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Human Sperm Aster Formation and Chromatin Configuration in Rabbit Oocytes Following Intracytoplasmic Sperm Injection Using a Piezo-Micromanipulator

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ABSTRACT

In human fertilization, the sperm centrosome nucleates a radial array of microtubules called the sperm aster. The sperm aster is responsible for apposition of male and female pronuclei, and later gives rise to the first meiotic spindle. The objective of this study was to determine microtubule assembly and chromatin configuration in rabbit oocytes following intracytoplasmic injection with human sperm by piezo-driven pipette. Oocytes were collected from superovulated dose 14-15 h after hCG injection and were fertilized by injection of a single human sperm into the ooplasm of each oocyte without additional activation treatment. Four hours post heterologous intracytoplamic sperm injection (ICSI), rabbit eggs were fixed and microtubule organization and chromatin configuration were examined by immunofluorescence microscopy. In unfertilized oocytes, microtubules were present only in the metaphase-arrested second meiotic spindle. Following human sperm injection, an aster of microtubules formed adjacent to the sperm head, around mid-piece, and sperm aster was enlarged and assembled around male and female pronuclei. During pronuclear centration, male and female pronuclei were surrounded by a microtubule array without nucleation sites. With fertile human sperm, the sperm aster formation rate was 54.6%. From our data we concluded that human spermatozoa can be injected successfully into rabbit oocytes, resulting in a reasonable survival rate, and that rabbit oocytes provide a reliable tools for assessing human sperm centrosomal function using the Piezo-ICSI system. *Iran. Biomed. J. 8 (2): 63-68, 2004*

Keywords: Centrosome, Fertilization, Intracytoplamic sperm injection (ICSI), Rabbit eggs

INTRODUCTION

Intracytoplamic sperm injection (ICSI) is useful when there are too few sperms for a successful *in vitro* fertilization, or when the sperm quality is so poor that they are unable to initiate oocytes interaction to permit sperm penetration. Normal human offspring are now routinely produced by the injection of a single sperm cell into an egg [1]. However, the physiological basis of fertilization after ICSI is not fully understood. The fertilization ability of spermatozoa is a complex phenomenon involving many molecular and cellular events [2].

In human fertilization, sperm introduces the centrosome and incorporated sperm centrosome organizes the radially arrayed microtubules, or sperm aster. This structure attracts maternal proteins, including γ tubuline and the other proteins [3, 4]. These events facilitate pronuclear mobilization, unite paternal and maternal genome (syngamy) and form the meiotic spindle in preparation for cleavage [5, 6].

Since many ICSI experiments can not be performed in humans, for ethical reasons, animal models are desirable. Fertilization in rodents such as rat, mouse and hamster is poor model owing to their

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maternal inheritance zygotic centrosome that is in strong contrast with the paternal contributed assembly of human sperm centrosome during fertilization [7, 8].

Some causes of fertilization failure may be due to the defect in the sperm centrosome. These data demonstrated that there are several points at which fertilization arrest occurs, which seems to be the result of improper centrosome function [9-12]. Additionally, there is some evidence in cattle where the centriolar complex is paternally inherited, and sperm aster size differs with sperm quality, affecting fertilization and live birth rates [13]. Although, rabbits are similar to human from the stand point of meiotic potential inheritance, information on post fertilization events in rabbits are lacking. In rabbit fertilization, the centrosome is paternally derived into the egg, and sperm centrosome organized the sperm aster, as in human fertilization [14, 7]. Rabbit oocytes provide a useful model for assessment of sperm centrosomal function by heterologouse ICSI in which human sperms are microinjected into rabbit eggs [15].

However, activation rates of rabbit egg after ICSI are quite low [15]. Peizo-driven micromanipulators have also contributed to improving micro insemination techniques in mouse and other laboratory animals by increasing the oocyte survival rates and fertilization efficiency [16-19]. In this report we applied a piezo-ICSI system for successful heterologous ICSI using rabbit eggs and human sperm.Therfore, we determined the sperm aster formation rate and male pronuclear formation in rabbit eggs following human sperm injection.

