Sperm Aster Formation and Pronuclear Decondensation During Rabbit Fertilization and Development of a Functional Assay for Human Sperm

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ABSTRACT

Microtubule organization and chromatin configurations in rabbit eggs after in vivo rabbit fertilization and after intracytoplasmic injection with human sperm were characterized. In unfertilized eggs, an anastral barrel-shaped meiotic spindle, oriented radially to the cortex, was observed. After rabbit sperm incorporation, microtubules were organized into a radial array from the sperm head, and cytoplasmic microtubules were organized around the male and female pronuclei. These microtubules extended from the decondensed sperm head participated in pronuclear migration, and organization around the female pronucleus may also be important for pronuclear centration. Support for these observations was found in parthenogenetically activated eggs, in which microtubule arrays were organized around the single female pronucleus. We found microtubule organization to be higher in human babies following ICSI [2]. Clinically, the rate of sex chromosomal anomalies has been reported to be higher in human babies following ICSI (reviewed by Schatten et al. [1]).

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a widely used clinical assisted reproductive technique. However, little is known about the molecular and cellular mechanism of fertilization following ICSI (reviewed by Schatten et al. [1]). The rate of sex chromosomal anomalies has been reported to be higher in human babies following ICSI [2]. Clinically, ICSI is applied in the case of repeated fertilization failures following in vitro fertilization (IVF). Several reports indicate that many eggs diagnosed in the clinic as “unfertilized” actually initiate aspects of the fertilization process but arrest at specific stages. Furthermore, some of the fertilization failures arise from defects in the zygotic centrosome, the oocyte’s microtubule organizing center, after insemination [3, 4]. For such cases, ICSI may not be an effective alternative to achieve fertilization. If so, then a novel assay for the detection of centrosomal defects might be developed in a relevant animal model for the purpose of examining male factor or idiopathic infertility.

During fertilization, the centrosome is typically introduced by the sperm in primates [4, 5], cattle [6], pigs [7], and sheep [8]. Function of the zygotic centrosome varies among bulls during IVF, and this variation affects male fertility [9]. In contrast to most mammals, rodent fertilization is accomplished by maternally inherited centrosomes [10–13]. In mice, the paternal centrosome degenerates during spermiogenesis [14], and sperm asters are not observed at the base of the incorporated sperm head (reviewed by Schatten [15]). Astral microtubules are absent from the human sperm centrosome injected into hamster eggs [13], indicating that rodent eggs are probably not a suitable model for assessing the sperm centrosomal function for nonrodent sperm.

Rabbits are lagomorphs, and centrosomal inheritance has been characterized to follow the paternal pattern of centrosomal inheritance because of the presence of a monoastral sperm aster [16, 17]. In addition, isolated sperm heads without midpieces failed to nucleate sperm asters in rabbit eggs [18]. These reports suggest that rabbit eggs may be a suitable tool for assessing human sperm centrosomal function. Conversely, SzoÈllo Èsi et al. [19] reported perinuclear distribution of centrosomal-like structures by electron microscopy in rabbit zygotes. These observations conflict with the interpretation of a strict contribution of the paternal centrosome during rabbit fertilization.

Although a number of sperm centrosomal proteins have been characterized, interactions between centrosomal proteins and centrosomal functions are still elusive [20]. γ-Tubulin is an essential centrosomal protein serving both to nucleate microtubules and to define their intrinsic polarity [20–22]. Furthermore, a key step in the transformation of the inherited sperm centrosome into a functional zygotic centrosome capable of recruiting the necessary maternal cytoplasmic constituents for assembling the sperm aster is the attraction of maternal γ-tubulin protein [23]. Detection of γ-tubulin at the centrosome may, therefore, serve to indicate sperm centrosomal function in the early zygote.

To assess the centrosomal inheritance in rabbit fertilization, we examined microtubule organization and chromatin configurations in rabbit eggs after in vivo fertilization and artificial activation by injection of a crude sperm factor isolated from the rhesus monkey. We also observed astral mi-
cortubule assembly and γ-tubulin expression in the human sperm centrosome after intracytoplasmic injection of human sperm into rabbit eggs.

