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Cat fertilization by mouse sperm injection

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Summary

Interspecies intracytoplasmic sperm injection has been carried out to understand species-specific differences in oocyte environments and sperm components during fertilization. While sperm aster organization during cat fertilization requires a paternally derived centriole, mouse and hamster fertilization occur within the maternal centrosomal components. To address the questions of where sperm aster assembly occurs and whether complete fertilization is achieved in cat oocytes by interspecies sperm, we studied the fertilization processes of cat oocytes following the injection of cat, mouse, or hamster sperm. Male and female pronuclear formations were not different in the cat oocytes at 6 h following cat, mouse or hamster sperm injection. Microtubule asters were seen in all oocytes following intracytoplasmic injection of cat, mouse or hamster sperm. Immunocytochemical staining with a histone H3-m_2K9 antibody revealed that mouse sperm chromatin is incorporated normally with cat egg chromatin, and that the cat eggs fertilized with mouse sperm enter metaphase and become normal 2-cell stage embryos. These results suggest that sperm aster formation is maternally dependent, and that fertilization processes and cleavage occur in a non-species specific manner in cat oocytes.

Keywords: Centrosome, ICSI, Interspecies, Mouse embryo, Sperm injection

Introduction

Fertilization is a series of processes that occur as the egg and the sperm create a new life. Fertilization begins with sperm entry into mature oocytes, and a union of male and female genomes occurs inside the egg, followed by progression to mitotic metaphase, and then to 2-cell cleavage. In most animals, including humans, cats, pigs and sea urchin, after the sperm enters the cytoplasm of an oocyte, a radial microtubule array, the so-called 'sperm aster', is organized from the sperm centrosome (Schatten, 1994). Sperm aster formation seems to be essential for pronuclear movement resulting in the union of the male and female genomes (Schatten, 1994; Simerly *et al.*, 1995). In contrast, in rodents (mouse and hamster), the sperm centriole is not introduced into oocytes during fertilization. Acentriolar mouse zygotes accomplish early embryonic cleavage using a microtubular organization center (MTOC). The differences between rodents and other animals with respect to centrosome introduction from sperm during fertilization are evolutionarily puzzling.

Interspecies intracytoplasmic sperm injection has been carried out to understand species-specific differences in oocyte environments and sperm components during fertilization. In rabbit oocytes following human sperm injection, astral microtubules radiated from the sperm neck and enlarged as the sperm head underwent pronuclear decondensation (Terada *et al.*, 2000). Beaujean *et al.* (2004) showed, using interspecies sperm injection with mouse sperm and sheep eggs, that the sperm demethylation process in the mouse is not dependent on the oocyte environment. In pig oocytes, normal pronuclear formation, DNA synthesis

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and metaphase entry were observed following mouse sperm injection (Kim *et al.*, 2003). Human sperm injection into mouse and rabbit oocytes has been used widely for studies related to assisted human reproduction (Yanagimachi, 2005; Yamazaki *et al.*, 2007). Successful 2-cell division of mouse oocytes following the injection of human and acrosome less hamster has been observed (Morozumi & Yanagomachi, 2005). However, it is not clearly understood which of the processes of normal metaphase entry and cleavage are maintained during interspecies fertilization by sperm injection.

To address whether sperm aster assembly is mediated by the maternal components and whether interspecies sperm are able to achieve complete fertilization of cat oocytes, we first examined microtubule assembly in cat oocytes following cat, hamster or mouse sperm injection. In this study, we saw normal pronuclear formation, sperm aster assembly and metaphase entry in cat oocytes after mouse sperm injection. In order to confirm the incorporation of mouse and hamster sperm in the entry to mitotic metaphase and 2-cell stage division, we used immunocytochemical staining with a histone H3-K9 antibody that differentially stains male and female components.

Materials and methods

Generation of oocytes

For collection of cat oocytes, 1–8-year-old female cats were induced to superovulate with 400 IU of pregnant mare serum gonadotropin (PMSG, Daesung Microbiological Labs, Co., Ltd., Seoul, Korea), followed by 100 IU of human chorionic gonadotropin (hCG; Daesung Microbiological Labs) 96 to 100 h later. Animals were treated according to the guidelines of the Chungbuk National University Institutional Animal Care and Use Committee. Expanded cumulus cell oocyte complexes (COCs) were collected 24 to 27 h after hCG injection from ovaries by slicing the ovarian cortex in feline-optimized culture medium (FOCM; Herrick *et al.*, 2007) supplemented with HEPES (FOCMH; Sigma-Aldrich Co.) and 5–10 U/ml heparin (Yin *et al.*, 2006), and washing twice in FOCMH without heparin. Unfertilized metaphase II eggs (MII) were collected (day 0) after 6–9 h of *in vitro* maturation and the cumulus cells were removed. Oocytes with visible polar bodies and superior morphology were centrifuged at high speed and used for injection.

