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Onset of Pronuclear Formation and DNA Synthesis in Porcine Oocytes following Intracytoplasmic Injection of Porcine or Murine Spematozoa

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ABSTRACT

The onset of pronucleus formation and DNA synthesis in porcine oocytes following the injection of porcine or murine sperm was determined in order to obtain insights into species-specific paternal factors that contribute to fertilization. After 44h *in vitro* maturation, spermatozoa was injected into the cytoplasm of oocytes. After injection, all oocytes were transferred to NCSU23 medium and cultured at 39℃ under 5% CO_2 in air. Similar frequencies of oocytes with female pronuclei were observed after injection with porcine sperm or with murine sperm. In contrast, male pronuclei formed 8 to 9 h following the injection of porcine sperm, and 6 to 8 h following the injection of murine sperm. After pronucleus formation maternally derived microtubules were assembled and appeared to move both male and female pronuclei to the oocyte center. A few porcine oocytes entered metaphase 22 h after the injection of murine sperm, but normal cell division was not observed. The mean time of onset of S-phase in male pronuclei was 9.7 h following porcine sperm injection and 7.4 h following mouse sperm injection. These results suggested that DNA synthesis was delayed in both pronuclei until the sperm chromatin fully decondensed, and the sperm nuclear decondensing activity and microtubule nucleation abilities of the male centrosome are cell cycle dependent.

(Key words : Pronucleus formation, DNA synthesis, Microtubule, Centrosome)

Ⅰ. INTRODUCTION

The sperm nucleus-decondensing activity of the oocytes appears to be cell cycle dependent. In mice, immature oocytes do not promote sperm nuclear decondensation prior to germinal vesicle breakdown (GVBD); the oocyte cytoplasm only acquires this

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ability after GVBD and loses it a few hours after fertilization (Usui and Yanagimachi, 1976; Borsuk and Tarkowski, 1989; Szollosi et al., 1990). Using conventional *in vitro* fertilization techniques, Wang and Niwa (1997) observed in the pig that spermatozoa could penetrate into immature and maturing oocytes with a high incidence of polyspermy. The spermatozoa in immature oocytes were partially decondensed soon after penetration, and could be transformed into metaphase chromatin when the oocytes were cultured to the metaphase stage. Although sperm penetration in pre-activated oocytes in pigs has been reported (Funahashi et al., 1993), little information is available on the fertilization processes, such as pronuclear formation and movement, in pre-activated pig oocytes.

During fertilization, morphological and molecular events that affect male and female chromatin are under precise temporal control. In conventional *in vitro* fertilization of the mouse, meiosis is completed within 2 h of insemination, and male and female pronuclei form between 4 to 7 h and 5 to 8 h post-insemination, respectively (Howlett and Bolton, 1985). DNA synthesis is initiated randomly beginning about 11 h post-insemination, and S phase last 6~7 h in both male and female pronuclei (Howlett and Bolton, 1985; Bouniol-Baly et al., 1997). The time of onset of replication in the mouse seems to be controlled by maternal factors stored in oocytes, which likely influence the formation of a functional nuclear membrane (Blow and Laskey, 1988). In contrast, in cattle, sperm influences the onset of DNA synthesis during fertilization (Comizzoli et al., 2000). Replication initiates earlier and lasts longer in zygotes fertilized by sperm of high *in vivo* potency, while replication terminates at a time that is independent of the fertility of the bull (Eid et al, 1994; Comizzoli et al., 2000). However, little is known about the influence of paternal effects on the onset of pronucleus formation and DNA synthesis following the injection of sperm into porcine oocytes. In the present study we compare these landmarks of fertilization in porcine oocytes injected with porcine and murine sperm in order to gain insights into species-specific paternal factors that contribute to fertilization.

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 Dulbeccos 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 sulfate (mDPBS). Cumulus-oocyte complexes (COCs) were aspirated from follicles with an 18-gauge needle into a disposable 10 ml syringe. Fifty porcine COCs were matured in 500 ml of a defined protein-free medium consisting of Tissue Culture medium 199 (TCM 199) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 10 ng/ml epidermal growth factor, 0.1% polyvinyl alcohol, 0.57 mM cysteine, 0.5 mg/ml LH, and 0.5 mg/ml FSH under paraffin oil at 39℃ for 40 to 44 h. After maturation, the cumulus cells were removed by vigorous pipetting of the complexes in the presence of 0.3 mg/ml hyaluronidase.