MATERIALS AND METHODS

Oocyte and Zygote Collection

All animal procedures were approved by the Oregon Health Sciences University (OHSU) Institutional Animal Care and Use Committee. Mature female New Zealand White rabbits were superovulated with six intramuscular injections of 0.3 mg FSH from porcine pituitary (Vetpharm, London, ON) followed by a single i.m. injection of 75 IU hCG (Profasi; Serono, Randolph, MA). Oocytes were collected by follicular aspiration 4–6 h post-hCG under anesthesia by tracheal intubation and maintenance with isoflurane gas (2.5%). Oocytes were cultured overnight in CMRL medium (Gibco BRL, Gaithersburg, MO) containing 20% inactivated fetal calf serum (FCS; Hyclone, Logan, UT), 10 mg/ml pituitary FSH, and 5 IU/ml hCG. Cumulus cells were removed by a brief incubation in 2 mg/ml hyaluronidase (Sigma, St. Louis, MO) in Tyrode’s albumin lactate pyruvate solution with 20 mM Hepes (TALP-Hepes), and only eggs that had arrested at the second meiotic metaphase were used for this study. Fertilized rabbit zygotes were collected from superovulated females 14–16 h postcoitum, by flushing the oviducts after the rabbits were anesthetized by tracheal intubation and maintenance with isoflurane gas. Zonae pellucidae were removed with 0.5% Pronase (Calbiochem, San Diego, CA) after a brief exposure to acidified Tyrode’s solution (pH 2.5) at 37°C. After a 30-min recovery at 37°C, eggs and zygotes were extracted by buffer M [25% [v:v] glycerol, 50 mM KCl, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, 50 mM imidazole hydrochloride, and 1 mM 2-mercaptoethanol, pH 6.8] and fixed in cold methanol according to the methods of Simmerly and Schatten [27]. Microtubules were labeled with a mixture of monoclonal antibody to β-tubulin (E7; diluted 1:5; Developmental Studies Hybridoma Bank, Iowa City, IA) and acetylated α-tubulin (clone 6-11-B1; diluted 1:100; Sigma) for the detection of the incorporated sperm axoneme. Primary antibodies were detected by fluorescein- or rhodamine-conjugated goat anti-mouse immunoglobulin G (IgG; diluted 1:40; Zymed, San Francisco, CA). Eggs and zygotes were then labeled with a polyclonal antibody raised against the C terminus of Xenopus laevis γ-tubulin (diluted 1:100; Babco, Richmond, CA) and detected using appropriate fluorochrome-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:40; Sigma). DNA was detected after labeling with 10 mg/ml Hoechst 33258. Coverslips were mounted in anti-fade medium (Vectashield; Vector Labs, Burlingame, CA) and examined using a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Thornwood, NY) or a scanning confocal microscope (Leica TCS NT; Leica Microsystems, Heidelberg, Germany). Images were pseudocolored using Adobe Photoshop 4.0 (Adobe Systems Inc., Mountain View, CA) and printed on a Sony dye sublimation video-printer (Sony, Carson, CA) as described previously [6].

RESULTS

Microtubule Organization and Chromatin Configuration During Rabbit In Vivo Fertilization

Microtubule and chromatin configurations during in vivo rabbit fertilization are presented in Figure 1. In unfertilized eggs (Fig. 1A), an anastral barrel-shaped meiotic spindle (green), oriented radially to the cortex, was observed, and chromosomes (blue) were aligned at the metaphase plate as previously reported [17]. After sperm incorporation, microtubules formed a radial aster from the base of the sperm head (Fig. 1B; arrow). As the sperm aster continued to enlarge, microtubules were organized around the male (Mpn) and female pronuclei (Fpn; Fig. 1C). During pronuclear centration, microtubule arrays without a distinct nucleation site were observed around the Mpn and Fpn (Fig. 1D; arrow: sperm tail). A confocal image demonstrates the perinuclear distribution of microtubules around both pronuclei (Fig. 1E). At prophase, an array of microtubules was observed around the condensing Mpn and Fpn (Fig. 1F). Conical images of microtubules (green) around pronuclei (blue) at similar stages indicated the presence of perinuclear
FIG. 1. Microtubule (green) and chromatin (blue) configurations during rabbit fertilization. A) In the fresh, unfertilized rabbit egg, the only microtubule organization observed was in the anastral, barrel-shaped second meiotic spindle. B) Sperm aster formation (arrow) at the base of the sperm head (14 h post-hCG), indicating the inheritance of a paternal centrosome. The midbody structure connecting the decondensing female chromosomes with the second polar body nucleus is seen in the right side of this image. C, D) Rabbit zygotes at 16 h and 18 h post-hCG, respectively (arrow: sperm tail). Note the extensive microtubule organization around both the Mpn and the Fpn. E) A confocal image of the microtubule patterns observed around the Mpn and Fpn during centration (18 h post-hCG; arrow: sperm tail). F) Prophase rabbit zygote (20 h post-hCG), with an extensive sheath of microtubules around the adjacent Mpn and Fpn. G) First mitotic prometaphase (22 h post-hCG; arrowheads: spindle poles). Note that the sperm axoneme remains associated with one of the developing mitotic spindle poles (arrow: sperm tail). H) Rabbit two-cell stage embryo (24 h post-hCG; arrow: sperm tail), demonstrating extensive microtubule organization around the daughter nuclei. Bar = 10 μm.
microtubule arrays without the distinct bipolar centrosomal arrays (Fig. 2), observations consistent with those in other species [15]. In the first mitotic prometaphase (Fig. 1G), an anastral barrel-shaped spindle (green) was formed with broad spindle poles (arrowheads). The sperm tail (arrow) was observed in close association with the spindle pole. At the two-cell stage (Fig. 1H), daughter nuclei were surrounded by a dense microtubule array, and the sperm tail (arrow) was found in one blastomere.