Preparation of spermatozoa

Preparation of spermatozoa was performed using the methods of Kim *et al.* (1998). Frozen–thawed semen was induced by capacitation and stained with MitoTracker fluorochrome (Molecular Probes) before injection. For ICSI with ejaculated cat spermatozoa, we used alternatively frozen and thawed semen from three proven breeder males (one male/replicate). Motile ejaculated spermatozoa were selected by swim-up processing in complete FOCMH medium. Mouse and hamster sperm mass was taken from the cauda epididymis and placed at the bottom of a 1.5 ml tube containing 200 μl of M2 medium for 30 min at 37°C to allow spermatozoa to swim-up through the medium.

Intracytoplasmic sperm injection (ICSI)

ICSI was carried out according to the methods of Kimura and Yanagimachi (1995) with some modifications. An aliquot (10 μl) of sperm suspension was mixed thoroughly with FOCMH containing 10–12% (wt/vol) polyvinylpyrrolidone (Sigma). A microdrop of suspended spermatozoa was placed on a dish, and the dish was placed on a Nikon Differential Interference Contrast inverted microscope equipped with Nikon micromanipulators. High quality spermatozoa were selected and each sperm was injected using a piezo-micromanipulator (MB-U; Prim Tech) after immobilization by touching the sperm tail with the injection pipette and aspiration into the injection pipette. Using the holding pipette (i.d. 120 mm), metaphase II oocytes were held by negative pressure with the polar body at the 6 or 12 o'clock position. The injection pipette (i.d. = 7 mm) was pushed through the zona pellucida and into the cytoplasm of the oocyte at the 3 o'clock position. After a minimal amount of cytoplasm had been aspirated into the injection pipette to ensure plasmalemma breakage, the immobilized spermatozoon was deposited into the oocyte. After injection, oocytes were cultured as previously described.

Activation

After injection, oocytes were cultured in FOCM supplemented with 4.0 mg/ml BSA at 38.7°C in 6% CO_2 in room air under mineral oil. Some groups of oocytes were activated by 5 min exposure to 7% (v/v) ethanol or 5 μM ionomycin in IVC medium at 3 h post-ICSI (Kim *et al.*, 1996; Nakamura *et al.*, 2001). Oocytes were then fixed and stained at 3 h, 6 h, 9 h and 15 h post-ICSI. A total of three replicates were performed.

Analysis of DNA synthesis

Cat oocytes following sperm injection were pulse-labelled *in vitro* with 5-bromo-deoxyuridine (BrdU) in order to evaluate antibody accessibility. The zygotes were incubated in IVC medium supplemented with 100 mM BrdU (Sigma) for 1 h at 38.7°C in 6% CO_2

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in room air. After incubation, the zygotes were rinsed in PBS (phosphate-buffered saline), fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature.

Antibodies

The following antibodies were used in this study: mouse anti-α-tubulin (Sigma), rabbit anti-dimethyl lysine 9 in histone H3 (H3K9; Santa Cruz), and BrdU (Invitrogen and Sigma).

Immunocytochemical staining

To determine the expression and distribution of proteins, cat embryos were fixed with 4% formaldehyde for 20 min. In the case of α-tubulin, embryos were incubated for 5 min in 0.1 μM taxol medium, and incubated with Buffer M (50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ethylene glycol bis, and 1 mM β-mercaptoethanol) for 5 min, before being fixed in –20°C methanol for 10 min. Embryos were then permeabilized with 0.2% Triton X-100 for 10 min and incubated with primary antibodies for 1 h followed by incubation with fluorescein isothicyanate (FITC)- or Alexa-labelled secondary antibodies. Propidium iodide (PI) or Hoechst 33343 was used to stain the nuclei. Slides were examined using laser-scanning confocal microscopy performed using a Leica DM IRB equipped with a krypton–argon ion laser for the simultaneous excitation of fluorescence for proteins and DNA.

Statistical analysis

The general linear models (GLM) procedure in the SAS program (SAS User's guide, 1985, SAS, Inc., Cary, NC, USA) was used to analyse the data from all experiments. Significant differences were determined using Tukey's multiple range test (Steel and Torrie, 1980) and p-values < 0.05 were considered significant.

