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Ultrastructure in Porcine Oocytes following Intracytoplasmic Injection of Murine Spermatozoa

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ABSTRACT

Although successful pronuclear formation and apposition were seen in porcine oocytes following mouse sperm injection, little is known on the morphology of male and female pronuclei following sperm injection. The objective of this study is to describe the ultrastructure of porcine zygote following murine sperm injection in relation to the chronology of pronuclear S phase. At 40h ~ 44h following *in vitro* maturation, Cumulus cells were removed in TCM-HEPES with 0.1% hyaluronidase. Then, 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. Oocytes were fixed in 2% glutaraldehyde in Dulbeccos phosphate-buffered saline and observed by Transmission Electron Microscopy. Nuclear precursor bodies were observed in each pronucleus. A cluster of large and small granules was attached in the nucleolus precursor body. After the apposition of male and female chromatin, chromatin condensation was observed throughout the nucleoplasm and nucleolus precursor bodies and condensed chromatin in contact with clusters of small and large granules and the nuclear envelope were found in apposed pronuclear regions. These results suggest that non-species specific nuclear cytoplasmic interactions take place during pronuclear formation and apposition following sperm injection.

(Key words : Ultrastructure, ICSI, Pronuclear formation, Fertilization)

Ⅰ. INTRODUCTION

Embryo quality is an important determinant of the success of embryo transfer procedures. Several methods for embryo evaluation have been reported. Dye exculsion tests (Kardymowicz, 1972), measures of enzyme activity (Schilling et al., 1979), glucose uptake (Renard et al., 1980), and live-dead stains (Schilling et al., 1979) are useful in predicting em-

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bryo survival following transfer, but these methods require complex equipment and a lengthy *in vitro* embryo culture period.

Understanding morphological changes of the chromatin, microtubule organization and DNA synthesis in the first cell cycle of the oocytes are of great significance for the effective use of cytoplasts for embryo. One of the important question regarding the success in the development of embryos is how the oocytes use stored components to form the spindles for the development. The most prominent ultrastructural entities of the pronuclei were the nucleolus prenucursor bodies (NPBs). During the S- and G2-phases, the NPBs spatially associated with clusters of interchromatin-like granules. The two components were firmly attached to each other by an electron-dense reticulum(Laurincik et al., 1998). The NPBs determined chronological nuclei cell cycle.

Ultrastructural abnormalities of bovine embryos have been observed that may be related to capacity for further embryonic development (Hyttel et al., 1989; Shamsuddin et al., 1994). Linars and Ploen (1981) have examined the ultrastructure of 7-day -old bovine embryos (blastocyst) collected from spontaneously ovulatory cows. The embryos were classified into three groups: normal, morphologically deviating or degenerated. This study suggested that only those embryos classified as normal blastocysts are likely to undergo further development. However, this observation was limited to the blastocyst stage. A drastic change in ultrastructural features in the development of bovine embryos was first observed at the morula stage (Abe et al., 1999; Plante and King, 1994). At the morula stage, the maturation of mitochondria, elongation of microvilli, and development of Golgi apparatus occured in the blastomeres. These findings suggest that the physiological activities of embryos increase at this stage, and these ultrastructural changes may relate to embryo development and survival following transfer. Therefore, it seems important to compare the ultrastructure of embryos classified by the widely-used embryo evaluation method and to attempt to relate ultrastructural features with physiological parameters that are crucial for embryo quality.

In the pig, sperm penetration is observed 3 to 5 h after mating and male and female pronuclei form 5 to 8 h after mating (Hunter, 1972, 1974). The synchronous development of paternal and maternal pronuclei *in vivo* fertilized zygotes has been demonstrated, but details concerning the onset of pronucleus formation and DNA synthesis have not been described (Laurincik et al., 1995). Previously, we reported successful pronucleus formation and apposition in porcine oocytes following injection with either porcine or murine sperm (Kim et al., 1999; Lee et al., 2002). We observed pronucleus formation around 6 h and pronuclear apposition beginning around 9 h following the injection of either porcine or murine sperm. However, little is known about the ultrastructure of embryos following the injection of sperm into porcine oocytes. In the present study we describe the ultra-structure of porcine zygote following murine sperm injection in relation to the chronology of pronuclear Sphase.

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 (TCM199) 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% polyvinyl-pyrrolidone 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. Transmission Electron Microscopy

Oocytes were fixed in 2% glutaraldehyde in Dulbeccos phosphate-buffered saline. The specimens were then postfixed in 2% OsO4 for 1 h, dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultra-thin sections were cut with a diamond knife and post-stained first with 1% uranyl acetate in 30% ethanol and then with Reynolds lead citrate. A transmission electron microscope (Hitachi 600, Hitachi Ltd, Kashiwa, Japan) was used to determine chromatin configuration in porcine oocytes following sperm injection. Negative film was digitalized with a scanner (Epson GT-9000) and archived on an erasable magnetic diskette.

