Electrical Activation Enhances Pre-Implantation Embryo Development Following Sperm Injection into *In Vitro* Matured Pig Oocytes

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ABSTRACT. The objective of this study was to evaluate the effect of electrical stimulation (EST) on pronuclear formation, chromosomal constitution, and developmental capability among *in vitro* matured pig oocytes following intracytoplasmic sperm injection (ICSI). After ICSI, the oocytes were randomly distributed and cultured into 3 groups: the EST activated ICSI group, non-activation ICSI group, and *in vitro* fertilization (IVF) group. The proportion of oocytes in which 2 pronuclei were formed in ICSI groups was significantly higher in the former groups than in the IVF group (96.2 and 93.5 vs. 64.5%, respectively, $P<0.05$). The cleavage rate was significantly higher in EST activated ICSI group (78.6%) than in the IVF and non-activated ICSI groups (51.8 and 46.0%, respectively, $P<0.05$), as was the proportion of oocytes that developed to the blastocyst stage at day 7 (18.9 vs. 11.6 and 9.1%, respectively, $P<0.05$). Diploid blastocysts were observed in 52.4, 63.0, and 65.2% of oocytes in the IVF, activated, and non-activated ICSI groups, respectively. Eight out of 23 gilts (34.8%) were confirmed to be pregnant in activated ICSI groups, but none of these pregnancies were carried to term. These results show that oocyte activation after ICSI is effective in elevating the cleavage rate and blastocyst development, while ensuring normal chromosome composition. Further research is needed to determine the pregnancy maintenance requirements for ICSI-embryos in pigs.
KEY WORDS: activation, ICSI, IVM, oocyte, swine.

Intracytoplasmic sperm injection (ICSI) is a valuable assisted reproduction technology for studying fertilization, treating human infertility, and producing transgenic animals. In humans, ICSI has become a widely applied means of overcoming infertility. In addition to its clinical usefulness, ICSI can be a valuable research tool for studying the fundamental aspects of interaction between 2 gametes during fertilization. Since the first report of the use of ICSI in mammals [38], several successful attempts to produce live offspring with ICSI using both *in vivo* matured [27, 37] and *in vitro* matured [2] oocytes have been reported. In pigs, successful production of offspring after ICSI has been reported mostly with *in vivo* matured oocytes [12, 21]. The use of ICSI-fertilized porcine *in vitro* matured oocytes resulted in the death of a piglet immediately after birth [17]. Recently, Nakai *et al.* [24] reported 3 healthy piglets produced by ICSI for the first time using *in vitro* matured porcine oocytes. In several species such as rabbits [19], hamsters [5], and mice [15, 26], injection

of the sperm itself is apparently sufficient to activate the oocyte, as the sperm nucleus can undergo decondensation and form a pronucleus (PN).

Compared with other species, the ICSI procedure has not been well established in pigs, partially because of activation of *in vitro* matured oocytes after ICSI, which lowers the fertilization rate and results in abnormal development of ICSI-fertilized porcine and bovine oocytes [18]. Thus, it appears that sperm preparation and post-ICSI oocyte activation affect the fertilization and development of ICSI-derived embryos [3, 11, 35]. Spermatozoa derived from several mammalian species are also unable to activate oocytes, mainly due to lack of oocyte activation factors. Recent studies have shown that species differences exist in oocyte activation after ICSI. An additional activation procedure is required to allow oocyte fertilization following spermatozoa injection in cattle [35] and pigs [18], whereas injection alone is sufficient to activate oocytes in mice and humans [37]. Although mechanical stimulation by the injection pipette occasionally results in activation, further stimulation is needed for porcine oocytes. Activation of mammalian oocytes in several species has been attempted using various chemicals, as well as physical and enzymatic stimuli [7]. Similar attempts have been made to improve ICSI results in pigs and cattle [11, 35], and stimuli such as ethanol [13, 22, 23], calcium ionophore [4, 11, 40] and

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EST [1, 34, 40] have been found to activate oocytes. In pigs, the blastocyst formation rate of ICSI-fertilized oocytes is significantly enhanced by electrical activation [17, 18], and EST is a potent activator of porcine oocytes [31, 40, 41]. Since an electrical stimulus can increase the oocyte Ca^{2+} concentration, it has often been used to activate porcine oocytes after nuclear transfer [20, 25, 32] and ICSI [17], and in parthenogenetic oocytes [41]. However, little is known about the effect of EST on embryo development in pigs.