Results

Male and female pronuclear formations were examined in cat oocytes at 6 h following ICSI of a cat, mouse, or hamster spermatozoon. The rate of male and female pronuclear formation in cat oocytes following mouse or hamster sperm ICSI was not different from that in cat oocytes with cat sperm ICSI (Fig. 1). No DNA synthesis had occurred in cat oocytes at 4 h following cat, mouse or hamster sperm injection (Fig. 2). However, DNA synthesis was observed in cat oocytes at 6 h following injection of cat (5/6), mouse (4/4) or hamster (4/5) sperm, and at 8 h following

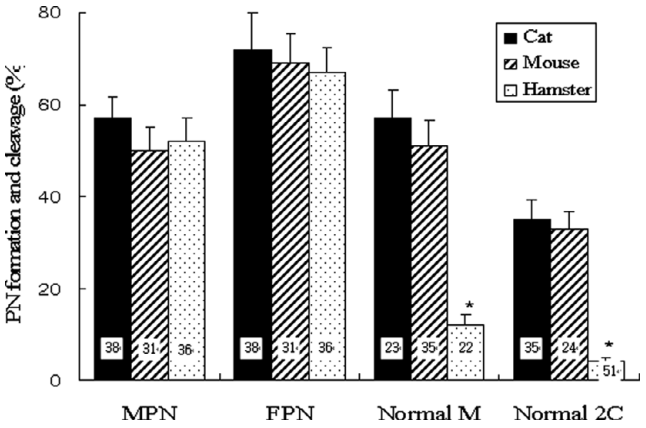

Figure 1 Percentage of pronuclear formation (1PN, 2PBs), metaphase entry and 2-cell division in cat oocytes following cat, mouse or hamster sperm injection. The data represent the means ± SEM of four independent experiments ($p < 0.05$).

injection of cat (3/3) or mouse (5/5) sperm (Fig. 2). The microtubule organization and chromatin configuration in cat eggs after ICSI with mouse or hamster sperm are shown in Fig. 3 (microtubules, green; DNA, blue; sperm tail, red). At 3 h and 6 h post-ICSI, the sperm aster, a radial microtubule array extending from the sperm centrosome, was seen in cat oocytes after cat (6/23, 26%), mouse (7/20, 35%) or hamster ICSI (2/13, 15%). No microtubules were observed to be organized around the female pronucleus (Fig. 3).

Mitotic metaphase and the 2-cell division stage were observed in cat oocytes at 14–15 h and 15–16 h after the injection of cat, mouse or hamster sperm (Figs. 1, 4 and 5, respectively). Immunocytochemical staining with a histone H3-m_2K9 antibody was used to confirm male and female components. A previous study had shown that male chromatin was rapidly demethylated in oocytes, but not female chromatin (Oswald *et al.*, 2000), suggesting the existence of discrimination among male and female chromatin. Fig. 4 shows that throughout metaphase, chromatin was stained with histone H3-K9 in the parthenotes. In contrast, in mitotic metaphase in oocytes, only half of the chromatin was stained with H3-K9 following cat or mouse sperm injection (Fig. 4). Similarly, half of the chromatin was stained in cat oocytes at the time of 2-cell division after cat or mouse sperm injection (Fig. 5). The incidence of normal mitotic metaphase (57%) and 2-cell division (35%) in cat oocytes following cat sperm injection was not different from that (normal metaphase, 51%; 2-cell division, 33%) following mouse sperm injection. A few cat oocytes progressed to normal mitotic metaphase (12%, $p < 0.005$) or 2-cell division (4%, $p < 0.001$) following hamster sperm injection (Fig. 1). However, many deformed 2-cell stage and arrested 1-cell stage embryos were observed among cat oocytes subjected to hamster sperm injection (Figs. 1 and 5).