Ⅲ. RESULTS

1. Ultrastructure of Porcine Zygotes Produced by Murine Sperm Injection

Between 10 and 13 h after sperm injection, both the paternal and maternal pronuclei are surrounded by an essentially complete nuclear envelope. Nuclear precursor bodies were observed in each pronucleus (Fig. 1 A&B). Most of the chromatin was dispersed, but small portions of condensed chromatin were distributed throughout the nucleoplasm. The status of chromatin was not specifically related to the presence of nuclear precursor bodies. A cluster of large and small granules was attached in the nucleolus precursor body (Fig. 1a). After the apposition of male and female chromatin, chromatin condensation was observed throughout the nucleoplasm (Fig. 1 B&b). Well-developed smooth endoplasmic recticulum, mitochondria, and intranuclear annulate lamellae could be seen (Fig. 1 B&b).

At 15 to 18 h after injection, in some cases the spherical fibrillar bodies were associated with less electron dense material and contained a single large vacuole, conferring a ring-shaped appearance in sectioned profiles (Fig. 2 A&a). The nuclear envelopes adopted an undulating appearance (Fig. 2a).

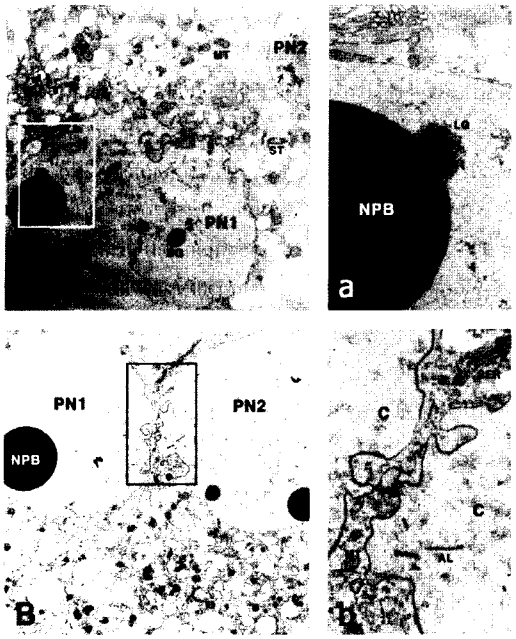

Fig. 1. Ultra-structure of two pronuclei in porcine oocytes following murine sperm injection. A. Two pronuclei (PN1 & PN2) can be seen. The midpiece of the spermatozoon (ST) was associated pronucleus. Mitochondria (MT) were also seen. Soft granules (SG) and chromatin (arrows) are present in the pronucleus. a. The boxed area from A showing a nuclear precursor body (NPB) that associated with large granules (LG). B. The two pronuclei were separated by a narrow cytoplasmic bridge containing well -developed mitochondria (MT), and smooth endoplasmic recticulum (SER). b. The boxed area from B showing the midpiece of sperm (arrow) remain associated in the pronucleus. Chromatin (c) and intranuclear annulated lamellae (AL) were seen.

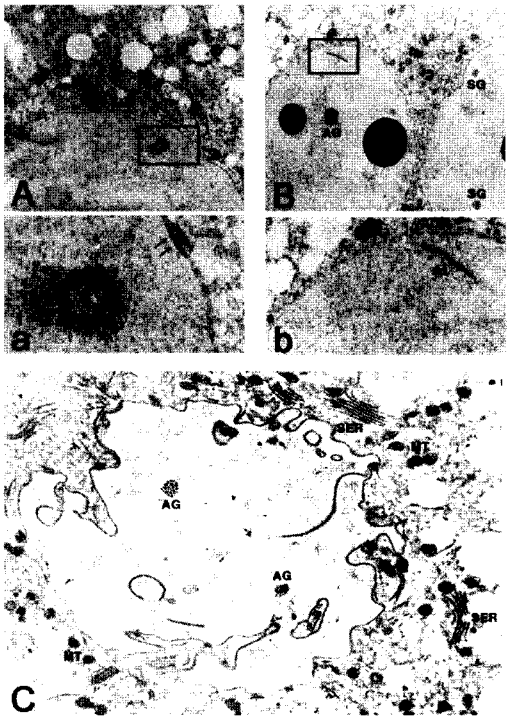

Fig. 2. A. Detailed of an S phase pronucleus. a. The boxed area from A showing a compact, electron-dense fibrillar body with a single large vacuole (V) in contact with chromatin (C). The nuclear envelopes became undulating in this area (arrows). B. Two apposed pronuclei together. b. The boxed area from B showing annulated lamellae. C. Dynamic interchanges between nucleus and cytoplasm can be seen in porcine oocytes 24 h following murine sperm injection. Aggregated granules (AG) were seen in nucleoplasm. Numerous smooth endoplasmic recticulum (SER), mitochondria (MT) were also observed in the vicinity of nucleus.