In this study, we investigated the effect of electrical stimulation (EST) after sperm injection into *in vitro* matured pig oocytes on early and later embryo development. The observations indicate that EST activation of porcine oocytes after ICSI plays a crucial role in the formation of male and female pronuclei and the subsequent cleavage and development of *in vitro* matured oocytes. In this study, we present evidence that the efficiency of porcine ICSI can be improved by oocyte activation by EST: we describe PN formation, cleavage rate, and the chromosomal constitution of embryos and their development *in vitro*.

MATERIALS AND METHODS

In vitro maturation of oocytes: Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 30°C in Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 5.54 mM D-glucose, 0.33 mM sodium pyruvate, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulphate (mDPBS). Cumulus-oocyte complexes (COCs) were aspirated with an 18-gauge needle into a disposable 10-ml syringe and follicles 3–6 mm in diameter were collected. The COCs were washed 3 times with TL-HEPES medium [30]. Collections of 10–20 COCs were matured in 100 μl of BSA-free NCSU 23 medium [29] supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 0.5 μg/ml FSH, 0.5 μg/ml LH, 1 μg/ml E$_2$, and 10 ng/ml EGF. After the first 20–22 hr of incubation, oocytes were cultured for a further 20–22 hr in the same maturation medium without hormone supplementation. After culture, expanded cumulus cells were removed in NCSU 23 medium containing 0.1% hyaluronidase by vortexing for 3 min.

Preparation of spermatozoa: Epididymal spermatozoa were obtained by castration of 3 boars of proven fertility. The spermatozoa were removed from the distal segment of the caudae epididymides by retrograde flushing from the ductus deferens. Spermatozoa were washed twice with TL-HEPES and then suspended in 10 ml of TL-HEPES. To induce capacitation, the sperm were resuspended in 10 ml of mTBM containing 2.5 mM caffeine in a 15-ml conical tube and kept at 39°C for 30–60 min [12].

In vitro fertilization: Twenty matured oocytes were cultured with spermatozoa at a final concentration of 5×10^5/ ml in 50 μl droplet of mTBM supplemented with 2.5 mM caffeine sodium benzoate and 4 mg/ml BSA for 6 hr at 39°C in an atmosphere of 5% CO_2 in air. The eggs were then transferred to 50 μl droplet of NCSU23 medium supplemented with 4 mg/ml BSA, and cultured for 8 days at

39°C in an atmosphere of 5% CO_2 in air.

Spermatozoa injection into oocytes: Spermatozoa were centrifuged at 400 g for 5 min and resuspended with TL-HEPES: 10% polyvinylpyrrolidone solution (1:2). Oocytes with visible polar body and excellent morphology were used for this experiment. Oocytes were centrifuged for 6 min at 12,000 g in 500 μl TL-HEPES medium in 1.2 ml Eppendorf centrifuge tube. The injection of spermatozoon into the oocyte cytoplasm was performed using the method of Kim *et al.* [8]. Briefly, the injection needle used was 6–7 μm inner and 8–9 μm outer diameters. The polar body was at 6 or 12 o'clock and the point of injection at 3 o'clock. An oocyte was penetrated by the injecting micropipette, small amount of cytoplasm was drawn into the micropipette, and then the cytoplasm together with the sperm cell and small amount of medium was expelled into the oocyte. After ooplasm injection, the injecting micropipette was withdrawn immediately, and the oocytes were released from the holding pipette to reduce the intracytoplasmic pressure exerted to the oocyte.

