—Mini Review—

Post-ICSI Cytoskeletal Dynamics during Fertilization

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Abstract: *Although intracytoplasmic sperm injection (ICSI) is an innovative treatment for male infertility, a significant number of clinical cases of fertilization failure remain. ICSI overcomes the difficulty in fertilization of sperm entry into the egg cytoplasm. The goal of fertilization, however, is the union of the male and female genomes; sperm incorporation into the oocyte is only the start of fertilization. Human sperm must perform three vital functions after entering the egg: it contributes to the male genome, awakens the quiescent egg, and crystallizes the motility apparatus that unites the sperm and egg nuclei, consummating the fertilization process. During fertilization in most mammalian species, including humans, the sperm introduces the centrosome, which acts as a microtubule organizing center (MTOC). By promoting pronuclear apposition and mitotic spindle formation, the sperm plays the leading part in the induction of "motility", post-ICSI, in fertilization. The present review introduces the challenges remaining in functional assessment of the human sperm centrosome and discusses the biparental (e.g. rabbit) and maternal (e.g. parthenogenesis) centrosomal contributions to microtubule organization during development.*

Key words: *Cytoskeleton, Fertilization, Sperm centrosome, Assisted reproductive techniques*

Introduction

Assisted Reproductive Technique (ART) is an innovative tool for the treatment of human sterility [1]. Little information exists, however, about the molecular and cellular events occurring in ART in humans [4]. From the gamete to the neonate, human reproduction involves a series of cell motility events, including both cell movement and morphological changes. The cytoskeleton plays a critical role in cell motility.

During human fertilization, the sperm introduces a centrosome, a microtubule organizing center (MTOC), into the oocyte and the radial array of microtubules emanating from the sperm centrosome, called the "sperm aster", is essential for both pronuclear movement and formation of the first mitotic spindle [3].

In this article, we review recent discoveries in the mechanisms governing cytoskeletal dynamics during fertilization. We also discuss our investigations into human sperm centrosomal function in detail. With regard to the MTOC in oocyte cytoplasm without sperm a centrosome, we discuss cytoskeletal dynamics in both rabbit fertilization, which exhibits biparental inheritance of centrosomes, and the parthenogenesis of bovine eggs.

Post-ICSI Sperm Centro Somal Function during Human Fertilization and a Functional Assay for Human Sperm Centrosomes by Heterologous ICSI

While numerous studies have assessed the fertility of human sperm, most only measure the sperm's ability to enter the egg cytoplasm. Fertilization, however, requires not only sperm entry, but also the union of the male and female genomes at the metaphase of the first mitosis. During human fertilization, the sperm introduces a centrosome, which serves as the microtubule organizing center (MTOC), allowing microtubules to be nucleated within the inseminated

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oocyte by the sperm centrosome. Organization of microtubules from the sperm centrosome is essential for both the movement and fusion of male and female pronuclei [5, 6]. The paternal centrosome replicates during the first cell cycle to form the two poles of the mitotic spindle that is required for cleavage.

Abnormal microtubule organization in human zygotes that have been clinically diagnosed as "unfertilized" suggests that centrosomal dysfunction contributes significantly to fertilization failure after proper sperm entry [7]. Rawe *et al.* [2] reported that of 150 human oocytes that failed to fertilize after ICSI, approximately half displayed activation failure, of which approximately 30% exhibited defects in pronuclear formation/ migration. The function of the zygotic centrosome varies among bulls during *in vitro* fertilization (IVF) and this variation affects male fertility [8]. These results suggest that sperm centrosomal function impacts on fertility in humans. As proper function of the human sperm centrosome is essential for human fertility, an appropriate assay examining centrosomal inheritance and function would likely benefit ART immensely. Direct assessment of human sperm centrosomal function, however, remains difficult. Recently, a novel method examining sperm centrosomal function using the heterologous ICSI system was reported, in which human sperm are microinjected into either rabbit [9, 10] or bovine [11–13] eggs. After incorporation of the human sperm into the eggs, we observed that the sperm aster was organized from the sperm centrosome and that the sperm aster enlarged as the sperm nuclei underwent pronuclear formation [14]. The sperm aster formation rate at 6 h post-ICSI was 60.0% in bovine eggs and 36.1% in rabbit eggs[9, 11]. In these systems, microtubule organization was derived from the paternal centrosome during fertilization, a similar process to the events of microtubule organization functioning in human fertilization. In rabbit eggs, human sperm aster formation rate correlated with the rate of cleavage, but did not reflect the rate of pronuclear formation in clinical IVF [10]. These results indicate an intimate relationship between infertility and sperm centrosomal dysfunction.

