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Fertilization Process in Porcine Oocytes Following Intracytoplasmic Injection of Porcine, Human, Bovine or Mouse Spermatozoon

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돼지, 사람, 소 및 생쥐 정자 미세주입에 의한 돼지난자의 수정과정

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요 약

본 연구에서는 돼지 난자 내에 돼지, 사람, 소 및 생쥐의 정자를 미세 주입한 후 전핵형성과 전핵의 이동을 관찰하였다. 핵과 미세소관은 정자 주입 후 간접면역 형광염색을 실시한 후 공초점주사현미경으로 관찰하였다. 돼지 난자 내에 돼지정자를 직접 주입하였을 경우 일반적인 수정과정과 동일하게 정자 중편부에서 성상체가 형성되었고, 이 성상체에 의해서 웅성 및 자성 전핵의 이동(44%), 유사분열(3%) 및 2-세포기(13%)까지 정상적인 수정이 이루어지는 것을 관찰할 수가 있었다. 반면에 이종(사람, 소 및 생쥐)의 정자를 돼지난자에 직접 주입하였을 경우 단위발생시 난 활성이 유도된 난자와 같이 난자자체에서 형성된 미세소관에 의해 전핵이 이동(47, 30 및 17%)하는 것을 볼 수가 있었다. 하지만, 접합체 형성 및 2-세포기로의 분리되는 과정은 관찰할 수 없었다. 이러한 결과로 돼지 난자 내에 이종의 정자가 주입되었을 때 정자의 핵은 비특이적으로 전핵으로 발달되고 난자 중심부로 이동된다는 것을 보여주는 것인데, 이때 전핵을 움직이는 것은 정자에서 유래된 중심체에 의한 것이 아니라 난자세포질 자체의 미세소관에 의한 것으로 관찰되었다.

(Key words : Porcine oocytes, ICSI, Fertilization, Centrosome)

I. INTRODUCTION

In most animals, the penetrating sperm introduces paternal centrosome as well as genetic material into the egg. The paternal centrosome organizes a functional microtubular aster called "sperm aster" in combination with the maternal centrosomal components. Previous results showed that the sperm aster lead to union of male and female pronuclei and the formation of spindle for the first cell division (reviewed by Schatten, 1994).

Recently we demonstrated successful fertilization following either intracytoplasmic sperm or isolated sperm head injection in the pig (Kim et al., 1998). The microtubule organization following sperm injection is similar with that during conventional fertilization. In contrast, following sperm head injection, microtubules were organized from the cortex of the oocytes and concentrated to the pronuclei, which seems to move both male and female pronuclei to the center of oocyte. This result suggested that

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pronuclear apposition can occur in the absence of male derived centrosomes.

Little information is available on the fertilization processes following injection of spermatozoa from different species. In this study we demonstrated, for the first time, pronuclear formation and apposition in porcine oocytes following intracytoplasmic injection of porcine, human, bovine and mouse spermatozoon. We examined microtubule organization and chromatin configuration in porcine oocytes during pronuclear formation and apposition.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* maturation

Prepubertal porcine ovaries were collected from a local slaughter house and transported to the laboratory at 35℃ in Dulbecco's phosphate buffered saline supplemented with 5.54 mM D-glucose, 0.33 mM sodium pyruvate, 75 ㎍/ml potassium penicillin G and 50 ㎍/ml streptomycin sulphate (mDPBS). Cumulus-Oocyte complexes (COCs) were aspirated with an 18-gauge needle into a disposable 10-ml syringe from follicles 3 to 6 mm in diameter. The COCs were washed three times with TL-HEPES (Prather et al., 1995) medium. Groups of 50 COCs were matured in 500 ㎕ of BSA-free NCSU 23 (Petters and Wells, 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 48 to 50 h.

2. Preparation of spermatozoa

1) Porcine sperm collection

Sperm-rich fraction (15 ml) was collected from a boar by gloved hand method, and after adding antibiotic-antimycotic solution (Sigma), the semen sample was kept at 20℃ for 16 h. Semen was washed two times by centrifugation with TL-HEPES supplemented with 10 mg/ml BSA (fraction V; Sigma). Sperm were suspended in 1.5 ml of TL-HEPES for use.