MATERIALS AND METHODS

Oocyte and zygote collection. Mature female New Zealand white rabbits were superovulated with six consecutives s.c. injections of 0.5 IU FSH (Antrin; Denka pharmaceutical Co.Ltd, Tokyo) given 12 h apart followed by a single i.v injection of 75 IU hCG (Sankyozoki Co. Ltd, Tokyo). All oocytes were collected 14-15 h post hCG injection by flushing the oviducts after rabbits were killed by 10% KCl. Cumulus cells were removed by a brief incubation in 0.1%hyaluronidase (Sigma .St.louis .Mo) in M2 culture medium and corona cells were removed by repeated gentle pipetting with a small-bore pipette.

ICSI with human sperm using a Peizo micromanipulator. Human sperm samples were obtained from four fertile donors under informed consent and the sperm samples were frozen and thawed in a water bath at 37°C. Then, sperms were washed with modified human tubal fluid medium (Irvine scientific Co. Sant Ana, CA) supplemented with 10% serum substitute (Irvine Scientific) by centrifugation at 500 ×g for 5 min.The sperm pellet was resuspended and then added to M2 culture medium with 10% (v/v) polyvinylpyroline.

Immediately before sperm injection, a motile spermatozoon was selected and each sperm was injected using a Piezo-micromanipulator (MB-U; PrimTech.Tsuchiura, Japan) after immobilization by touching the sperm tail with the injection pipette.

In Piezo-ICSI, the zona pellucida was penetrated using several Peizo-pulses. Then the needle was allowed to penetrate deeply into the ooplasm without applying piezo pulse. The puncture of the oolema was confirmed by the ability of the oolema, which had been brought into the oocyte to return to its original position [16].

After injection, eggs were cultured in Hepes-buffered M 199 supplemented with 10% (v/v) FCS at 38.5°C with 5% CO_2 in air under mineral oil. After ICSI, the eggs were fixed and stained at 2, 3, 4, 6 and 8-10 hours.

Immunocytochemical detection of microtubules centrosome and DNA. Zona pellucidae were removed with 0.75% pronase (Sigma, St. Louis, Mo) after a brief exposure to acidified tyrodes solution (pH 2.5) at 37°C. After a 30-min recovery at 37°C, eggs were extracted by buffer M (25% v/v glycerol, 50 mM KCL, 0.5 mM $MgCL_2$, 0.1 mM EDTA, 1 mM EGTA, 50 mM imidazole hydrochloride and 1 mM 2-mercaptoetanole, pH. 6.8) and fixed in cold methanol according to the methods of Hewiston *et al.* [2]. Fixed eggs were then permeabilized overnight with 0.1 M PBS containing 0.1% (v/v) Triton X-100 detergent.

Microtubules were labeled with a mixture of monoclonal antibody against β-tubulin (clon 2-28-33; diluted 1:100 Sigma, St. Louis, Mo) and acetylated α-tubulin (clone 6-11-B1; diluted 1:100 Sigma, St. Louis, Mo). The primary antibodies were detected by fluorescin-conjogated goat anti-mouse immuno-globulin G (IgG; diluted 1:40; Zymed, Sanfrancisco, CA). The DNA was detected after labeling with 10 mg/ml of Hoechst 33342. Coverslips were mounted in antifad medium (Vectashied; Vector Labratories, Burlingame, CA) and examined using conventional epifluorescence microscopy (Lica, Germany). The images were recorded digitally and archived on magnet optical disk .

RESULTS

Microtubule and chromatin pattern in unfertilized rabbit oocytes. The organization of microtubules, centrosomal components and DNA were detected after immunocytochemical labeling and epifluorescence microscopy. The only microtubules present in the mature unfertilized oocytes are those found in the meiotic spindle (Fig. 1A, bright green).The chromosomes were aligned on the metaphase plate (Fig. 1A, blue). The meiotic spindle is arrested at second metaphase, and is oriented radially to the cell cortex with one spindle pole at the oocytes surface. No other microtubules are detected in the cytoplasm of unfertilized rabbit oocytes.

Microtubule and chromatin organization in rabbit oocyte after intracytoplasmic injection of human sperm. Microtubule and DNA configuration in rabbit oocytes were detected after the intracytoplasmic injection of human sperm. At 2-3 h post sperm injection (Fig. 1B).

The microtubular aster was organized from the neck of the spermatozoon. The sperm showed no signs of decondensation where as small female pronucleus had begun to develop. At 4 h, post sperm injection as sperm aster continued to enlarge; microtubules were organized around the male pronucleus and female pronucleus (Fig. 1C).