Activation and further development of the oocytes: The procedure for EST of porcine oocytes was conducted as described by Kim *et al.* [8]. EST to induce activation was performed with a BTX Electro Cell Manipulator (Biotechnologies and Experimental Research, Inc., San Diego, CA, U.S.A.) to a chamber with 2 parallel platinum wire electrodes (200 μm o.d.) spaced 1 mm apart overlaid. After 30 min sperm injection, oocytes were stimulated by a 10-sec pulse at 0.48 KV/cm AC followed by a 30 μsec pulse at 1.26 KV/cm DC at room temperature (25°C). After stimulation, all oocytes were washed with NCSU23 medium and transferred to a 50 μl NCSU23 medium supplemented with 4 mg/ml BSA, and cultured for 8 days at 39°C in an atmosphere of 5% CO_2 in air.

Assessment of pronuclear formation and cell number of blastocysts: Pronuclear formation was classified 18 hr after start of IVF, sperm injection, and EST, respectively. Embryos were mounted on slide and fixed in 25% (v:v) acetic alcohol for 48 to 72 hr at room temperature, then stained with 1% (w:v) orcein in 45% (v:v) acetic acid. The pronuclear stages of eggs were examined under a phase-contrast microscope at a magnification of ×400. After 168 hr of *in vitro* embryo culture, blastocysts were fixed at room temperature for 1 hr with 4% paraformaldehyde and stained with 0.05 mM *bis*benzimide (Hoechst 33342). The cell numbers of blastocysts were examined at ×200 and ×400 magnification by epifluorescence microscope (Olympus, Tokyo, Japan).

Chromosomal analysis: At 144 hr after injection, blastocysts were cultured in culture medium in the presence of colcemid (0.05 μg/ml) for 5 hr. The blastocysts were then placed in a hypotonic solution of sodium citrate (1%) for 5 min, and fixed individually on microscope slides by the air-drying method [36]. Blastocysts were stained with 10% Giemsa at pH 6.8. The chromosome constituent of each spread was determined at ×1,000 magnifications under oil.

Transfer of zygotes and embryos produced by ICSI: Induction of estrus was accomplished by i.m. injection of

Table 1. Pronuclear formation of porcine oocytes after IVF and ICSI with and without activation

Treatment	No. of oocytes	Pronuclear formation (%)*			
		1PN	2PN	1PN+sPN	Other
Control (IVM-F-C)	62	12 (19.4)	40 (64.5)[b]	4 (6.5)	6 (9.7)
ICSI (activation**)	52	0	50 (96.2)[a]	0	2 (3.8)
ICSI (non-activation)	46	0	43 (93.5)[a]	3 (6.5)	0

a), b) Within column, percentages with different superscripts differ significantly, $P<0.05$. *2PNC, male and female pronuclear chromosomal structure (PNC); 1PN+sPN, condensed or swelling male chromatin; other, plate and unidentified multiple pronuclei. **Activation; 30 min after ICSI, 85 volts, 30 μsec, 1 pulse.

Table 2. *In vitro* development of porcine zygotes cultured for 7 days after IVF and ICSI

Treatment	No. of oocytes used	No. (%) of embryos cleaved	No. (%) of embryos developed to blastocysts
Control (IVM-F-C)	731	379 (51.8)[b]	85 (11.6)[b]
ICSI (activation**)	355	279 (78.6)[a]	67 (18.9)[a]
ICSI (non-activation)	496	228 (46.0)[b]	45 (9.1)[b]

*Percentages with different superscripts within a column differ significantly, $P<0.05$. **Activation; 30 min after ICSI, 85 volts, 30 msec, 1 pulse.

