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In Vitro Development of Porcine Oocytes Following Intracytoplasmic Injection of Round Spermatid

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원형정자 미세주입에 의한 돼지 난자의 체외 배 발달

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요 약

본 연구에서는 돼지난자내 원형정자 주입후 수정율과 체외 배 발달을 조사하였다. 원형정자 주입 2시간전에 인위적 전기자극을 주고 원형정자를 주입했을 때 난자들의 수정율이 주입 직후에 전기자극을 준 것들과 전기 자극을 주지 않은 것들보다 수정율이 높았다. 원형정자와 원형 정자핵을 각각 주입 한 후 전핵 형성율과 전핵 이동율을 조사하였으나 유의차를 발견할 수 없었다. 전핵의 형성과 이동중 미세소관의 움직임을 간접형광면역법 및 공촛점 현미경을 사용하여 조사해본 결과, 난활성 직후 난자의 표층에서 미세소관이 발생해서 이것에 의해 웅성 및 자성전핵이 난자 중심부로 이전됨을 볼 수 있었다. 원형정자와 원형정자핵 주입후 배양 6일째에 각각 25%와 27%의 배반포로 형성되었고, 8일째 형성된 배반포를 염색하여 관찰한 결과 세포수가 각각 평균 87에서 99개가 형성되었음을 알 수 있었다. 이러한 결과는 체외 성숙된 돼지 난자 내에 원형정자 혹은 원형정자 핵을 미세 주입하는 방법에의해 정상적인 배발달을 하는 돼지 수정란 생산이 가능한 것을 보여주는 것이다.

(Key words : Porcine, Oocyte, IVD, Round spermatid, Intracytoplasmic injection)

I. INTRODUCTION

Assessment of normal fertilization and offspring production by injecting round spermatids could offer exciting opportunities not only for studying gamete physiology during fertilization, but also for the clinical treatment of male infertility due to defective spermiogenesis. Unlike spermatozoa, spermatids are unable to activate oocytes, mainly due to lack of the oocyte activation factors. Therefore additional activation procedure is required to allow fertilization following spermatid injection in the mouse (Kimura et al., 1995), rabbit (Sofikitis et al., 1996) and pig (Lee et al., 1998).

Kimura and Yanagimachi (1995) reported that injection of 'undenuded' mouse spermatid resulted in 14 % fertilization with a single electrical stimulation. In contrast, injection of spermatid nucleus into oocytes that have been parthenogenetically activated 1 h before injection resulted in much higher rate of fertilization. This suggests that experimental conditions employed affect fertilization rates following round spermatid or round spermatid nucleus injection. Despite importance of various animal models for the round spermatid injection in the human clinic, little information is available on this subject for any species other than the mouse.

Concerns have been raised on the significance of paternal original centrosome during spermatid fertilization. Because the distal centriole gives rise to the axoneme during spermiogenesis (Holstein and Roosen-Runge, 1981; Russel et al., 1990), spermatids or earlier male germ cells may not provide a complete centrosome. However, success in achieving human, hamster and rabbit fertilization using intact spermatid or denuded round spermatids (Sofikitis et al., 1996) indicated that mammalian oocytes may not need the centrosome of mature spermatozoa for the successful fertilization. In the present study we determined microtubule organization following spermatid injection, and compared fertilization rates and developmental ability following injection of round spermatid and round spermatid nuclei with different activation times.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* maturation

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25℃ in Dulbecco's phosphate buffered saline supplemented with 5.54 mM D-glucose, 0.33 mM sodium pyruvate, 75 mg/ml potassium penicillin G and 50 mg/ml streptomycin sulphate (mDPBS). Cumulus-Oocyte complexes (COC) were aspirated with an 18-gauge needle into a disposable 10-ml syringe from follicles 3 to 6 mm in diameter. The COC were washed 3 times with TL-HEPES (Prather et al., 1995) medium. Groups of 50 COC were matured in 500 μℓ of BSA-free NCSU 23 (Petters et al., 1993) medium supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 10 IU/ml PMSG (Sigma, St. Louis, MO, USA) and 10 IU/ml hCG (Sigma) under paraffin oil at 39℃ for 40 to 44 h.