By 6 h post injection, the male and female pronuclei had enlarge and moved toward a more central position but were not opposed (Fig. 1D). In this stage, male and female pronuclei were surrounded by a microtubule array without nucleation sites. During decondensation of human sperm within rabbit oocytes, the sperm tail remain (Fig. 1C and E, arrow). Microtubules extending from the male pronucleus appear to contact the surface of the female pronucleus and microtubules emanating from pronuclear region are not detected. By 8-10 h post injection, the male and female pronuclei opposed to each other in the center of oocytes (Fig. 1E). During the development of pronuclei, the sperm aster continued to enlarge until it filled the entire cytoplasm. The sperm aster was not oriented preferentially toward the female pronucleus but instead assumed a perinuclear distribution around both parental pronuclei and, at this stage, no distinct nucleation sites were visible (Fig. 1D). After syngamy, the male pronucleus can be distinguished by their larger size compared to female pronucleus (Fig. 1E). The sperm aster may be between the two pronuclei (not shown), but more often the aster is positioned eccentrically on the male pronucleus.

Sperm aster formation rate. Rabbit eggs were examined for sperm aster formation at 4 h post-ICSI. As the human sperm head decondensed in rabbit egg cytoplasm, microtubule arrays were organized around the male pronucleus (Fig. 1B and C). We observed that in 41 of 75 (54.6%) eggs, the sperm aster was organized after human sperm injection.

DISCUSSION

In the present study, we demonstrated the patterns of microtubule and chromatin organization following human sperm injection in rabbit eggs by using a piezo-driven pipette. During fertilization in human, cow, monkey, rabbit, pig, sheep, the sperm introduces the centrosome and the microtubule organizing center of the cell [5, 7, 17, 20].The centrosome initiates polymerization of an aster of microtubules. These microtubules act as structural elements and guide the male and female pronuclei to a position tightly apposed to one another, in the center of the egg.

It has shown that some of human fertilization failures were the result of centrosome dys function to form sperm aster [5, 12]. Asch *et al.* [9] reported that some fertilization failures arise due to the specific defects within either gamet after sperm penetration, particularly in the microtubule ability of sperm centrosome. Recent improvement in fixation techniques for immunofluorescence microscopy allowed initial observations to be made about microtubule dynamics [21].

However, establishment of a hetrologous system to assess sperm centrosomal function might lead to improve the diagnosis and treatment for male and unexplained infertility. Fertilization in rodent is depend on maternal centrosome where as in humans the centrosome is paternally derived [7]. This is especially relevant with regard to hetrologous ICSI to assay sperm function [22], since human spermatozoa injection into the hamster oocytes will not nucleate microtubules [8], as they do after injection into the human oocytes [9]. In rabbit, the sperm midpiece is paternal component responsible for aster formation, since isolated sperm heads failed to produce similar results upon oocyte activation [14]. Microtubule and chromatin configuration of *in vitro* fertilized rabbit oocytes have been studied by Terada *et al.* [15]. They demonstrated that sperm aster is a temporal

structure and the microtubule organization around pronuclei may be important for pronuclear centration. In this study, we also showed that male derived centrosome nucleate microtubule and sperm are the paternal component for aster formation and also some midbody structure around the female pronucleus in 4 h post ICSI. Previous reports showed that sperm introduced the centrosome in rabbit egg during the fertilization [14, 15]. Szollosi *et al.* [23] reported perinuclear distribution of centrosomal-like structures by electron microscopy in rabbit zygotes. Sperm aster formation was observed at the sperm head in rabbit eggs after intracytoplasmic injection with human sperm (Fig. 1B and C).