**FIG. 2.** Serial optical sections of microtubules (green) around pronuclei (blue) in the first mitotic prophase of an embryo as observed by confocal microscope. Both pronuclei are surrounded by dense microtubule arrays without nucleation sites, suggesting perinuclear distribution of maternal centrosomes. Bar = 10 μm.

**Microtubule Organization and Chromatin Configuration During Parthenogenetic Activation of Rabbit Eggs by Microinjection of Rhesus Sperm Factor**

Sperm factor injection into rabbit eggs induced egg activation effectively, and the observed microtubule and DNA configurations are shown in Figure 3. At the pronuclear stage, microtubule arrays (green) similar to the patterns of microtubules observed after in vivo fertilization were organized around the Fpn (blue) that formed after activation (Fig. 3, A and B). In the first mitotic prometaphase, a bipolar anastral barrel-shaped spindle was observed (Fig. 3C). During late telophase, microtubules were mostly observed at the poleward face of the separating, decondensing daughter chromosomes (Fig. 3D).

**Microtubule Organization, Chromatin Configuration, and γ-Tubulin Detection in Rabbit Eggs After Intracytoplasmic Injection with Human Sperm**

Microtubules, DNA configurations, and γ-tubulin were observed in rabbit eggs after intracytoplasmic injection with human sperm (Fig. 4). We observed that 26 of 72 (36.1%) eggs were activated with decondensed sperm nuclei following ICSI. Beginning 3–4 h postinjection, an astral array of microtubules (green) from the base of the microinjected human sperm head could be observed (Fig. 4A). γ-Tubulin (red) was detected in the sperm centrosome (Fig. 4B). As the human sperm head decondensed in the rabbit egg cytoplasm, microtubule arrays were organized around both the Mpn and Fpn (Fig. 4C; arrow: sperm tail), and γ-tubulin was still detected (Fig. 4D; arrowhead).

**DISCUSSION**

**Microtubule Organization and Chromatin Configuration During Rabbit Fertilization**

In rabbit fertilization, the presence of astral microtubules around the sperm head (sperm aster) has been observed by electron microscopy [16] and immunofluorescence [17, 18]. Our study also shows a sperm aster, indicating the inheritance of a paternal centrosome, in addition to cytoplasmic microtubules organized around the Fpn, which is without a distinct nucleation site. We have also observed microtubule assembly in aged unfertilized rabbit eggs (cytasters; our unpublished observations) as well as around the Fpn in parthenogenetically activated rabbit eggs (see below). Confocal images of early pronuclear-stage zygotes confirm the dual source of microtubule organization within the cytoplasm. Collectively, these data suggest that centrosomal inheritance is biparental in rabbits, with both maternal and paternal contributions during early insemination. Interestingly, Szöllösi et al. [19] reported the presence of para-
crystalline structures and striated rootlets around rabbit pronuclei and suggested that these structures were new morphological forms of maternal centrosomes.

From these observations, we hypothesize that the sperm aster is a temporal structure, and that microtubule organization around the pronuclei, probably controlled by perinuclear distribution of maternal centrosomal constituents, may be important for pronuclear centration. The first mitotic spindle was barrel-shaped and anastral, with broad mitotic poles, suggesting a maternal contribution of centrosomal constituents in the first mitotic cycle. Conversely, a sperm axoneme was involved with a single spindle pole (Fig. 1G), suggesting the participation of the sperm centrosome in the formation of the first mitotic spindle. In the first mitotic spindles of humans [4], rhesus monkeys [5], and cattle [6], anastral poles are observed, and the sperm axoneme remains associated with one spindle pole, indicating the contribution of a paternal centriole in the first mitotic cycle.