2. Isolation of round spermatids

The isolation of spermatids was performed using the method of Lee et al. (1998). Briefly, tesicular tissue was washed with Hank's Balanced Salt Solution (HBSS, Gibco, Grand Island, NY, USA) supplemented with 12.5 mM HEPES (Sigma). After washing, testicular tissue was minced into small pieces with a pair of scissors and the cells in the seminiferous tubules were released into the medium by repeated pipettings. The cell suspension was filtered through a mesh (50 μm, Nylon mesh) to remove cell aggregates and tissue debris, treated for 1 min with 0.1~0.2 mg/ml Pronase (Sigma) in HBSS and centrifuged at 400×g for 5 min. This treatment eliminated most elongating (flagellum-generating) spermatids and mature spermatids from the cell population by agglutinating them in sticky masses. Round spermatids were identified by their size (Lee et al., 1998). The isolated cells could be stored for several hours at 4℃ without losing their viability.

3. Oocyte activation

The procedures for electrical stimulation of porcine oocytes were as described by Kim et al (1996b). Electrical stimulation to induce activation was delivered with a BTX Electro Cell Manipulator (Biotechnologies and Experimental Research, Inc., San Diego, CA) to a chamber with two parallel platinum wire electrodes (200 μm o.d.) spaced 1 mm apart overlaid. Cumulus cell denuded oocytes were stimulated by a 10 sec pulse at 0.48 KV/cm AC followed by a 30 ms pulse at 1.26 kV/cm DC at room temperature (25℃).

4. Round spermatid and round spermatid nuclear injection

Spermatogenic cell fractions were centrifuged (400g, 5 min) and resuspended in TL HEPES :

10% polyvinylpyrrolidone solution (1:1). A microdrop (5 μl) of this suspension was placed in a slide, and the slide was placed in Leitz Differential Interference Contrast inverted microscope equipped with Leitz micromanipulators. The oocytes were denuded cumulus cells by repeated pipetting. Oocytes with visible polar body and of excellent morphology were used for this experiment. Oocytes were centrifuged for 10 min in an Eppendorf centrifuge at 12,000 g in 50 ml TL-HEPES medium in 1.2 ml Eppendorf centrifuge tube. The injection of spermatids into the oocyte cytoplasm was performed using the method of Lee et al. (Lee et al., 1998). Briefly, the injection needle used was of 6~7 μm inner and 8~9 μm outer diameter. 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, a small amount of cytoplasm was drawn into the micropipette, and then the cytoplasm together with the spermatid and a small amount of medium was expelled into the oocyte. In a series of experiments of round spermatid nuclear injection, a spermatid was drawn in and out of an 4~5 μm inner diameter pipette until plasma membrane was ruptured. The spermatid nucleus without plasma membrane was then injected using same method mentioned above. Immediately after ooplasmic injection, the injecting micropipette was withdrawn quickly, and the oocytes were released from the holding pipette to reduce the intracytoplasmic pressure exerted to the oocyte. After injection, all of the oocytes were transferred to NCSU23 medium and cultured for at 39℃ under 5% CO_2 in air.

5. Assessment cell number of blastocysts

At specific time points, the eggs were fixed, stained with 0.05 mM Hoechst 33342 and examined nuclear morphology and numbers at 200× and 400× magnification with epifluorescence microscopy.