Nucleolus precursor bodies and condensed chromatin in contact with clusters of small and large granules and the nuclear envelope were found in apposed pronuclear regions (Fig. 2 B&b). Dynamic changes in nucleoplasm components were seen in late pronuclear stage zygotes, including well-developed smooth endoplasmic reticulum and numerous mitochondria in the vicinity of the pronucleus, but nuclear precursor body was not present at this stage. Small granule clusters, and an accumulation of large granules and intranuclear annulate lamellae

were seen (Fig. 2C).

Ⅳ. DISCUSSION

During the transit of spermatozoa through the epididymis, sperm nuclei are made very stable by an extensive cross-linking of protamines, sperm -specific basic proteins (Bedford and Calvin, 1974). Following sperm penetration into the oocyte cytoplasm, the protamines are removed and replaced by histones, and the sperm nucleus is remodeled into a pronucleus with assembly of the nuclear envelope. The process of pronuclear formation is not fully understood at present. Reduction of the sulfate bond by glutathione (Sutovski et al., 1996) and nucleoplasmin from the germinal vesicle (Philpott et al., 1991; Maeda et al., 1998) seem to play key roles in the decondensation of sperm and the formation of the male pronucleus. Previously, sperm penetration into the zona pellucida of GV-stage oocytes and sperm decondensation in these oocytes have been reported in dog, cow, and pig (Mahi and Yanagimachi, 1976; Niwa et al., 1991; Wang et al., 1994). However, the degree of sperm nuclear decondensation appears different among these species; in the dog and cow, sperm nuclei are fully decondensed, but in the pig they are only partially decondensed. It seems that the amount or activity of the factor, such as reduced glutathione (Wiesel and Schultz, 1981), that is contained in the cytoplasm of GV oocytes may vary in different species. Much lower amounts of glutathione is contained in GV-stage porcine oocytes as compared to fully matured oocytes (Yoshida et al., 1993), which may induce incomplete progress of the decondensation of male chromatin following sperm injection into GV-stage oocytes.

The nucleous is the site of ribosomal RNA synthesis and processing. In the bovine embryos, the appearance of functional nucleoli is preceded by the presence of dense nucleolar precursor bodies (NPBs) until the two-to four-cell stage, when nucleologenesis begins (Plante and King, 1994; King et al., 1989; Kopecny et al., 1989). The morphological changes that characterize nucleogenesis occur in a stepwise manner (King et al., 1989), and are after used to monitor transcriptional activity in developing embryos (Kopecny and Niemann. 1993). Nucleogenesis progressed through the four stages of development from a dense NPB to a fully-activity fibrillogranullar nucleolus (King et al., 1989).

Ultrastructural studies showed that dense spherical nucleus precursor bodies are observed in both female and male pronuclei of porcine oocytes after the injection of murine sperm. It has been proposed that human nuclear precursor bodies are formed by the aggregation of small dense bodies, which arise in association with condensed chromatin (Tesarik and Kopecny, 1989). However, this phenomenon is not seen in porcine zygotes (Laurincik et al., 1995). In the present study we found intranuclear granules of varying size and shape as early as the pronuclear stage, similar to what is observed in the pronuclei of porcine zygotes *in vivo* (Laurincik et al., 1995).

Conspicuous intranuclear particles or granules have been seen in pronuclei during mammalian fertilization (Szollosi et al., 1990; Laurincik et al., 1995), and are thought to be involved in RNA/ribonucleoprotein metabolisms in early embryos (Fakan and Odartchenko, 1980; Szollosi et al., 1990). The present study revealed that annulate lamellae develop in mature pronuclei. Intranuclear annulate lamellae have been reported in normally fertilized human (Soupart and Strong, 1974), bovine (Crozet, 1984), and porcine (Szollosi and Hunter, 1973) zygotes. In human ova, annulated lamellae are predominantly found in proximity to the pronuclei (Soupart and Strong, 1974), and they are more frequent in oocytes with polyspermic pronucleus

development. In rabbit oocytes they are not seen until the fully developed pronuclei migrate to the oocyte center. As suggested earlier, lamellae may result from the overproduction of nuclear envelope fragments, with which they share structural similarities (Soupart and Strong, 1974; Thompson et al., 1974).

As observed for fertilization with isogenic species sperm (Laurincik et al., 1995), characteristic undulating of the nuclear envelope is seen in porcine oocytes following murine sperm injection. This structural change may be required for specialized nucleocytoplasmic transport. Extensive development of Golgi complex, cisternae of endoplasmic recticulum and annulated lamellae in the vicinity of the apposed pronuclei is seen following murine sperm injection (Szollosi and Hunter, 1973), which is a common feature of mammalian zygotes. Collectively, in the present study we did not see any critical ultra structures differences between male and female pronuclei following murine sperm injection, suggesting that non-species specific nuclear cytoplasmic interactions take place during pronuclear formation and apposition.

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