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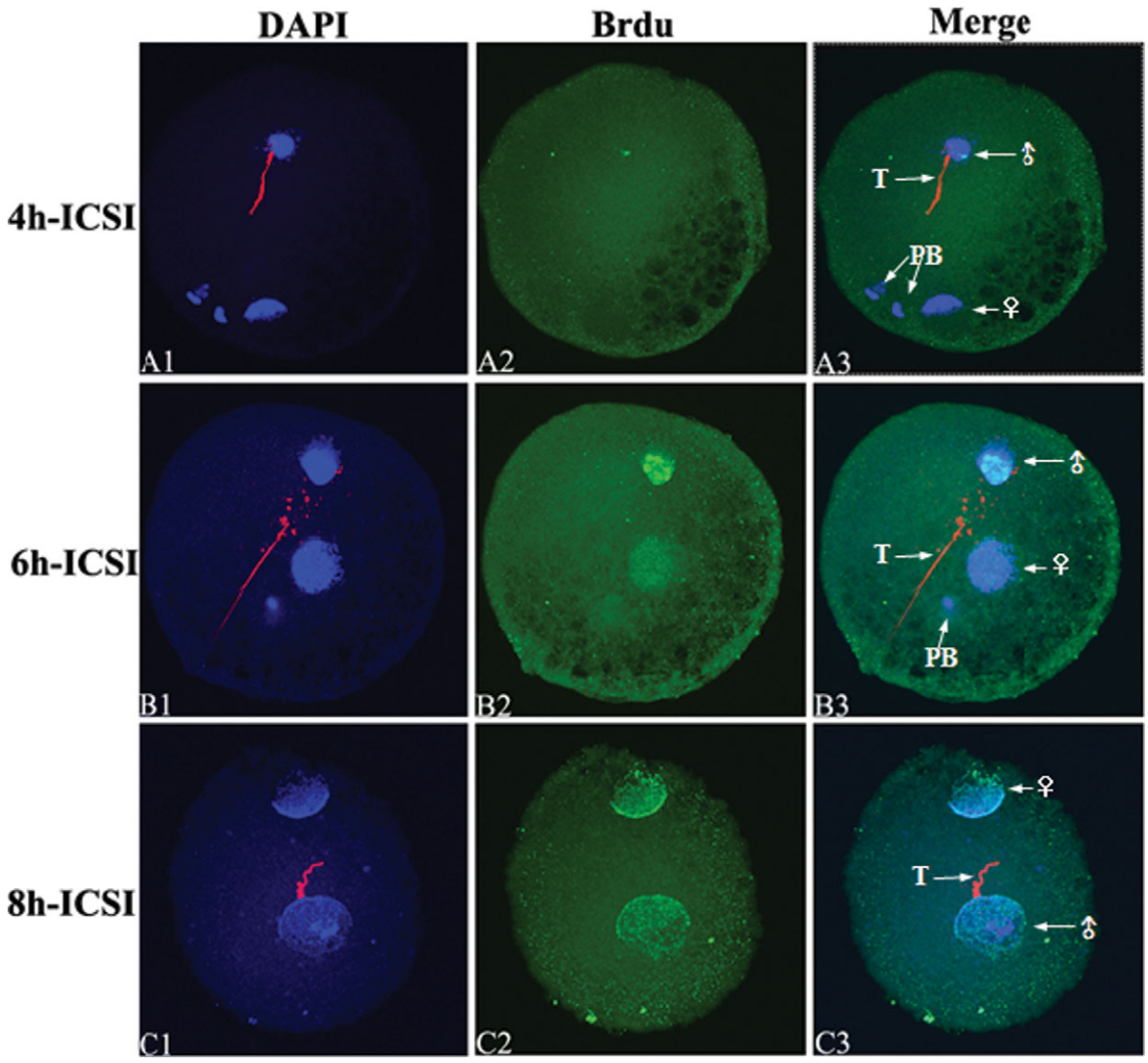

Figure 2 Representative laser-scanning confocal microscopic images of DNA synthesis in cat oocytes following injection of cat, mouse or hamster sperm (×630). Blue staining, chromatin; red staining, sperm tail; green staining, DNA synthesis; ♂, male pronucleus; ♀, female pronucleus; T, tail. (The sperm tail is detected by MitoTracker.) (*A*) At 4 h following ICSI (cat, mouse or hamster sperm), DNA synthesis had not been initiated in any pronuclei. (*B*) At 6 h following ICSI, DNA synthesis had initiated in both male and female pronuclei. (*C*) At 8 h following ICSI, DNA synthesis was complete in fully developed pronuclei. PB, polar body.

Discussion

Previous studies have shown that interspecies ICSI in pig oocytes (Kim *et al.*, 1999, 2003) and in mouse oocytes (Kimura *et al.*, 1998; Fulka *et al.*, 2008) can result in the successful production of male and female pronuclei. Similarly in our study, the incidence of male and female pronuclei was not different in cat oocytes subjected to cat sperm injection than in cat oocytes receiving interspecies sperm injection. To address the question of whether pronuclei from an interspecies sperm component are functional in cat oocytes, we observed DNA synthesis in cat oocytes following cat, mouse or hamster sperm injection. DNA replication in all groups had a similar onset 6 h after ICSI, and completely normal male and female pronuclei were observed at 8 h after ICSI. The similarity of pronuclear formation and onset time of DNA replication observed in cat oocytes following cat, mouse or hamster sperm injection suggests that the formation of functional pronuclei is a non-species specific process.