6. Microtubule detection

Microtubules and DNA were detected by indirect immunocytochemical techniques described by Kim et al. (1998a). Briefly, the oocytes were permeabilized in a modified Buffer M (Kim et al., 1996a) for 20 min, fixed in methanol at −20℃ for 10 min and stored in phosphate-buffered saline (PBS) containing 0.02% sodium azide and 0.1% bovine serum albumin for 2~7 days at 40℃. Microtubule localization was performed using anti-α-tubulin monoclonal antibody (Sigma). The sperm tails were detected by applying anti-acetylated α-tubulin antibody (Sigma). Fixed oocytes were incubated for 90 min at 39℃ with antibody diluted 1:300 in PBS. After several washes with PBS containing 0.5% Triton-X 100 and 0.5% BSA, oocytes were incubated in a blocking solution (Simerly and Schatten, 1993) at 39℃ for 1 h. The blocking was followed by incubation in FITC labeled goat anti-mouse antibody (Sigma). DNA was fluorescently detected by exposure to 50 mg/ml propidium iodide (Sigma) for 1 h. Stained oocytes were then mounted under a coverslip with antifade mounting medium (Universal Mount, Fisher Scientic Co., Huntsville, AL, USA) to retard photobleaching. Slides were examined using laser-scanning confocal microscopy. Laser-scanning confocal microscopy was performed using a Bio-Rad MRC 1024 equipped with a Krypton-argon ion laser for the simultaneous excitation of fluorescein for microtubules and propidium iodide for DNA. The images were recorded digitally and archived on an erasable magnetic optical disk.

7. Statistical Analyses

The data were pooled from at least four rep-

lications. Differences in the percentages of oocytes developing to a particular stage were determined by Chi-square procedures.

Ⅲ. RESULTS

Activation was determined as either second polar body extrusion or pronuclear formation. The spermatid injection alone did not induce activation of oocytes as compared with sham injection (Table 1). The oocytes with two large pronuclei and two polar bodies (2PN+2PB) were classified as normal fertilization at 9 to 12 h following round spermatid injection (Table 1). Electrical stimulation at 2 h before spermatid injection significantly enhanced the incidence of normal fertilization as compared to those following injection with no stimulation or with stimulation immediately after injection. Table 2 showed pronuclear formation and apposition in porcine oocytes following injection of round spermatid and spermatid nucleus. The incidences of two pronuclear formation and apposition were not different between following intracytoplasmic spermatid and spermatid nucleus.

A dense network of microtubules in the cytoplasm was organized from the cortex following spermatid injection. During pronuclear movement, the maternally derived microtubules filled the whole cytoplasm, which appeared to move male and female chromatin (Fig. 1A & B). Mitosis and 2-cell division were observed at 20 to 24 h after spermatid injection with electrical stimulation. At mitotic metaphase, the microtubular spindle had focused astral poles, and chromosomes were aligned on the spindle equator (Fig. 1C). During mitosis, asters were assembled at each spindle pole, and they filled the cytoplasm.

At 6 days following injection the incidences of blastocoele formation following round spermatid (25%) and spermatid nucleus (27%) were high-

Table 1. Chromatin configuration of porcine oocytes at 9 to 12h following intracytoplasmic injection of round spermatid

Electrical stimulation	No. of oocytes				
	Injected (r)	Activated	2PN + 2PB	1PN + sPN*	Others
None	95(10)	25(26)[a]	5(20)	15(60)	5(20)
2h before injection	96(10)	82(85)[b]	36(44)	16(20)	30(37)
After injection	97(10)	85(88)[b]	20(24)	19(22)	46(54)

* Condensed or swelling male chromatin.

[a,b] Others include eggs with metaphase plate, unidentified pronuclei ($P<0.05$).

Table 2. Fertilization of porcine oocytes at 15 to 18 h following intracytoplasmic injection of round spermatid or round spermatid nuclei

Type of cell injected	No. (%) of oocytes				
	Successfully injected(r)	2 PN + 2PB	apposed PN + 2PB	1PN + sPN*	Others**
Round spermatid	123(7)	42(34)	32(26)	9(7)	40(33)
Round spermatid nuclei	102(7)	38(37)	30(29)	12(12)	22(22)

* Condensed or swelling male chromatin

** Others include eggs with metaphase plate, unidentified multiple pronuclei.

Table 3. *In vitro* development of pig zygotes cultured for 6 and 8 days following round spermatid and round spermatid nuclei