2) Human sperm collection

Semen were isolated from ejaculation. Semen were mixed with an equal volume of HEPES- or bicarbonate-buffered HTF-PS and centrifuge for 5~10 min at 1,800 g. The pellet was suspended in 1.5 ml of HEPES-buffered HTF-PS for use.

3) Mouse sperm collection

Sperm was isolated from a mature male mouse (ICR, 8~13 weeks old) couda epididymides. Sperm were washed twice with TL-HEPES and then suspended in 1.5 ml of TL-HEPES.

4) Bovine sperm collection

Sperm for ICSI were prepared from frozen-thawed semen. Sperm were washed twice with TL-HEPES and then suspended in 1.5 ml of TL-HEPES. The sperm was resuspended to 1 ml with heparin-containing (10 ㎍/ml) TL-HEPES in a Effendorf tube and kept at 39℃ for 30~60 mins to induce capacitation.

3. Oocyte activation

The procedures for electrical stimulation of porcine oocytes were as by Hagen et al. (1991). At 10 to 30 min before sperm injection, the matured oocytes were denuded of cumulus cells, washed and preincubated 5 min in pulse medium: 0.25 M mannitol supplemented with 0.01% polyvinyl alcohol, 0.5 mM HEPES, 100 ㎛M $CaCl_2 \cdot H_2O$ and 25 ㎛M $MgCl_2 \cdot 6H_2O$ with pH 7.2. Electrical stimulation to induce activation was delivered with a BTX Eletro Cell Manipulator to a chamber with two parallel platinum wire electrodes (200 ㎛ o.d.) spaced 1 mm apa-

rt overlaid with pulse medium. Electrical pulse (10 sec pulse at 0.48 KV/cm AC followed by a 3 μs pulse at 1.26 KV/cm DC at room temperature (25℃) after oocytes were equilibrated with pulse medium for 3 min wes used for electrical stimulation. After a two min recovery the oocytes were transferred to 500 μl of NCSU 23 medium and cultured at 39℃ in an atmosphere of 5% CO_2 in air until porcine, human, bovine and mouse spermatozoon injection.

4. Spermatozoon injection into oocytes

Spermatozoa were centrifuged (400 g, 5 min) and resuspended in TL-HEPES : 10% polyvinylpyrrolidone solution (1:1). A microdrop (5 ml) of this suspension was placed a slide, and the slide was placed in Leitz Differential Interference Contrast inverted microscope equipped with Leitz micromanipulators. The oocytes were denuded of cumulus cells by repeated pipetting. Oocytes with visible polar body and of excellent morphology were centrifuged for 10 min in an Eppendorf centrifuge at 12,000 g in 50 μl TL-HEPES medium in 1.2 ml Eppendorf centrifuge tube. The injection of spermatozoon into the oocyte cytoplasm was performed according to the method of Lee et al. (1998). Briefly, the injection needle used was of 6~7 mm inner and 8~9 mm 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 spermatozoon and a small amount of medium was expelled into the oocyte. 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 NCSU 23 medium and cultured for at 39℃ under 5% CO_2 in air. The oocytes were fixed at 10 to 15 h and 20 to 24 h following sperm cell injection.

5. Immunofluorescence microscopy

Mictotubule and DNA were detected by indirect immunocytochemical techniques described by Kim et al. (1996a). Briefly, the oocytes were permeablized in modified Buffer M (Simerly and Schatten, 1993; 25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 1 mM B-mercaptoethanol, 50 mM imidazole, pH 6.7, 3% Triton X-100, and 25 mM phenylmethylsulfonyl fluoride) 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 day at 4℃. Microtubule localization was performed using anti-α-tubulin monoclonal 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 blocking solution (0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered milk, 0.5% BSA and 0.02% sodium azide) at 39℃ for 1h. The blocking was followed by incubation in FITC labeled goat antimouse antibody (Sigma). DNA was fluorescently detected by exposure to 5 mg/ml propidium iodide (Sigma) for 1 h. Stained oocytes were then mounted under a coverslip with antifade mounting medium (Universal Mount, Fisher Scientific 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.