Table 3. Chromosomal analysis of porcine embryos produced by IVF or ICSI

Treatment	No. of blastocysts identified	No. (%) of Ploidy		
		Haploid	Diploid	Other*
Control (IVM-F-C)	21	0	11 (52.4)	10 (47.6)
ICSI (activation**)	27	0	17 (63.0)	10 (37.0)
ICSI (non-activation)	23	0	15 (65.2)	8 (34.8)

*Other includes embryos with unidentified and mixed multiple chromosomes. **Activation; 30 min after ICSI, 85 volts, 30 msec, 1 pulse.

1,000 IU of equine chorionic gonadotropin (eCG; Intergonan, Intervet, Boxmeer, Netherlands) followed by 500 IU of human chorionic gonadotropin (hCG; Ovogest, Intervet) 72 hr later. Sperm-injected eggs were transferred to the oviducts. Fifty hours after the hCG injection as described by Wallenhorst and Holts [39]. Thirty eight recipients received 3,166 embryos of 1 to 4-cell-stage and pregnancy was determined on 25 and 50 days after embryo transfer by ultrasonography. In detail, 1,252 embryos of non-activated ICSI group were transferred into 15 surrogated mothers and 1,914 embryos of activated ICSI group were transferred into 23 surrogated mothers.

Statistical analysis: All percentage data were transformed to the arc-sine of their square roots. The one-way ANOVA test was used for the comparison of group mean difference. Unless otherwise stated, statistical significance was determined at $P<0.05$.

RESULTS

Pronuclear formation among in vitro fertilized and ICSI-fertilized oocytes: In Table 1, we compare pronuclear formation among 3 different groups (*in vitro* fertilization control, ICSI followed by activation, and ICSI without activation). The proportion of oocytes with 2 pronuclear formations was significantly higher in the ICSI groups, irrespec-

tive of EST, than in the IVF group (Table 1, 96.2 and 93.5 vs. 64.5%, respectively, $P<0.05$). Some of the PN found among IVF oocytes possessed only a female PN (19.4%).

Development of in vitro fertilized and ICSI-fertilized oocytes: Following injection of the spermatozoa, the eggs were allocated to the EST activation or non-activation groups, as described in Materials and Methods, and their cleavage and developmental rates were compared with those in the IVF group (n=7, Table 2). The EST activation group had a significantly higher cleavage rate (78.6%) than the IVF and non-activation ICSI groups (51.8 and 46.0, respectively, $P<0.05$). Development to the blastocyst stage at 7 days was also significantly higher in the activation group than in the IVF and non-activation groups (18.9 vs. 11.6 and 9.1%, respectively, $P<0.05$).

Chromosomal analysis of embryos: Ploidy analysis of blastocysts was performed to examine the incidence of diploidy in each of the treatment groups. Among the IVF, activation, and non-activation groups, 52.4, 63.0, and 65.2% of embryos, respectively, were diploid (Table 3).

Cell number of IVF and ICSI oocytes: The average number of blastocyst nuclei at day 8 was 46.7 ± 2.9, 44.7 ± 4.2, and 41.9 ± 4.6 in the IVF, activation, and non-activation groups, respectively (Table 4).

Transfers of zygotes and embryos produced by ICSI: Thirty-eight gilts received 40–185 ICSI embryos, and preg-

nancy was determined by ultrasonography at days 25 and 50 of gestation. Eight out of twenty-three gilts (34.8%) were confirmed to be pregnant in activated ICSI groups and one out of fifteen gilts (6.7%) was pregnant in non-activated ICSI group. Butnone of these pregnancies were carried to term (Fig. 1).

DISCUSSION

In the present study, an EST procedure was added after ICSI in pigs. EST is a well known and potent activator of porcine oocytes in somatic cell nuclear transfer and ICSI [6, 14, 33, 40]. After electrical stimulation, cytoplasmic centrosomal material is activated and organizes a network with microtubules that move pronuclei to the center of the oocytes [9]. We found that 2 pronuclei were formed in similar proportions in ICSI-fertilized oocytes, irrespective of whether EST was applied or not. However, EST significantly increased the rates of oocyte cleavage and blastocyst development compared with oocytes that were not activated by EST.