Type of cell injected	No. of embryos examined (r)	No. (%) of oocytes developed to blastocysts		Cell number of blastocysts(n)
		at 6 days	at 8 days	
Sham injection	67(5)	0(0)[a]	3(9)[a]	29 ± 6 (3)[a]
Spermatid	150(10)	38(25)[b]	45(30)[b]	99 ± 15 (21)[b]
Spematid nuclei	150(10)	41(27)[b]	52(35)[b]	87 ± 13 (22)[b]

* Nuclear number of blastocysts at day 8 examined in each experimental group is given in parenthesis.

[a,b] Different superscripts within columns denote significant differences ($P<0.05$).

er than following sham injection control (0%, Table 3). Some oocytes following sham injection developed to the blastocyst stage at 8 days, but the incidence of development is lower than those following spermatid or spermatid nucleus. The average nuclear numbers of blastocysts at 8 days following spermatid and spermatid nucleus injection were 87 and 99, respectively (Fig. 1D and Table 3).

Ⅳ. DISCUSSION

Present study demonstrated that additional activation procedure is required for the successful fertilization with round spermatid. This result extended to the pig the requirement of activation procedure following round spermatid injection in mice (Kimura et al., 1995), humans (Fishel et al., 1997), rabbit (Sofikitis et al., 1996) and cattle (Goto et al., 1996). Previous studies indicated that the oocyte activation factors, probably oscillin, is a 33 kDa protein residing in the equatorial segment region of the acrosome (Parrington et al., 1996). In the mouse oocyte activating factor(OAF) appears (or becomes active) in spermiogenesis and is located in the perinuclear material of sperm head (Kimura et al., 1998). Our previous study in the pig showed that injection of either an intact spematozoa or isolated sperm head activated oocytes, suggesting that oocyte activation factor may be presented in the plasma membrane of porcine sperm head (Kim et al., 1998). Collectively, in the pig, like as shown in the mouse, oocyte activating factor(s) seems to be assembled during spermiogenesis.

Electrical stimulation at 2 h before injection enhanced the incidence of fertilization of oocytes following round spermatid injection. This suggests that the timing of spermatid injection and oocyte activation is important for the normal development of pronuclei and for the formation of syngamy. While the injected spermatid is in G2, the unfertilized its oocytes at metaphase II is M phase. Unlike the sperm DNA, the spermatid DNA is not protected against an immediate action of oocyte cytoplasmic factors by the association with protamines. When spermatids are injected into oocytes, metaphase promoting factor (MPF, Masui et al., 1971), which maintains the oocyte chromosomes in the metaphase of the second meiotic division, may also drive the spermatid nuclei to condense. Lee et al. (1998) also reported that inadequate activation following round spermatid injection induce decondensation of male chromatin. Fishel et al. (1997) suggested that the problem of cell cycle imbalance between the spermatid and the metaphase II oocyte can be avoid by artificially activating the oocytes several hours before in-