Ⅲ. RESULTS

The oocytes with two large pronuclei and two polar bodies (2 PN + 2 Pb) and two large pronuclei and one polar bodies (2 PN + 1 Pb) were observed at 12 to 15 h following porcine, human, bovine and mouse sperm injection (Table 1). The incidences (54, 38, 44 and 43%) of pronuclear formation were not different among oocytes following porcine, human, bovine and mouse sperm injection, respectively. Following porcine sperm injection, the sperm aster was organized from the neck of spermatozoon, and filled the whole cytoplasm (Fig. 1AB). In contrast, sperm aster was not organized following human, bovine and mouse sperm injection. Instead, microtubules were organized from the oocyte cortex and then filled the whole cytoplasm in all cases in normally fertilized oocytes (Fig. 1C). This organization is similar to what has been shown previously in the parthenogenetically activated oocytes (Kim et al., 1996 bc, 1997) or in the oocytes following round spermatid injection (Lee et al., 1998).

Table 2 summarize chromatin configuration in porcine oocytes at 20 to 24 h after porcine, human, bovine and mouse sperm injection. Pronuclear apposition observed in the oocytes following 44, 47, 30 and 17% , respectively. The incidence of mitosis (3%) and two cell division (13%) were observed following porcine sperm injection, but human, bovine and mouse sperm injection were unfertilized. During pronuclear movement the sperm aster filled the whole cytoplasm following ICSI, suggesting their role for the pronuclear apposition. In contrast, following human, bovine and mouse sperm injection, the maternal original microtubules filled the whole cytoplasm, which seems to move male and female chromatins (Fig. 1D).

Ⅳ. DISCUSSION

In the present study we demonstrated, for the first time, pronuclear formation and pronuclear apposition in porcine oocytes following injection of different species spermatozoa. Previously, Yanagimachi et al. (1991) reported that nuclei isolated from spermatozoa of various species such as, golden hamster, mouse, human, rooster and the fish tilapia could develop into pronuclei.

Table 1. Chromatin configurations in porcine oocytes at 12~15h following intracytoplasmic injection of porcine, human, bovine or mouse sperm

Parameter assessed	Type of cells injected			
	Porcine	Human	Bovine	Mouse
No. of oocytes				
successfully injected (replication)	60 (5)	26 (3)	27 (3)	28 (3)
with two PN and two PB (%)	31 (52)	4 (15)	3 (11)	8 (29)
with two PN and one PB (%)	1 (2)	6 (23)	9 (33)	4 (14)
with one PN, one PB and decondense sperm (%)	0 (0)	8 (31)	6 (22)	0 (0)
with one PN, sperm head and two PB (%)	9 (15)	0 (0)	0 (0)	0 (0)
with three PN and one PB (%)	5 (8)	0 (0)	0 (0)	0 (0)
with metaphase Ⅱ and sperm head(%)	2 (3)	4 (15)	3 (11)	12 (43)
with others(%)	12 (20)	4 (15)	6 (22)	4 (14)