Induction of oocyte activation by injection of intact or isolated spermatozoa suggests that an oocyte activation factor may be present in the plasma membrane of the porcine sperm head [9]. In the pig, as in mice, oocyte-activating factors appear to be assembled during spermatogenesis. Recently, Parrington *et al.* [28] reported that the sperm-borne oocyte-activating factor is a 33-KDa protein, oscillin, residing in the equatorial segment of the acrosomal region. It has been suggested that an additional activation procedure is not required for normal ICSI fertilization. In mice, isolated spermatazoa heads, which lose all of their components except for the nucleus and the perinuclear materials, can activate oocytes as efficiently as intact spermatozoa when injected [15]. Kim *et al.* [9] demonstrated that both a spermatozoon and an isolated sperm head could activate oocytes without additional activation when injected into the cytoplasm in pigs. However, the present study demonstrated that sperm injection procedure itself without oocyte activation treatment is not sufficient to activate porcine oocytes. In this study, shame injection (three replicates) also has been conducted (data not shown in table). Forty out of eighty-five (47.1%) oocytes were cleaved and 1 out of 85 oocytes (2.5%) was developed to blastocyst stage. It seems that embryo development of ICSI without activation has been affected partially by physical injection procedure. EST followed by ICSI showed a significantly higher cleavage rate than the ICSI group that was not subjected to EST. We also observed that pronuclear formation was higher in post-ICSI oocytes compared with those fertilized by IVF. However, in our study, similar observations were reported by Lee *et al.* [18] that the cleavage failure rate was higher in ICSI alone group compared to ICSI + EST activation or EST activation + ICSI at 168 hr after ICSI. When ICSI was combined with EST, a significantly higher blastocyst development rate was seen in activated COCs compared with ICSI alone group. EST after injection of a spermatozoon enhanced the incidence of fertilization of *in vitro* matured

Table 4.   Number of blastomeres of *in vitro*-developed porcine blastocysts derived from IVF or ICSI

Treatment	No. of blastocysts*	No. of blastomeres	
		Mean ± SE	Ranges
Control (IVM-F-C)	21	46.7 ± 2.9	22–68
ICSI (activation)	10	44.7 ± 4.2	20–64
ICSI (non-activation)	10	41.9 ± 4.6	27–52

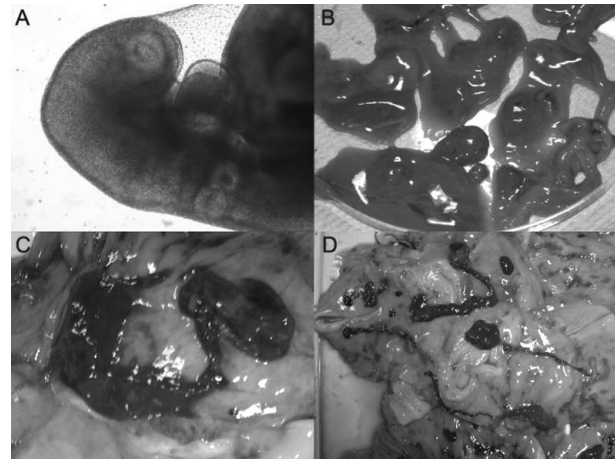*Examined at 168 hr post-injection or insemination.

Fig. 1.   Representative photographs of live and mummified porcine fetuses. A) Live fetus at 18 days. B), C), and D) Mummified fetuses at 54, 96, and 111 days of gestation.

porcine oocytes, suggesting that the timing of sperm injection and oocyte activation are important for the normal development of pronuclei and for syngamy [10, 18]. Thus, an additional electrical activation procedure after ICSI should enhance fertilization and subsequent embryonic development in porcine oocytes.