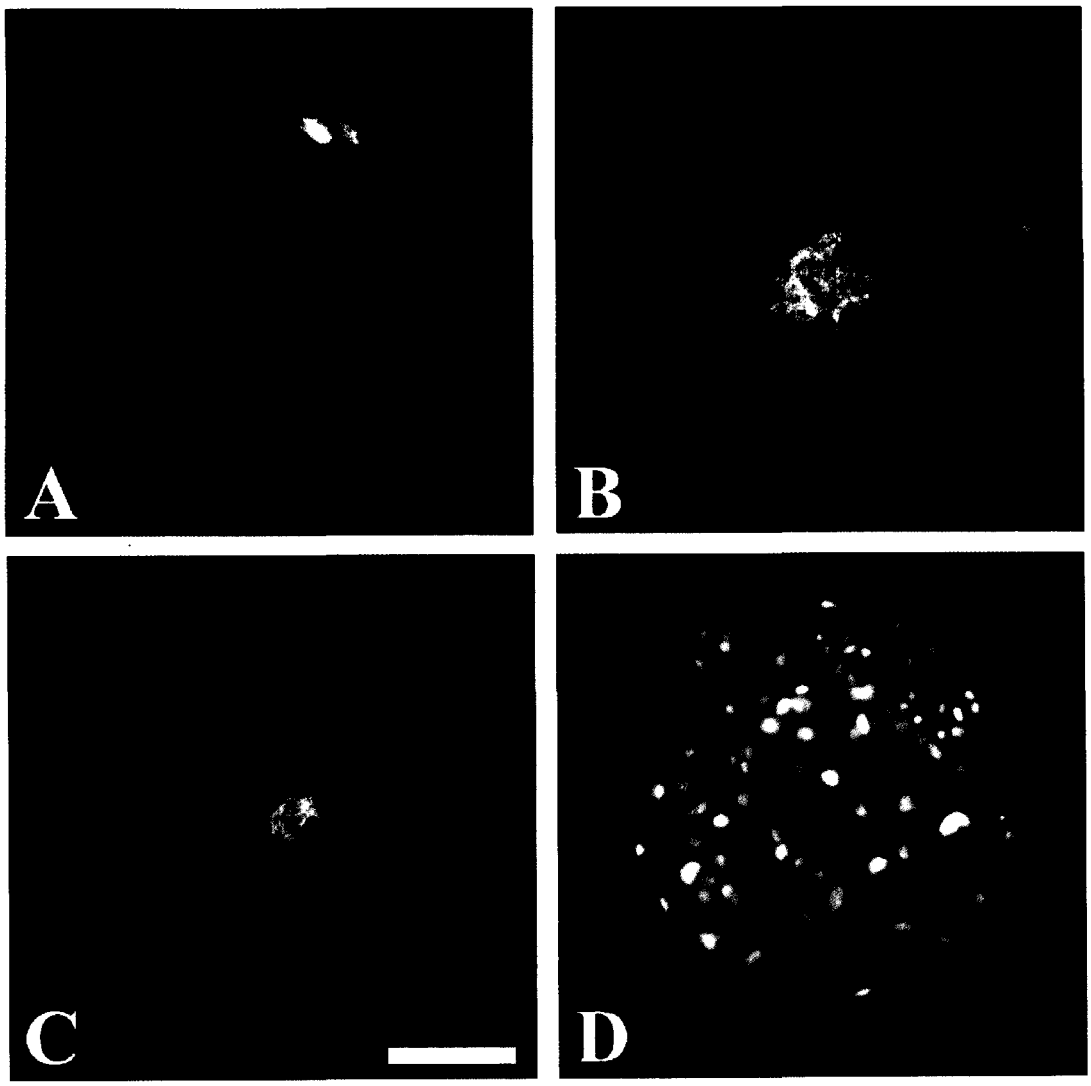

Fig. 1. Laser scanning confocal image (A,B,C) and nuclear staining (D) of porcine blastocysts following round spermatid injection in the pig. Green, microtubules; red chromatin; yellow, overapped image of green and red. A. Two pronuclei were seen in the porcine oocyte at 10 to 12 h following injection. Microtubules were organized from the oocyte cortex B. Microtubules were filled the whole cytoplasm and seems to move both pronuclei. C. Microtubules were concentrated to the condensed chromatin during mitotic metaphase. D. Nuclei of a blastocyst following round spermatid injection.

jection. Therefore, synchronization of cell cycle following spermatid injection by activating several hours before injection would enhance the incidence of fertilization and subsequent embryonic developments in porcine oocytes.