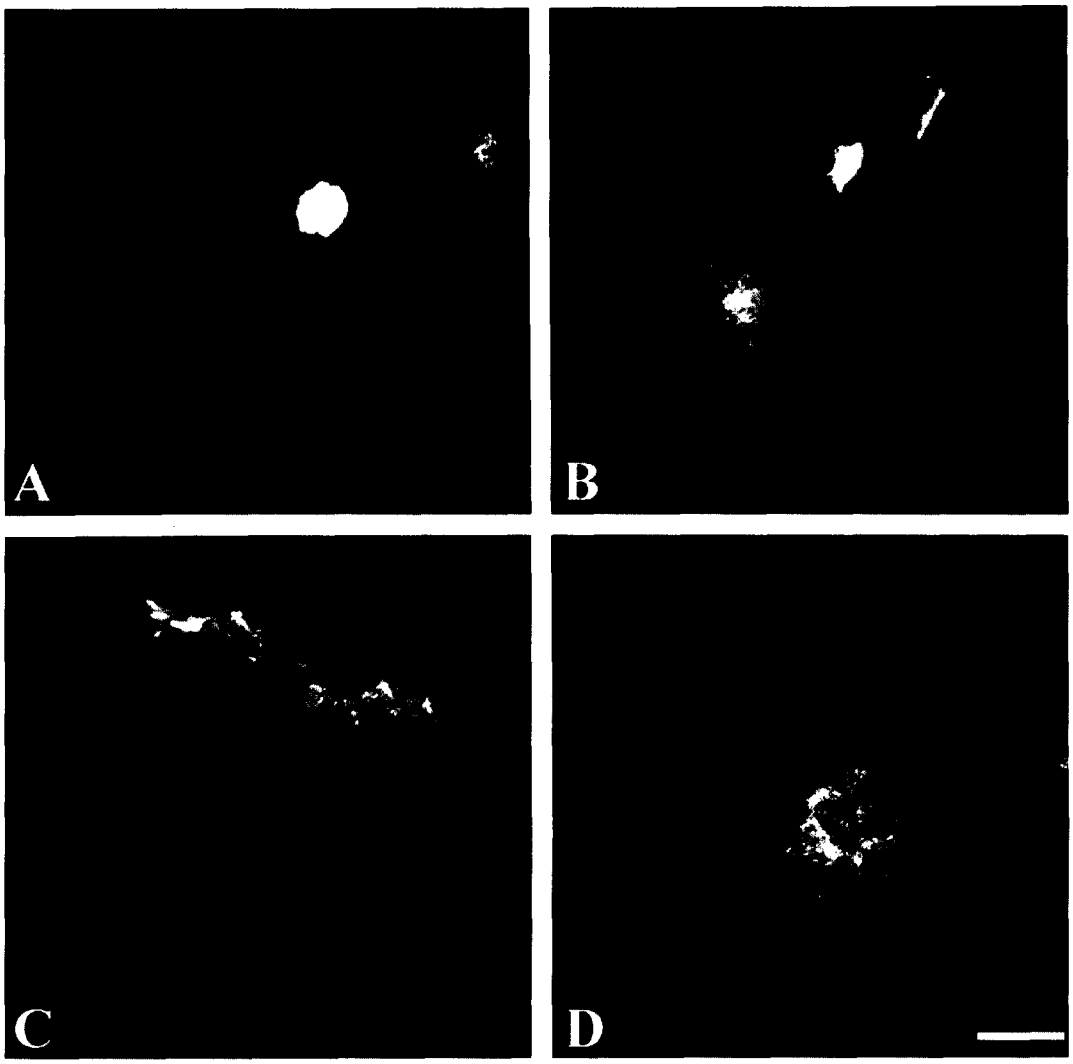

Fig. 1. Laser scanning confocal microscopic images of microtubules and chromatin in porcine oocytes following spermatozoon injection (x 630). Green, microtubules; red, chromatin. yellow, overlap image of microtubules and chromatin, Bar = 20㎛. A. Soon after sperm injection, sperm aster was seen in the neck of sperm. B. Sperm aster enlarge, and no microtubules were observed in the female chromatin. C. Dense microtubule networks was observed from the cortex and started to fill the whole cytoplasm following injection of different species spermatozoon. D. At the time of pronuclear apposition the microtubules filled the whole cytoplasm

However, in our knowledge, it has not been reported that the pronuclear apposition following injection of spermatozoon from different species. Because paternal derived centrosome during

Table 2. Chromatin configurations in porcine oocytes at 18~21 h following intracytoplasmic injection of porcine, human, bovine or mouse sperm

Parameter assessed	Type of cells injected			
	Porcine	Human	Bovine	Mouse
No. of oocytes				
successfully injected (replication)	61 (5)	39 (3)	30 (3)	36 (3)
Two-cell (%)	8 (13)	0 (0)	0 (0)	0 (0)
Mitosis (%)	2 (3)	0 (0)	0 (0)	0 (0)
closely apposed two PN (%)	27 (44)	18 (47)	9 (30)	9 (25)
with one PN, decondens sperm and two Pb (%)	0 (0)	0 (0)	3 (10)	7 (19)
with one PN, one Pb and sperm head (%)	2 (3)	3 (8)	9 (30)	14 (39)
with one PN, two Pb and sperm head (%)	9 (15)	6 (15)	3 (10)	3 (8)
with others (%)	13 (21)	9 (23)	6 (20)	3 (8)

fertilization, concerns have been raised on the microtubule organization during pronuclear formation and movement following injection of different species spermatozoon into oocyte cytoplasm.