In cat eggs fertilized by sperm injection, the resulting zygote has a large sperm aster after ICSI with ejaculated sperm (Murakami *et al.*, 2005; Comizzoli *et al.*, 2006). Sperm asters are observed during fertilization in most animals, with the exception of

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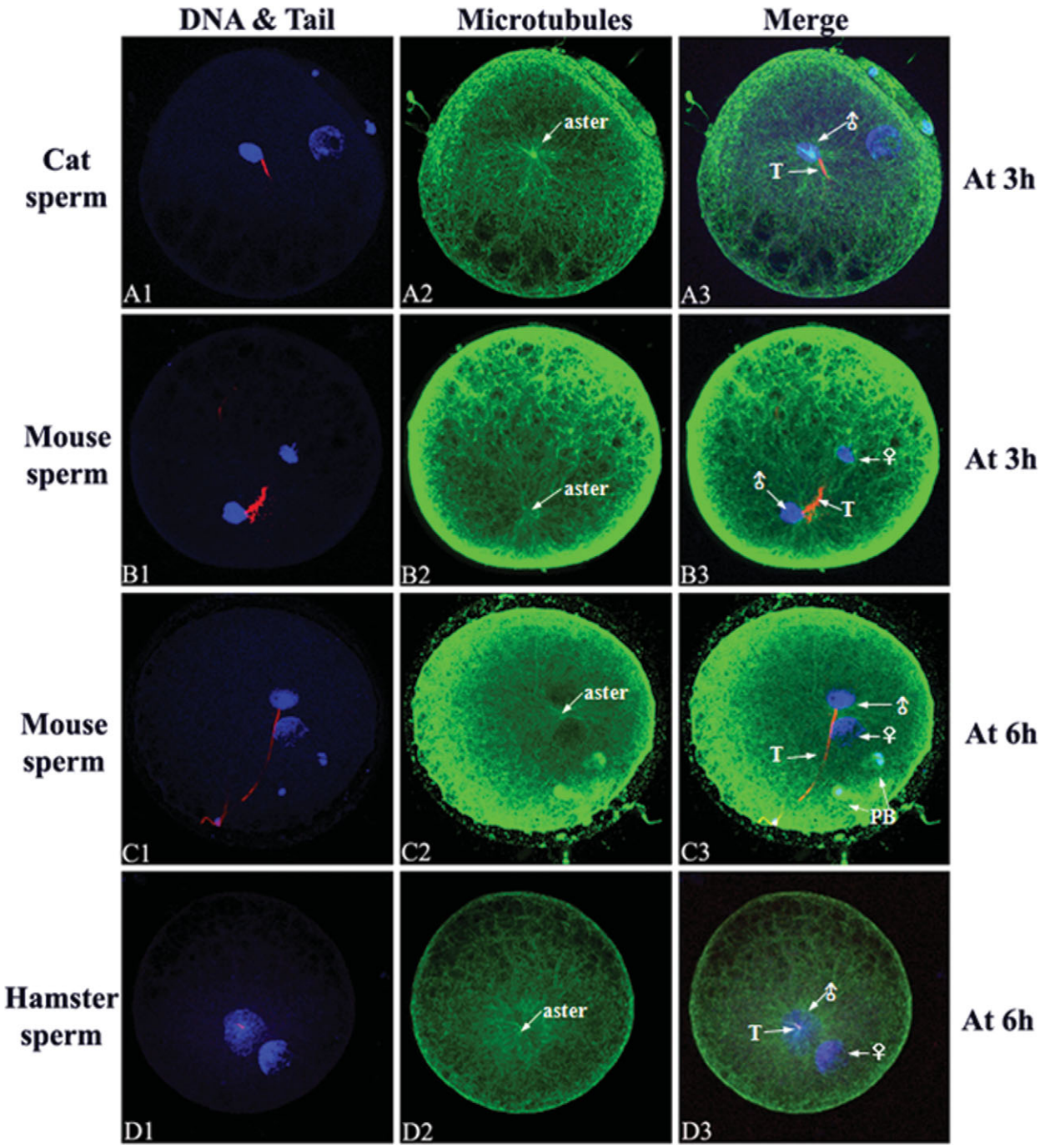