In the mouse injection of spermatid nuclei resulted in 77 % fertilization while injection of undenuded nuclei resulted in the 14% fertiliza-

tion (Kimura et al., 1995). This suggested that some cytoplasmic components, which persist some time after injection, inhibit male pronuclear formation. In this study the incidence of fertilization and *in vitro* development to the blastocyst stage were not different between following injection of round spermatid and round spermatid nucleus in the pig. The importance and role of cytoplasmic components of the spermatid during fertilization are poorly understood. Since the spermatid has just completed meiosis and contains a complete haploid set of the chromosome in it's nucleus, the nucleus could mingle with female chromatin and develop further to offspring. However, concerns have been raised towards the fertilization processes with spermatid or spermatid nucleus. Unlike mouse and hamster, most animals including pig and human showed paternal inheritance of centrosome /centrioles during fertilization (Kim et al., 1996a,b,c, 1997a, Schatten et al., 1994). Recently we reported successful fertilization in porcine oocytes following injecting either isolated sperm head or spermatid. The functional microtubules for male and female pronuclear movements were organized from the oocyte cytoplasm during fertilization with spermatid or isolated sperm head (Kim et al., 1998b; Lee et al., 1998). Fertilization with spermatid nucleus confirmed our previous findings that fertilization processes can be occurred in the absence of paternal derived centrosome in the pig.

In the present study, for the first time, we demonstrated that porcine oocytes following injection of round spermatids are capable of developing to the blastocyst stage. Although a few sham injected oocytes developed to the blastocyst stage, the incidence of development is very low. The development to the blastocyst stage following sham injection is due to parthenogenetic activation. The number of nuclei in blastocysts following round spermatid injection was examined to determined to assess whether the embryos had a sufficient number of viable cells and whether the dual culture system increased the cell numbers. Blastocysts obtained by spermatid injection had a similar number of cells compared with those of *in vitro* developed embryos following collecting from oviduct indicating a good quality of embryos (Davis et al., 1985; Rath et al., 1995 Kim and Menino, 1997; Kim et al., 1997b,c).

V. SUMMARY

In this study we determined fertilization processes and developmental ability of porcine oocytes following injection of round spermatid in the presence and absence of artificial activation. Electrical stimulation at 2 h before spermatid injection significantly increased the incidence of normal fertilization as compared to those following injection without stimulation or with stimulation immediately after injection. The incidences of two pronuclear formation and apposition were not different in oocytes between following intracytoplasmic spermatid and spermatid nucleus injection. Indirect immunocytochemistry and laser scannning confocal microscopy study revealed that microtubuels were organized from the oocyte cortex following round spermatid injection, and this seemed to move both male and female pronuclei into the oocyte center. At 6 days following injection blastocoele formation was seen in the eggs following round spermatid (25%) and round spermatid nucleus injection (27%). However, none of oocytes developed to the blastocyst stage at 6 days following sham injection. The average cell numbers of blastocysts at 8 days following injection of spermatid and spermatid nucleus were 87 to 99. These results suggested that either round spermatid or it's nu-

cleus can be used to produce viable embryos by injection into unfertilized oocytes in the pig.