Microtubule mediated events in pig oocytes have been studied during fertilization and parthenogenesis (Kim et al., 1996 abc ; 1997 a). Following sperm penetration the microtubular aster was organized in the sperm neck area in combination with maternal centrosomal material, which moves male and female chromatin toward the center of oocytes. After electrical activation, in contrast, cytoplasmic centrosomal material is activated and organizes a network of microtubules which moves pronuclei to the center of eggs. In this study we observed that microtubule dynamics of porcine oocytes fertilized by ICSI were similar to those during conventional fertilization. Similar observations in microtubule assembly have been reported in the human and monkey oocytes following ICSI (Hewitson et al., 1996; Sutovsky et al., 1996). In contrast, following human, bovine and mouse sperm injection, microtubular aster was not seen in the male chromatin. Instead, maternal derived microtubules were organized from the cortex of oocytes following injection, which seemed to move pronuclei into the center. The organization of the microtubule matrix following human, bovine and mouse sperm injection is similar with those during parthenogenesis in the pig (Kim et al., 1996b & c) or those following spermatid injection (Lee et al., 1998). These results indicated that the microtubules organized by maternal componentstake over the roles for the pronuclear movements in the absence of male derived microtubules.

The mechanism whereby the maternal derived microtubules organize and move pronuclei to the center of oocytes during parthenogenesis (Navara et al., 1994; Kim et al., 1996 bc; 1997), following spermatid (Lee et al., 1998) or human, bovine and mouse sperm injection (present study) is elusive at present. Heald et al. (1996) developed an *in vitro* system in which beads coated with artificial chromosomes in Xenopus eggs. In the absence of centrosome, chromatin beads induce the assembly of functional microtubules in interphase, confident to transport nuclear substrates and replicate DNA. More recently Rodionov and Borisy (1997) observed formation of the radial microtubule array in the fish melanophore cells in the absence of centrosome. Their

results suggested that self organization mechanism for microtubule assembly would be presented in the cytoplasm, which possibly arrange chromatin in the proper position during mitosis. Taken together, the cell cytoplasm may have the ability to organize the appropriate microtubules for the chromatin dynamics during pronuclear apposition or mitosis, although it is poorly understood.

Although many evidences showed that microtubule give a leading role for the pronuclear movement. However microtubules may not be the only cytoskeletal element for pronuclear migration. In experiments using the microfilament inhibitor latrunculin also play a role in pronuclear movement. In mouse (Maro et al., 1984) and pig (Kim et al., 1996), microfilaments became concentrated around both pronuclei after fertilization. Microfilaments has been known to be involved in cortical granule reaction during fertilization and activation of porcine oocytes (Kim et al., 1996d, 1997b). Further studies are required to determine exact roles of microtubules and microfilaments during pronuclear apposition.

In summary, we demonstrated successful pronuclear formation and apposition in the porcine oocyte following intracytoplasmic injection of spermatozoa from different species. Microtubule organization and chromatin configuration of porcine oocytes fertilized by porcine sperm injection were similar to those during conventional fertilization. In contrast, following human, bovine and mouse sperm injection, maternally derived microtubules were produced which appeared to move both pronuclei into the center oocytes. These results suggested that the sperm nucleus of different species can form pronuclear and apposited in the porcine oocytes when they were injected into ooplasm.

V. SUMMARY

We demonstrated, for the first time, pronuclear formation and apposition in porcine oocytes following intracytoplasmic injection of porcine, human, bovine and mouse spermatozoon. Microtubule organization and chromatin configuration were investigated in these oocytes during pronuclear apposition. Following intracytoplasmic injection of porcine spermatozoon, the microtubular aster was organized from the neck of spermatozoon, and filled the whole cytoplasm. This male derived microtubules appear to move both pronuclei to the center of oocytes. In contrast, following injection of spermatozoa from different species such as human, bovine and mouse, microtubules were organized from the cortex of the oocytes and concentrated to the pronuclei, which seems to move both male and female pronuclei to the center of oocyte. This organization is similar to what has been shown in the parthenogenetically activated porcine oocytes. These results suggested that the porcine, human, bovine and mouse sperm chromatin can be formed pronucleus and apposited in the center of oocytes in the absence of male derived microtubule when they were injected into porcine oocytes.

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