2. Injection of Sperm into Oocytes

Spermatozoa were centrifuged (400×g, 5 min) and resuspended in TCM-HEPES: 10% polyvinylpyrrolidone solution (1:1). A microdrop (5 ml) of this suspension was placed on a slide, and the slide was placed in a Nikon Differential Interference Contrast inverted microscope equipped with Narsige micromanipulators. The oocytes were denuded cumulus cells by repeated pipetting. Oocytes with visible polar bodies and of excellent morphology were

used. Oocytes were centrifuged for 10 min in an Eppendorf centrifuge at 12,000 × g in 1.2 ml Eppendorf centrifuge tube. Spermatozoa were injected into the cytoplasm of oocytes using the method of Lee et al. (1998). Briefly, the injection needle used was of 6~7 mm inner and 8~9 mm outer diameters. The polar body was at 6 or 12 oclock and the point of injection at 3 oclock. 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 quickly withdrawn, and the oocytes were released from the holding pipette to reduce the intracytoplasmic pressure exerted to the oocyte. After injection, all oocytes were transferred to NCSU23 medium and cultured at 39℃ under 5% CO_2 in air.

3. Immunofluorescence Microscopy

Microtubules and DNA were detected by indirect immunocytochemical techniques as described by Kim et al. Briefly, the oocytes were permeabilized in modified Buffer M (Simerly et al., 1993) (25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 1 mM b-mercaptoethanol, 50 mM imidazol, 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 solution (PBS) containing 0.02% sodium azide and 0.1% bovine serum albumin for 2~7 days at 4℃. Microtubule localization was performed using anti-α-tubulin (Sigma #T-5168) or the γ-tubulin monoclonal antibody (Sigma #T-6557). Fixed oocytes were incubated for 90 min at 39℃ with a 1:100 dilution of anti-α-tubulin or a 1:10 dilution of the anti-γ-tubulin in PBS. After several washes with PBS containing 0.5% Triton-X 100 and 0.5% BSA, oocytes were incubated in a 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 1 h. The blocking was followed by incubation with 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 Scientific Co., Huntsville, AL, USA) to slow 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 fluorescence for microtubules and propidium iodide for DNA.

4. DNA Synthesis

DNA synthesis was measured by incorporation of 5-bromo-2-deoxyuridine (BrDU, Sigma). Oocytes were incubated with 100 μm BrDU in culture medium at 39℃ in 5% CO_2. After labelling oocytes were washed three times in PPB, which is DPBS-PVA and bovine serum albumin (BSA). The zona pellucidae of the oocytes were removed by a brief exposure to 0.5% pronase and then washed in PBS. Zona-free oocytes were fixed in methanol at −20℃ for 20 min, washed with PPB, and then permeabilized in 1% (v/v) Triton X-100 in PPB for 15 min and washed three times again in PPB. Oocytes were hydrolysed in 4N HCl for 30 min, washed in PPB, and incubated overnight with Monoclonal anti-BrDU (Sigma) diluted 1:10 in 1% FBS in PPB at 4℃ in a humidified box. They were then washed in PPB three times and incubated with FITC labeled goat anti-mouse antibody (Sigma) diluted 1:100 in 1% FBS in PPB for 1 h in the dark at 39℃ in 5% CO_2. Afterwards, oocytes were washed in PPB three times for 15 min, and the stained with propidium

iodide (200 μg/ml) in PPB for 1 h 39℃ in 5% CO_2, washed threes times in PPB again, mounted on slides in malinol, and view with a confocal laser scanning microscpe (Bio-Rad MRC 1024).

5. Statiscal Analysis

The effect of post injection time on the rates of female and male pronuclear formation, and DNA replication was expressed as a linear regression of the individual percentage and time following sperm injection (Comizzoli et al., 2000). The mean times of these events was defined as time at which half of the zygotes had a male or female pronucleus or were undergoing DNA synthesis. Data were pooled from at least four replicate experiments. Differences in the percentages of oocytes developing to a particular stage were determined by Chi-square procedures.

Ⅲ. RESULTS

1. Pronucleus Formation following the Injection of Porcine and Murine Sperm

Microtubules and chromatin configuration during pronuclear apposition, entry into metaphase, and cell division were monitored at 22 and 26 h following the injection of murine sperm, as shown in Fig. 1. Following pronucleus formation, maternally derived microtubules assembled and appeared to move both the male and female pronuclei into the oocyte center (Fig. 1A, Kim et al., 1999). Entry into metaphase was observed in a few oocytes 22 h following murine sperm injection (6/95, 6.3%). In all cases, chromatin debris was seen in metaphase oocytes (Fig. 1B). However, porcine oocytes injected with murine sperm did not cleave normally when they were fixed at 26 h (Fig. 1C&D, Kim et al., 1999).