Support for the maternal contribution of centrosomal constituents is found in parthenogenetically activated eggs following rhesus sperm factor injection (Fig. 3). Microtubule arrays were organized around the single Fpn (Fig. 3, A and B), and the first mitotic spindle was barrel-shaped with broad spindle poles (Fig. 3C). No organized microtubule arrays around the haploid Fpn can be seen in parthenogenetically activated eggs of rhesus monkeys [5] and cattle [6]. In rabbit parthenogenetically activated eggs, centrioles were not observed in earlier stages of development, with de novo formation of centrioles only observed as early as the morula or early blastocyst stages [28].

From these observations, we conclude that a biparental centrosomal contribution is needed for completion of rabbit fertilization. This is different from previous reports that suggest paternal inheritance of microtubule organization in rabbit fertilization [16, 17].

Can Rabbit Eggs Be a Tool for Assessing the Centrosomal Function in Human Sperm?

Our previous studies indicated that about 50% of the eggs clinically diagnosed as “unfertilized” had arrested at specific stages after sperm incorporation, and that some of these fertilization failures were the result of microtubule or centrosome dysfunction [3, 4]. In these cases, ICSI may not be an effective fertilization alternative. Establishment of a system to assess sperm centrosomal function might lead to improved diagnosis and treatment for male and unexplained infertility. However, there is currently no assay in use at fertility clinics to assess sperm centrosomal function in terms of the cytoskeletal events necessary to complete normal fertilization.

The injection of human sperm into hamster eggs is an inappropriate in vitro model for human fertilization because hamster fertilization relies on a maternal centrosome, and human sperm do not undergo microtubule assembly in hamster eggs [13]. Such assays should use eggs that have a paternally derived centrosomal system (e.g., cattle). However, activation rates of cow eggs after ICSI are quite low even when some novel methods are used [29], and manipulation is difficult [1].

The presence of a sperm aster in rabbit fertilization [16,
FIG. 4. Microtubule (green) and chromatin (blue) configuration and γ-tubulin (bright red) detection in rabbit eggs after intracytoplasmic injection with human sperm. **A** Beginning at 3–4 h post-ICSI, an astral array of microtubules (arrow) from microinjected human sperm heads (arrowhead) could be observed. **B** γ-Tubulin (arrow) was also detected in the sperm head (arrowhead). **C** During pronuclear formation, astral arrays of microtubules did not radiate into the entire cytoplasm, and both Mpn and Fpn were surrounded by a microtubule array detected by confocal microscopy (arrow: sperm tail). **D** During pronuclear centration, Mpn and Fpn were surrounded by a microtubule array without nucleation sites (arrow: sperm tail) and γ-tubulin was still detected in sperm centrosome (arrowhead). Bar = 10 μm.

17] and the absence of a sperm aster around microinjected sperm heads without the sperm midpiece in rabbit eggs [18] suggest that the rabbit egg may be a novel model for assessing human centrosomal function. Sperm aster formation was observed at the sperm head in rabbit eggs after intracytoplasmic injection with human sperm (Fig. 4A). At the pronuclear stage, microtubule astral structures were changed around the pronuclei (Fig. 4, C and D). The patterns of microtubule organization were almost the same as in the rabbit fertilization process described above.

Detection of γ-tubulin in fertilized human and rhesus monkey zygotes has been reported [4, 30]. γ-Tubulin in zygotes is thought to be largely composed of attracted maternal proteins with perhaps some paternal protein contribution [15, 31]. Furthermore, γ-tubulin assembles with pericentrin by a dynein-driven process and induces microtubule nucleation and spindle organization [32]. The development of an assay using *Xenopus laevis* egg extracts might be a test for centrosomal function using sperm from men with questionable fertility. In humans, γ-tubulin is largely inaccessible in mature sperm until after disulfate bond reduction. Exposure of disulfate-reduced sperm in cytostatic factor-arrested *Xenopus* cell-free extracts dramatically increases γ-tubulin detection at the sperm centrosome and is thought to be a principal step for sperm aster formation in vitro [23].
It is premature to conclude that centrosomal function in human sperm can be assessed using the rabbit system. However, we can assess the ability to nucleate a sperm aster and to undergo sperm decondensation, and the capacity to accumulate γ-tubulin, one of the representative functional centrosomal proteins, using rabbit eggs. Further studies will be needed regarding the efficacy of these assays as a potential human infertility assay; however, the correlation between the accumulation of centrosomal proteins like γ-tubulin, sperm aster formation, and the further development of zygotes can now be examined in a relevant animal model.

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