Chromosomal analysis of blastocysts showed a similar incidence of diploidy in the IVF control and ICSI groups, irrespective of whether EST was used or not. The proportion of oocytes with multiple chromosomal abnormalities was also similar among all 3 groups. It is possible that the occurrence of chromosomal abnormalities might be due to inhibition of extrusion of the second polar body as some nuclei presumably re-enter the S-phase of the cell cycle without having passed through metaphase.

The birth of live piglets following ICSI has been reported by 2 groups [12, 15, 16, 21]. These groups have succeeded in producing piglets from laparotomized or aspirated oocytes *in vivo*. In this study, activated ICSI embryos transferred gilts had higher pregnancy rate than non-activated ICSI embryos transferred gilts at days 25 and 50 of gestation. It suggested that activated ICSI group had higher *in vivo* embryo development than non-activated ICSI group in early implantation period like *in vitro* embryo development comparison. However, none of the pregnancies proceeded to term, suggesting that the viability of blastocysts

produced by ICSI is limited.

   This study showed that the activation of oocytes with EST after ICSI is effective in increasing the cleavage rate and subsequent blastocyst development, while maintaining a normal chromosome composition *in vitro*. Further studies regarding factors involving the development of embryos after ICSI and after implantation are needed to determine how to maintain pregnancies induced by ICSI-fertilized *in vitro* matured porcine oocytes.

REFERENCES

1. Campbell, K. H., Ritchie, W. A. and Wilmut, I. 1993. Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. *Biol. Reprod.* **49**: 933–942. [Medline] [CrossRef]

2. Catt, S. L., Catt, J. W., Gomez, M. C., Maxwell, W. M. and Evans, G. 1996. Birth of a male lamb derived from an in vitro matured oocyte fertilised by intracytoplasmic injection of a single presumptive male sperm. *Vet. Rec.* **139**: 494–495. [Medline] [CrossRef]

3. Devito, L. G., Fernandes, C. B., Blanco, I. D., Tsuribe, P. M. and Landim-Alvarenga, F. C. 2010. Use of a Piezo drill for intracytoplasmic sperm injection into cattle oocytes activated with ionomycin associated with roscovitine. *Reprod. Domest. Anim.* **45**: 654–658. [Medline]

4. Funahashi, H., Cantley, T. C., Stumpf, T. T., Terlouw, S. L. and Day, B. N. 1994. In vitro development of in vitro-matured porcine oocytes following chemical activation or in vitro fertilization. *Biol. Reprod.* **50**: 1072–1077. [Medline] [CrossRef]

5. Haigo, K., Yamauchi, Y., Yazama, F., Yanagimachi, R. and Horiuchi, T. 2004. Full-term development of hamster embryos produced by injection of round spermatids into oocytes. *Biol. Reprod.* **71**: 194–198. [Medline] [CrossRef]

6. Jolliff, W. J. and Prather, R. S. 1997. Parthenogenic development of in vitro-matured, in vivo-cultured porcine oocytes beyond blastocyst. *Biol. Reprod.* **56**: 544–548. [Medline] [CrossRef]

7. Kaufman, M. H. 1983. Methodology: in vitro and in vivo techniques. pp. 20–26. *In*: Early Mammalian Development: Parthenogenetic Studies (Kaufman M. H. ed.), Cambridge University Press, Cambridge.

8. Kim, N. H., Simerly, C., Funahashi, H., Schatten, G. and Day, B. N. 1996. Microtubule organization in porcine oocytes during fertilization and parthenogenesis. *Biol. Reprod.* **54**: 1397–1404. [Medline] [CrossRef]

9. Kim, N. H., Lee, J. W., Jun, S. H., Lee, H. T. and Chung, K. S. 1998. Fertilization of porcine oocytes following intracytoplasmic spermatozoon or isolated sperm head injection. *Mol. Reprod. Dev.* **51**: 436–444. [Medline] [CrossRef]