The frequencies of female pronucleus formation were similar in oocytes injected with porcine sperm

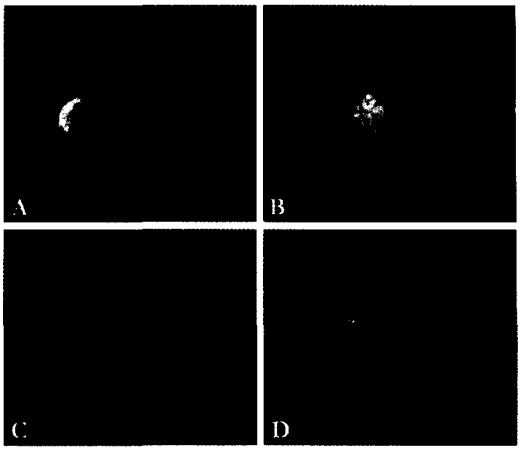

Fig. 1. Pronuclear apposition, metaphase entry and abnormal cleavage in porcine oocytes following injection of murine spermatozoa. Red, DNA; Green, microtubules. A. Pronuclear apposition. B. Metaphase entry in chromatin. Arrow indicates chromatin debris. C&D. Multiple pronuclear-like structures in a porcine oocyte (C) and abnormal cleavage (D) at 26 h following murine sperm injection.

(32 ± 8 and 61 ± 12% at 4 and 5 h, respectively) and murine sperm (45 ± 13 % and 72 ± 10 at 4 h and 5 h, respectively, Fig. 2). The calculated mean time at which 50% of oocytes had a female pronucleus was 4.6 h after porcine sperm injection, and

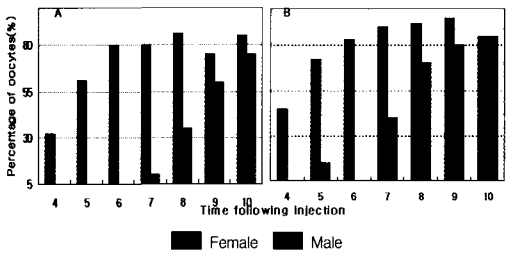

Fig. 2. The incidence (mean ± SEM) of oocytes with male (red bars) and female pronucleus (blue bars) formation in porcine oocytes following porcine (A) and mouse (B) sperm.

4.8 h after murine sperm injection (Fig. 2). In contrast, male pronuclei formed 8 to 9 h following porcine sperm injection and 6 to 8 h following murine sperm injection. The calculated mean time of male pronucleus formation was significantly later following porcine sperm injection (8.7 h) than following murine sperm injection (7.3 h, $P < 0.05$).

2. DNA Synthesis following Sperm Injection

The synthesis of DNA after sperm injection was evaluated by BrDU incorporation into pronuclei (Fig. 3). DNA synthesis occurred only after complete decondensation of the spermatozoon. Although a fully formed female pronucleus was present in oocytes before sperm decondensation, DNA synthesis was not seen in either pronucleus (Fig. 3 C&D). Oocytes with complete male and female pronuclei were assayed for DNA synthesis (Fig. 3). As shown in Fig. 4, the mean time of onset of S-phase in the male pronucleus was 9.7 h following porcine sperm injection, and 7.4 h following murine sperm injection. The mean time of onset of S-phase in parthenotes was 6.3 (n=25). Eight hours after sperm

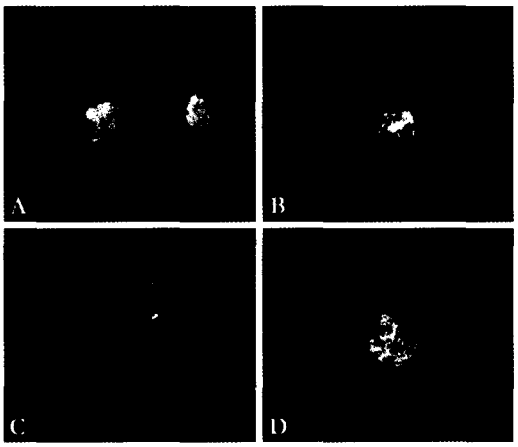

Fig. 3. DNA synthesis in porcine oocytes following porcine (A, B) and murine sperm injection (C&D). Red: chromatin, Green: BrDU.