VI. REFERENCES

1. Davis, D. 1985. Culture and storage of pig embryos. J. Reprod. Fertil., 33 (Suppl): 115-124.
2. Fishel, S., S. Green, A. Hunter, F. Lisi, L. Rinaldi, R. Lisi and H. McDermott. 1997. Human fertilization with round and elongated spermatids. Hum. Reprod., 12:336-340.
3. Goto, K., A. Kinoshita, Y. Nakanishi and K. Ogawa. 1996. Blastocyst formation following intracytoplasmic injection of in-vitro derived spermatids into bovine oocyte. Hum. Reprod., 11:824-829.
4. Holstein, A. F. and E. C. Roosen-Runge. 1981. "Atlas of Human Spermtogenesis." Berlin: Grosse Verlag, pp. 1-80.
5. Kim, N. H., H. Funahashi, R. S. Prather, G. Schatten and B. N. Day. 1996a. Microtubule and microfilament dynamics in porcine oocytes during meiotic maturation. Mol. Reprod. Dev., 43:248-255.
6. Kim, N. H., C. Simerly, H. Funahashi, G. Schatten and B.N. Day. 1996b. Microtubule organization in porcine oocytes during fertilization and parthenogenesis. Biol. Reprod., 54:1397-1404.
7. Kim, N. H., S. J. Moon, R. S. Prather and B. N. Day. 1996c. Cytoskeletal alterration in aged porcine oocytes and parthenogenesis. Mol. Reprod. Dev., 43:513-518.
8. Kim, N. H., K. S. Chung and B. N. Day. 1997a. The role and distribution of microtubule and microfilaments during fertilization and parthenogenesis. J. Reprod. Fertil., 111: 143-149.
9. Kim, N. H., J. T. Do, H. Song, D. B. Koo, J. H. Kim, H. T. Lee and K. S. Chung. 1997b. Involvement of adrenergic system on the cortical granule exocytosis and polyspermic penetration during *in vitro* fertilization of porcine oocytes. Theriogenology 48(8) 1351-1360.
10. Kim, N. H. and A. R. Menino. 1997. Effects of extracellular potassium concentration on blastocoel formation and expansion and hatching in porcine embryos developing *in vitro*. Journal of Reproduction and Fertility, 111:229-233.
11. Kim N. H, S. J. Uhm, J. Y. Ju, H. T. Lee and K. S. Chung. 1997c. Blastocoel formation and cell allocation to the inner cell mass and trophectoderm in haploid and diploid parthenotes developing *in vitro*. Zygote 5:365-370.
12. Kim, N. H., H. M. Chung, K. W. Cha and K. S. Chung. 1998a. Microtubule and Microfilament organization in maturing human oocytes. Human Reproduction. 13:45-52.
13. Kim, N. H., J. W. Lee, S. H. Jun, H. T. Lee and K. S. Chung. 1998b. Fertilization of porcine oocytes following intracytoplasmic spermatozoon or isolated sperm head injection. Mol. Reprod. Dev., 51:1-9.
14. Kimura, Y. and R. Yanagimachi. 1995. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development, 121: 2397-2405.
15. Kimura, Y., R. Yanagimachi, S. Kuretake, H. Bortkiewicz, A. C. F. Perry and H. Yanagimachi. 1998. Analysis of mouse oocyte activation suggests the involvement of sperm perinuclear material. Biol. Reprod., 58:1407-1415.
16. Lee, J. W., N-H. Kim, H. T. Lee and K. S. Chung. 1998. Microtubule and chromatin organization during the first cell cycle following intracytoplasmic injection of round spermatid into porcine oocytes. Mol. Reprod. Dev., 50:221-228.

17. Masui, Y. and C. L. Market, 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool., 177:129-146.
18. Parrington, J., K. Swann, V. I. Schevchenko, A. K. Sesay and F. A. Lai. 1996. Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. Nature. 379:364-368.
19. Petters, R. M. and K. D. Wells. 1993. Culture of pig embryos. J. Reprod. Fert., 48:61:73.
20. Prather, R. S., M. L. Boice, J. Gibson, K. E. Hoffman and T. W. Parry. 1995. *In vitro* development of embryos from sinclair miniature pigs: a preliminary report. Theriogenology, 43:1001-1007.
21. Rath, D., H. Niemann and C. R. L. Torres. 1995. *In vitro* development to blastocysts of early porcine embryos produced *in vivo* or *in vitro*. Theriogenology, 43:913-926.
22. Russell, L. D., R. A. Ettlin, A. P. S. Hikin and E. D. Clegg. 1990. Histological and Histopathological Evaluation of the Testis. Cache River Press, U.S.A.
23. Schatten, G. 1994. The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev. Biol., 165: 299-335.
24. Simerly, C. and G. Schatten. 1993. Techniques for localization of specific molecules in oocytes and embryos. Methods Enzymol 225:516-552.
25. Sofikitis, N. V., T. Toda, I. Miyagawa, P. M. Zavos, P. Pasyianos and E. Mastelou. 1996. Beneficial effects of electrical stimulation before round spermatid nuclei injections into rabbit oocytes on fertilization and subsequent embryonic development. Fertil. Steril., 65:176-185.

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