10. Kim, N. H., Shin, J. S., Kim, C., Jun, S. H., Lee, H. T. and Chung, K. S. 1999. Fertilization and in vitro development of porcine oocytes following intracytoplasmic injection of round spermatid or round spermatid nuclei. *Theriogenology* **51**: 1441–1449. [Medline] [CrossRef]

11. Kolbe, T. and Holtz, W. 1999. Intracytoplasmic injection (ICSI) of in vivo or in vitro matured oocytes with fresh ejaculated or frozen-thawed epididymal spermatozoa and additional calcium-ionophore activation in the pig. *Theriogenology* **52**: 671–682. [Medline] [CrossRef]

12. Kolbe, T. and Holtz, W. 2000. Birth of a piglet derived from an oocyte fertilized by intracytoplasmic sperm injection (ICSI). *Anim. Reprod. Sci.* **64**: 97–101. [Medline] [CrossRef]

13. Kubiak, J. Z. 1989. Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev. Biol.* **136**: 537–545. [Medline] [CrossRef]

14. Kure-bayashi, S., Miyake, M., Okada, K. and Kato, S. 2000. Successful implantation of in vitro-matured, electro-activated oocytes in the pig. *Theriogenology* **53**: 1105–1119. [Medline] [CrossRef]

15. Kuretake, S., Kimura, Y., Hoshi, K. and Yanagimachi, R. 1996. Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol. Reprod.* **55**: 789–795. [Medline] [CrossRef]

16. Lacham-Kaplan, O. and Trounson, A. 1995. Intracytoplasmic sperm injection in mice: increased fertilization and development to term after induction of the acrosome reaction. *Hum. Reprod.* **10**: 2642–2649. [Medline]

17. Lai, L., Sun, Q., Wu, G., Murphy, C. N., Kuhholzer, B., Park, K. W., Bonk, A. J., Day, B. B. and Prather, R. S. 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. [Medline] [CrossRef]

18. Lee, J. W., Tian, X. C. and Yang, X. 2003. Failure of male pronucleus formation is the major cause of lack of fertilization and embryo development in pig oocytes subjected to intracytoplasmic sperm injection. *Biol. Reprod.* **68**: 1341–1347. [Medline] [CrossRef]

19. Li, G. P., Chen, D. Y., Lian, L., Sun, Q. Y., Wang, M. K., Liu, J. L., Li, J. S. and Han, Z. M. 2001. Viable rabbits derived from reconstructed oocytes by germinal vesicle transfer after intracytoplasmic sperm injection (ICSI). *Mol. Reprod. Dev.* **58**: 180–185. [Medline] [CrossRef]

20. Macháty, Z. and Prather, R. S. 1998. Strategies for activating nuclear transfer oocytes. *Reprod. Fertil. Dev.* **10**: 599–613. [Medline] [CrossRef]

21. Martin, M. J. 2000. Development of in vivo-matured porcine oocytes following intracytoplasmic sperm injection. *Biol. Reprod.* **63**: 109–112. [Medline] [CrossRef]

22. Minamihashi, A., Watson, A. J., Watson, P. H., Church, R. B. and Schultz, G. A. 1993. Bovine parthenogenetic blastocysts following in vitro maturation and oocyte activation with ethanol. *Theriogenology* **40**: 63–76. [Medline] [CrossRef]

23. Nagai, T. 1987. Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. *Gamete Res.* **16**: 243–249. [Medline] [CrossRef]

24. Nakai, M., Kashiwazaski, N., Takizawa, A., Hayashi, Y., Nakatsukasa, E., Fuchimoto, D., Noguchi, J., Kaneko, H., Shino, M. and Kikuchi, K. 2003. Viable piglets generated from porcine oocytes matured in vitro and fertilized by intracytoplasmic sperm head injection. *Biol. Reprod.* **68**: 1003–1008. [Medline] [CrossRef]

25. Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H. and Perry, A. C. 2000. Pig cloning by microinjection of fetal fibroblast nuclei. *Science* **289**: 1188–1190. [Medline] [CrossRef]