However, we can assay the ability to nucleate

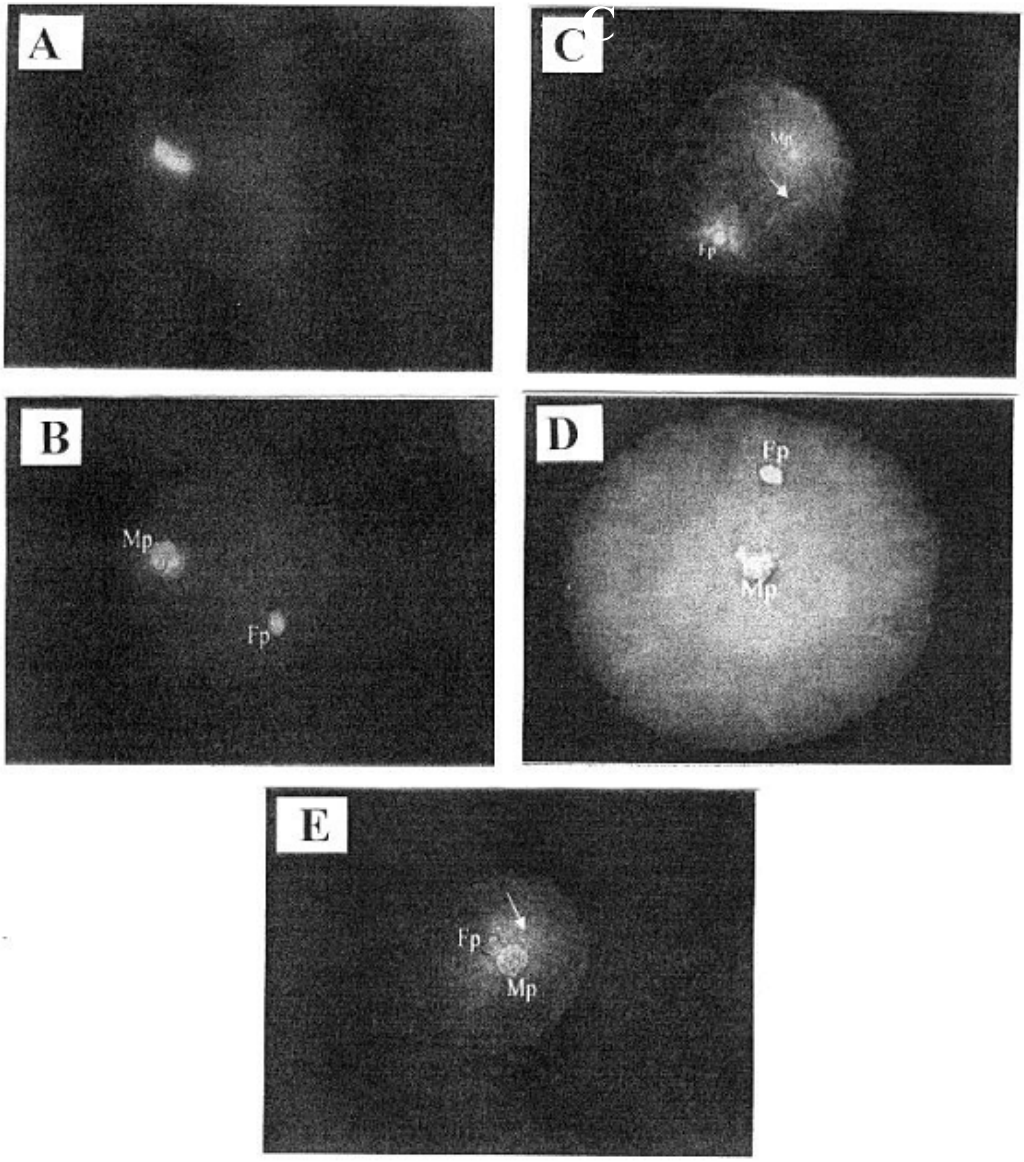

Fig. 1. Microtubule (green) and chromatin (blue) configuration in rabbit oocytes after intracytoplasmic injection with human sperm by a Piezo-driven pipette. **(A)** Within the unfertilized metaphase II-arrested oocytes, microtubules were confirmed to spindle, which held the maternal chromosomes along the meiotic plate. No other microtubules are detected in the cytoplasm of the unfertilized rabbit oocytes. At 2-3 h post ICSI, microtubules (arrow) are found in association with the incorporated sperm head. **(C)** As the male pronucleus continues to decondense in the cytoplasm male pronucleus, the microtubules of sperm aster enlarge circumscribing the male pronucleus (arrow: sperm tail). The midbody structure connecting the decondensing female chromosomes was observed (4 h). **(D)** During pronuclear centration, male pronucleus and female pronucleus were surrounded by a microtubules array (6 h). **(E)** The pronuclei move into apposition near the center of the oocytes and the sperm aster microtubule now span the entire cytoplasm. The sperm tail is associated with Mp (arrow). Bar = 25 μm.