Figure 3 Laser-scanning confocal microscopic images of microtubules and chromatin in cat oocytes following intracytoplasmic sperm injection (ICSI) of cat, mouse or hamster sperm (×630). Microtubules (green), sperm tail (red) and chromatin (blue) are shown. (*A*) Cat oocytes following cat sperm ICSI. At 3 h post-ICSI, a midbody structure (arrow) connecting the decondensing female chromosomes was observed. The sperm nucleus (arrowhead) was decondensed, and a radial array of microtubules (the sperm aster, arrow) was organized from the sperm centrosome. Microtubules were not organized around the female pronucleus. (*B*) Mouse sperm injection group at 3 h post-ICSI. At this time, the male and female pronuclei had decondensed, and the sperm aster was organized from the sperm centrosome. (*C*) Mouse sperm injection group at 6 h post-ICSI. At this time, the sperm aster had enlarged toward the male and female pronuclei. (*D*) Hamster sperm injection group. At 6 h post-ICSI, a small sperm aster was seen at the base of the hamster sperm head, the male and female pronuclei had decondensed and they had done so more fully than the cat or mouse sperm injection groups. PB, polar body; ♀, Female pronucleus; ♂, male pronucleus; T, tail.

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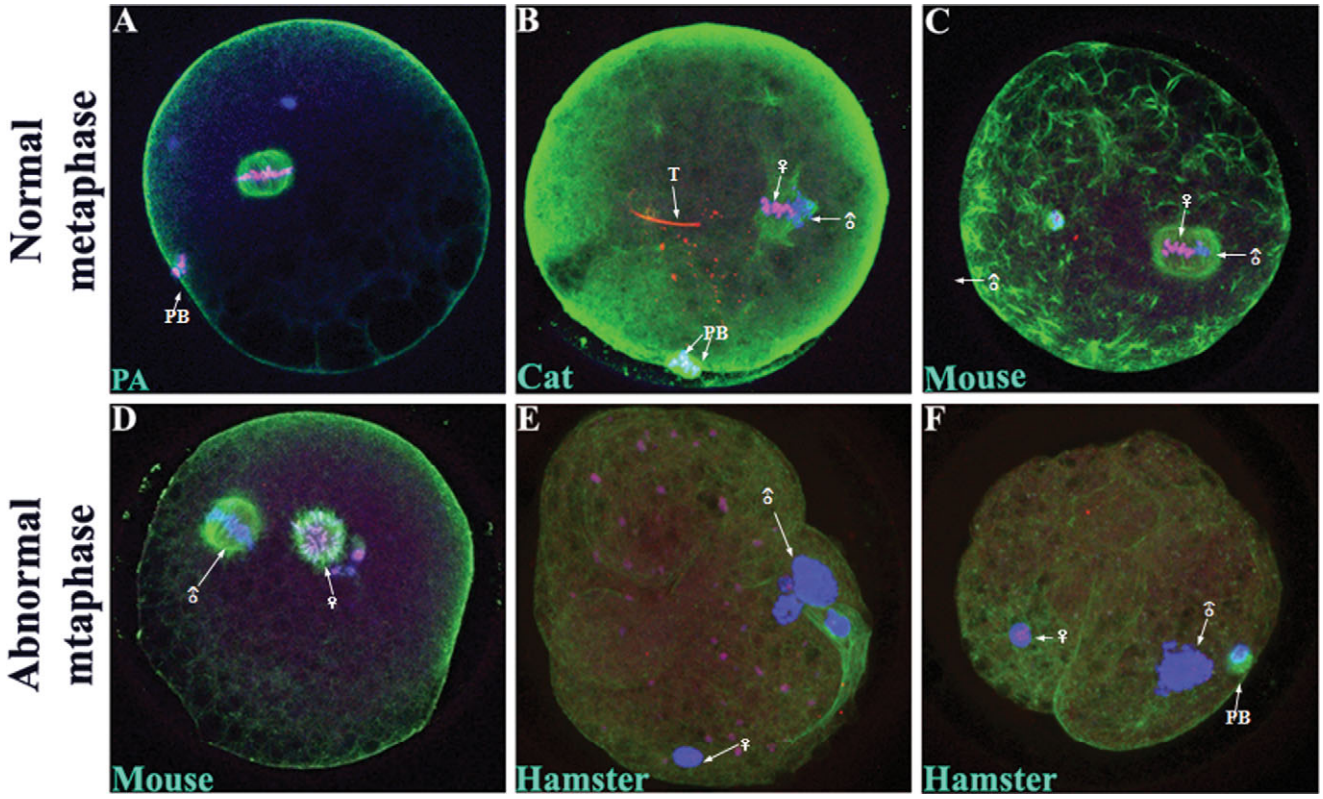