26. Ono, T., Mizutani, E., Li, C., Yamagata, K. and Wakayama, T. 2011. Offspring from intracytoplasmic sperm injection of

aged mouse oocytes treated with caffeine or MG132. *Genesis*
49: 460–471. [Medline] [CrossRef]

27. Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A. C.
1992. Pregnancies after intracytoplasmic injection of single
spermatozoon into an oocyte. *Lancet* **340**: 17–18. [Medline]
[CrossRef]

28. Parrington, J., Swann, K., Shevchenko, V. I., Sesay, A. K. and
Lai, F. A. 1996. Calcium oscillations in mammalian eggs trig-
gered by a soluble sperm protein. *Nature* **379**: 364–368. [Med-
line] [CrossRef]

29. Petters, R. M. and Wells, K. D. 1993. Culture of pig embryos.
J. Reprod. Fertil. Suppl. **48**: 61–73. [Medline]

30. Prather, R. S., Boice, M. L., Gibson, J., Hoffman, K. E. and
Parry, T. W. 1995. In vitro development of embryos from Sin-
clair miniature pigs: a preliminary report. *Theriogenology* **43**:
1001–1007. [Medline] [CrossRef]

31. Prather, R. S., Eichen, P. A., Nicks, D. K. and Peters, M. S.
1991. Artificial activation of porcine oocytes matured in vitro.
Mol. Reprod. Dev. **28**: 405–409. [Medline] [CrossRef]

32. Prather, R. S., Tao, T. and Machaty, Z. 1999. Development of
the techniques for nuclear transfer in pigs. *Theriogenology* **51**:
487–498. [Medline] [CrossRef]

33. Prather, R. S., Sims, M. M. and First, N. L. 1989. Nuclear
transplantation in early pig embryos. *Biol. Reprod.* **41**: 414–
418. [Medline] [CrossRef]

34. Procházka, R., Kanka, J., Sutovsky, P., Fulka, J. and Motlik, J.
1992. Development of pronuclei in pig oocytes activated by a
single electric pulse. *J. Reprod. Fertil.* **96**: 725–734. [Medline]

[CrossRef]

35. Rho, G. J., Kawarsky, S., Johnson, W. H., Kochhar, K. and Bet-
teridge, K. J. 1998. Sperm and oocyte treatments to improve
the formation of male and female pronuclei and subsequent
development following intracytoplasmic sperm injection into
bovine oocytes. *Biol. Reprod.* **59**: 918–924. [Medline] [Cross-
Ref]

36. Tarkowski, A. K. 1966. An air-drying method for chromo-
some preparation from mouse eggs. *Cytogenesis* **5**: 394–400.
[CrossRef]

37. Tesarik, J. 1996. Fertilization of oocytes by injecting sperma-
tozoa, spermatids and spermatocytes. *Rev. Reprod.* **1**: 149–
152. [Medline] [CrossRef]

38. Uehara, T. and Yanagimachi, R. 1976. Microsurgical injection
of spermatozoa into hamster eggs with subsequent transfor-
mation of sperm nuclei into male pronuclei. *Biol. Reprod.* **15**:
467–470. [Medline] [CrossRef]

39. Wallenhorst, S. and Holtz, W. 1999. Transfer of pig embryos to
different uterine sites. *J. Anim. Sci.* **77**: 2327–2329. [Medline]

40. Wang, W. H., Abeydeera, L. R., Prather, R. S. and Day, B.
N. 1998. Functional analysis of activation of porcine oocytes
by spermatozoa, calcium ionophore, and electrical pulse. *Mol.
Reprod. Dev.* **51**: 346–353. [Medline] [CrossRef]

41. Zhu, J., Telfer, E. E., Fletcher, J., Springbett, A., Dobrinsky,
J. R., De Sousa, P. A. and Wilmut, I. 2002. Improvement of
an electrical activation protocol for porcine oocytes. *Biol. Re-
prod.* **66**: 635–641. [Medline] [CrossRef]