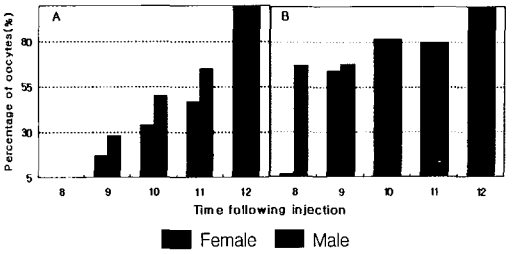

Fig. 4. Onset of DNA synthesis in male (red bars) and female pronuclei (blue bars) in porcine oocytes injected with porcine (A) and murine sperm (B). Number of zygotes examined in each experimental group is given in parentheses.

injection the frequency of male pronuclei undergoing DNA synthesis (67%) was higher than that of female pronucleus (7%, Fig. 4, $P < 0.01$). This result suggests that DNA synthesis is delayed in both pronuclei, until male pronucleus formation and entry into S phase.

Ⅳ. DISCUSSION

Previously, Funahashi et al. (1995) observed the onset of female and male pronucleus formation in porcine oocytes matured *in vitro* following conventional *in vitro* fertilization. After insemination, sperm penetration occurred as early as 3 h and female pronuclei had formed by 6 h with complete development by 12 h. Male pronuclei formed between 9 and 12 h after insemination. This result suggests that male pronucleus formation *in vitro* maturation/ fertilization systems occurs a few hours later than female pronucleus formation (Funahashi et al. 1995). Similarly, in the present study, we observed male pronucleus formation 7 to 9 h following sperm injection, a few hours later than female pronucleus formation. Since synchronized formation of male and female pronuclei has been reported for *in vivo* fertilized porcine and bovine zygote (Hunter, 1974;

Crozet, 1984), the delay in male pronuclear formation as compared to female pronucleus formation has been suggested to be due to inappropriate condition for *in vitro* maturation/fertilization in the pig (Funahashi et al., 1995, 1996; Kim et al., 1996). Another explanation for asynchronous pronucleus development is that male pronucleus formation following sperm injection may require additional time for the disassembly of sperm-specific structures such as acrosome and perinuclear theca (Sutovsky et al., 1996). Ramalho-Santos et al. (2000) observed delayed DNA decondensation in Rhesus monkey oocytes following sperm injection, which is probably related to the persistence of the sperm acrosome and perinuclear theca.

Howlett and Bolton (1985) reported that under conventional *in vitro* fertilization conditions, murine meiosis is completed within 2 h post-insemination, and male and female pronuclei form 4 to 7 h post insemination and 5 to 8 h post-insemination, respectively. In contrast to what is observed for the mouse, in the pig, sperm penetration and pronucleus formation appear to occur a few hours later (9 to 12 h). In the present study we observed that the mean time of male pronucleus formation following porcine sperm injection was longer than that following murine sperm injection. This suggests that species-specific components present in sperm may be important in initiating for sperm nuclear decondensation and pronucleus formation. The mechanism whereby sperm components induce pronuclear formation is elusive at present. Successful sperm decondensation and male pronucleus formation requires glutathione for the reduction of sulfate bond (Sutovsky et al., 1996) and nucleoplasmin from germinal vesicle (Philpott et al., 1991; Maeda et al., 1998) to mediate the replacement of sperm nuclear protamine with histone. Different levels of glutathione and/or nucleoplasmin in porcine and murine oocytes may explain why murine sperm triggers the decondensation of sperm chromatin more quickly than does porcine sperm.

The onset and duration of zygotic DNA synthesis has been studied in mice (Luthardt and Donahue, 1973) and cattle (Eid et al., 1994) as well as in the pig (Laurincik et al, 1995). In porcine zygotes produced by *in vivo* fertilization, S-phase occurs about 55 to 62.5 h after the injection of hCG, and comparable extents of autoradiographic labeling are observed for both pronuclei in almost all zygotes, suggesting that the maternal and paternal S-phase are synchronized (Laurincik et al., 1995). In the present study we observed that S-phase is initiated in both male and female pronuclei after the full development of the male pronucleus. Although a fully formed female pronucleus is present in oocytes before sperm decondensation, DNA synthesis is not seen in either nucleus. This result suggests that only paternal component influences the timing of the onset of replication. Similar research result in Rhesus monkey showed that the factor or factors contributed by paternal components are effective only after pronucleus formation, and they affect both pronuclei (Ramalho-Santos et al., 2000). However, it is unclear which component in sperm initiates DNA synthesis in porcine zygotes. As shown for mice (Howlett and Bolton, 1985), we also observed that in the absence of paternal components, activated oocytes could initiate DNA replication at a specific interval after pronucleus formation. This finding supports the theory of endogenous oocyte programming, set in train by the terminal events of oocyte maturation, may regulate the house keeping of the eggs, while sperm penetration super-imposed on the oocyte program to initiate subsequent embryogenesis (Howlett and Bolton, 1985; Ramalho-Santos et al., 2000).

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