy CN, Kuhholzer B, Park KW, Bonk AJ, Day BN, Prather RS (2001): Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into *in vitro* matured oocytes. Zygote 9:339-346.
13. Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C (1989): Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell 57:717-723.
14. Lavitrano M, Forni M, Bacci ML, Stefano CD, Varzi V, Wang H, Seren E (2003): Sperm mediated gene transfer in pig: Selection of donor boars and optimization of DNA uptake. Mol Reprod Dev 64:284-291.
15. Lee JW, Kim NH, Lee HT, Chung KS (1998): Microtuble and chromatin organization during the first cell cycle following intracytoplasmic injection of round sper-

matid into porcine oocytes. Mol Reprod Dev 50:221-228.
16. Lee JW, Yang X (2004): Factors affecting fertilization of porcine oocytes following intracytoplasmic injection of sperm. Mol Reprod Dev 68:96-102.
17. Lu JK, Fu BH, Wu JL, Chen TT (2002): Production of transgenic silver sea bream (*Sparus sarba*) by different gene transfer methods. Mar Biotechnol (NY) 4:328-337.
18. Maione B, Lavitrano M, Spadafora C, Kiessling AA (1998): Sperm-mediated gene transfer in mice. Mol Reprod Dev 50:406-409.
19. Miyoshi K, Taguchi Y, Sendai Y, Hoshi H, Sato E (2000): Establishment of a porcine cell line from *in vitro*- produced blastocysts and transfer of the cells into enucleated oocytes. Biol Reprod 62:1640-1646.
20. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997): 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407:313-319.
21. Palermo G, Joris H, Derde MP, Camus M, Devroey P, Van Steirteghem A (1993): Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. Fertil Steril 59:826-835.
22. Palermo G, Joris H, Devroey P, Van Steirteghem AC (1992): Induction of acrosome reaction in human spermatozoa used for subzonal insemination. Hum Reprod 7:248-254.
23. Perry AC, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y, Yanagimachi R (1999): Mammalian transgenesis by intracytoplasmic sperm injection. Science 284:1180-1183.
24. Szczygiel MA, Ward WS (2002): Combination of dithiothreitol and detergent treatment of spermatozoa causes paternal chromosomal damage. Biol Reprod 67: 1532-1537.

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