Figure 4 Laser-scanning confocal microscopic images of microtubules, dimethyl histone H3K9 and chromatin assembly are shown in normal and abnormal pronuclear and mitotic metaphase formations in activated oocytes, or after interspecies sperm injection of cat oocytes (×630). Blue staining, chromatin; red staining, methyl-H3K9 or sperm tail; green staining, microtubules; ♂, male chromatin; ♀, female chromatin; T, tail. Images were taken 14–15 h after activation of oocytes or injection of sperm. (*A*) Metaphase chromatin was stained with histone H3-m_2K9 in pathenogenic cat oocytes. (*B*, *C*) Metaphase chromatin from cat oocytes following cat or mouse sperm injection was seen in half of the chromatin stained with H3-m2K9. (*D*) Metaphase chromatin from cat oocytes following mouse sperm injection shows two metaphase chromatin strands, one was stained with H3-m2K9 and the other was not. (*E*, *F*) Abnormal chromatin and deformation of cytoplasmic embryos in cat oocytes following hamster sperm injection. PB, polar body.

rodents. In rodents, fertilization is accomplished by means of a maternally inherited centrosome. In mice, the paternal centrosome seems to be degenerated during spermiogenesis, and sperm asters are not observed at the base of the incorporated sperm head (Schatten, 1994). Interestingly, in the absence of functional sperm centrosomal components, such as parthenotes, the maternally derived microtubules are organized in the cytoplasm as is seen in mouse fertilization (Kim *et al.*, 1996; Terada *et al.*, 2000). In the starfish (Saiki & Hamaguchi, 1992, 1998), in the absence of sperm components, introduction of the maternal centrosome (polar body centrosome), which is involved in the meiotic progress of oocytes, is able to serve as an aster even during cleavage, suggesting an involvement of maternal components in sperm aster formation. Unexpectedly, we have demonstrated the existence of sperm aster formation in cat oocytes following mouse or hamster sperm injection. The organization of microtubule asters by mouse sperm in cat oocytes suggests that mouse sperm components do not completely lose their ability to form a functional centrosome during spermiogenesis, as opposed to the strictly maternal inheritance pattern in mice that has been suggested based on previous studies (Schatten, 1994).

In our study, we have demonstrated, for the first time, the complete incorporation of interspecies sperm components in cat oocytes. Previously, Kim *et al.* (2003) had demonstrated the ability of pig oocytes injected with mouse sperm to enter into metaphase. In this study, by staining H3K9, which stains female chromatin only, we have demonstrated the incorporation of male chromatin during metaphase entry and 2-cell division. This result suggests that these fertilization processes occur in a non-species specific manner. In the present study, we also found a high incidence of deformation of cat oocytes following hamster sperm injection. Similarly, Kimura *et al.* (1998) previously reported that mouse oocytes injected with acrosome-intact rabbit and hamster spermatozoa were deformed and did not develop into 2-cell embryos.