sperm aster to undergo sperm decondensation using rabbit eggs. The release of sperm centriole into the oocyte cytoplasm during rabbit Piezo-ICSI is likely to be contributed by special mechanisms. Phosphorylation and dephosphorylation of proteins in the sperm tail connecting piece have been implicated in the ability of rabbit sperm to organize a sperm aster [14]. The presence of centrin and γ-tubulin and the state of centrosomal phosphorylation in non-rodent eggs demonstrate steps in the normal sequence of events that transform the mature mammalian sperm into an active participant in the zygote [3, 4].

Studies on cattle [18], rabbit [15, 14] zygotes and portentous have also supported a bi-paternal contribution to the zygotic centrosome. In the current study, injected oocytes cause signs of activation such as; reorganization of the meiotic spindle and progression through meiosis II, within 6h of sperm injection. Although, the time between sperm incorporation and the formation of the aster is variable among different species, due to the differences in the length of the cell cycle and stage at which fertilization take place, it is believed that the sperm centrosome components are required to form the paternal aster soon after fertilization [2, 3, 7, 24]. Unfortunately, little is known about the events occurring as early as 6 h after sperm penetration *in vivo* [20].

The result of this study showed that rabbit oocyte can be good model for assessment of human centrosomal function. Although this is not the first report of rabbit hetrologous ICSI, Terada *et al.* [15] reported human aster formation following ICSI into the rabbit egg was very low (36%). The success rate in this study is considerably higher than that of conventional ICSI [15]. Sperm injection into rabbit oocyte was previously considered to be difficult by conventional ICSI. Since the oolema of rabbit is very flexible and ooplasm has low viscosity, the conventional pipette can not readily break it [25].

Our observations on microtubule nucleation in rabbit oocytes after Piezo-ICSI strongly support that this injection system is more effective than conventional ICSI in rabbit. Why dose Piezo-ICSI work better than conventional ICSI? In contrast to conventional ICSI, sperm injection via a Piezo-driven micropipette was far less traumatic to the oocyte than the conventional method using a mechanically driven pipette. Therfore, the piezo-driven pipette could break the oolema readily and consistently [16, 18, 19]. When the piezo actuator is used, the oolema is broken readily and a spermatozoon can be injected without causing the lysis of the oocytes.

Several reports showing high survival and fertilization rate in mice, bovine and human by Piezo-ICSI compared with those obtained by conventional ICSI, which was characterized by low survival and fertilization rate [6, 18, 26]. These studies were confirmed that Peizo-ICSI was superior to the conventional ICSI. In the present study, human aster formation in rabbit eggs after Peizo-ICSI was almost 54% without additional activation treatment. As compared with conventional ICSI, human sperm aster rate with this system was higher [15]. Because in Piezo-ICSI no suction of ooplasm is conducted at the sperm injection, it is thought that the damage caused by Piezo-ICSI is much less than that of conventional ICSI. In rabbit ICSI, the biggest problem is low survival rate. This problem may be related to mechanical manipulation and the low efficiency of oocytes activation after ICSI. In rabbit, plasma membrane is more fragile and has lower survival rates after micromanipulation [25]. As a result, the secure injection of the spermatozoon into the oocytes was considered difficult, and Kimura and Yanagimachi [16] reported an 80% survival rate and 75% fertilization rate to injected oocytes. This is in part, due to the effective sperm injection into the oocytes [15]. These data indicate that this hetrologous ICSI system may be a more relevant way to assess the human centrosomal function. Rabbits have been used to produce ICSI offspring [24] but are not routinely used as a model for studying human ICSI because of the limited success. Furthermore, rabbit oocytes require an electrical or chemical stimulus after ICSI to ensure the resumption of meiosis, unlike human [27].

We used a novel system for assessment of sperm centrosomal function by hetrologous ICSI in which human sperm is microinjected into the rabbit eggs using a Piezo micromanipulator. We conclud that Piezo-ICSI of rabbit oocytes with human spermatozoa results in a relatively high rate of sperm aster formation. This method can be a relevant assay for human sperm centrosomal function and may lead to the discovery of a new type of infertility that has been treated as "unexplained infertility".

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