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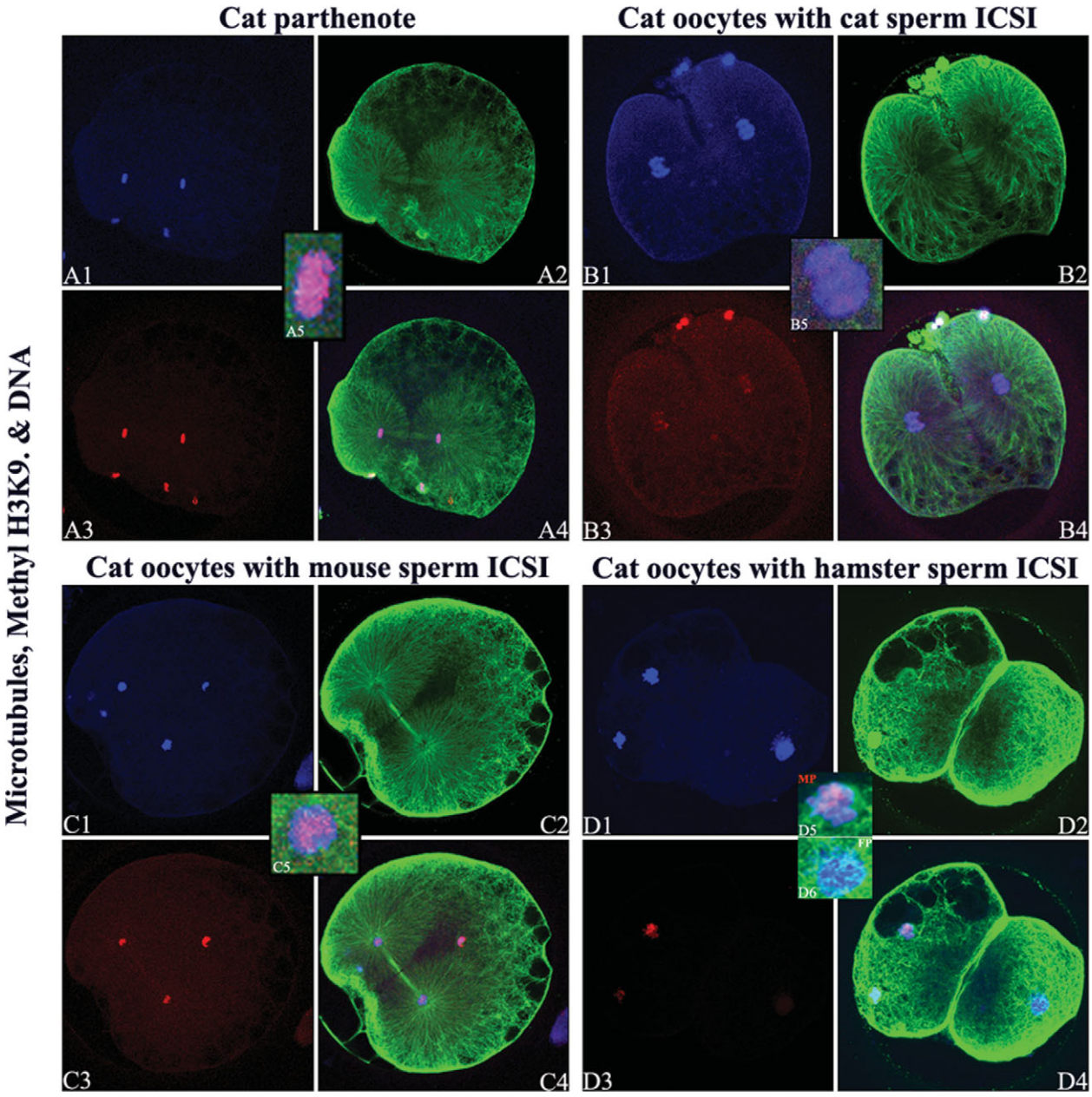

Figure 5 Laser-scanning confocal microscopic images of microtubules, dimethylation histone H3K9 and chromatin assembly are shown at the 2-cell division stage in activated oocytes and after sperm injection of cat oocytes (×630). Images were taken 15–16 h after the activation of oocytes or the injection of cat, mouse or hamster sperm, respectively (*A–D*). Blue staining, chromatin; red staining, methyl-H3K9; green staining, microtubules; ♂, male chromatin; ♀, female chromatin. (*A*) Two-cell division-stage chromatin was stained with histone H3-m_2K9 in pathenogenic oocytes. (*B*, *C*) Two-celled cat embryos from cat or mouse sperm injection showed half of their chromatin stained with H3-K9. (*D*) Two-celled cat embryos from hamster sperm injection. One blastomere showed staining with H3-K9 and the other did not.

When sperm heads were freed from the acrosome prior to injection, the oocytes did not deform (Yamauchi *et al.*, 2002; Morozumi & Yanagimachi, 2005). The observation of deformation in cat oocytes, which is similar to that seen in mouse oocytes following hamster sperm injection, suggests that the contents of acrosome may have detrimental effects on the cat fertilization following hamster ICSI.

In summary, we have demonstrated that there is a similar incidence of pronuclear formation and DNA synthesis in cat oocytes following cat, mouse or hamster sperm injection. This finding suggests the existence of non-species specific processes in functional male pronuclear formation during fertilization. The observation of sperm aster formation by mouse sperm in cat oocytes supports a maternal role in

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functional microtubule assembly in the cat. Successful incorporation of mouse sperm and progression of the zygote into metaphase and 2-cell cleavage suggests that complete fertilization occurs in a non-species specific manner in the cat oocyte.

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