Globozoospermia is a special feature of teratospermia, in which sperm are characterized by a round head, the lack of an acrosome and acrosomal enzymes, and a disorganized mid-piece. Round-headed sperm cannot penetrate the zona pellucida of an egg, so fertilization cannot be achieved, resulting in infertility [15]. We assessed sperm centrosomal function in round-headed sperm by heterologous ICSI, using bovine eggs. The rate of sperm aster formation in eggs injected with round-headed sperm was 15.8%, which is significantly lower than the rates observed for eggs injected with fertile donor sperm [12]. Ethanol activation after ICSI improved male pronuclear formation in eggs injected with round-headed sperm to 84.9%. In contrast, ethanol activation did not improve the rate of sperm aster formation following ICSI with round-headed sperm (32.3%), suggesting that sperm centrosomal function is independent of the sperm's ability to activate the egg [12].

Dysplasia of the fibrous sheath (DFS), a rare form of teratospermia, results in infertility. DFS sperm are immotile due to structural deformities from the midpiece to the tail [16, 17]. These sperm also exhibit centrosomal dysfunction and both abnormalities are potential causes of infertility [13]. Even after ISCI, failure of either fertilization or embryonic development continued in several patients [18]. The rate of human sperm aster formation in bovine eggs injected with DFS sperm was less than 10% [13].

These results indicate that sperm from men with congenital teratospermia exhibit centrosomal dysfunction. The cause of centrosomal dysfunction in these forms of teratozoospermia is uncertain, although morphological abnormalities in the midpiece of the sperm have been reported [16, 19]. We have assessed the expression of centrin, a functional centrosomal protein, in normal and DFS sperm. In normal sperm, centrin [20] was expressed in the sperm midpiece in 100% of sperm, as assessed by immunofluorescence. In contrast, centrin immunolabeling was positive in only 2% of DFS sperm [21]. These data suggest that a structural defect in the sperm centrosome results in a functional deficit in the centrosomes.

Is it possible to restore defective human sperm centrosomal function? Nakamura *et al.* [21] reported an attempt to restore defective human sperm centrosomal function using a heterologous ICSI system. Prior to ICSI, sperm were treated with dithiothreitol (DTT), which reduces the disulphide bonds within the head and pericentriolar regions of the sperm, thus unraveling the sperm centrosome. After ISCI, the bovine oocytes were treated with the cytoskeletal stabilizer, paclitaxel. The combination of DTT and paclitaxel treatment promoted microtubule organization in heterologous ISCI using dead sperm from a fertile donor, which could not induce microtubule organization without treatment. This treatment, however, was ineffective for DFS sperm. The safety of this method should be discussed carefully. While these initial experiments were unsuccessful at treating the sperm defects, it may be possible in the

future to reverse the failure of sperm centrosomal function post-ICSI.

Currently available methods of ART do not address sperm centrosomal dysfunction. Further cell and molecular biological advances are needed to overcome post-ICSI fertilization failure caused by sperm centrosomal dysfunction.

Is Sperm Centrosomal Function Essential for the Completion of Fertilization?

In human fertilization, centrosomal inheritance is paternal and sperm centrosomal function is important for events in development immediately post-ICSI. However, the pattern of centrosome inheritance during fertilization differs between species. Appearance of the MTOC shows its centrosomal inheritance. In contrast to most other mammals, rodents exhibit maternal inheritance of centrosomes during fertilization [22–25]. In mice, the paternal centrosome degenerates during spermiogenesis [26], and sperm asters are not formed at the base of the incorporated sperm head [27]. Microtubules are instead organized from multiple centrosomal foci that preexist in the cytoplasm of unfertilized eggs [23, 28]. Thus, centrosomal inheritance in rodent fertilization is maternal and the sperm centrosome is dispensable for the completion of fertilization. The number of reports of successful full-term births in cloned mammals [29], even of parthenogenesis [30], have been increasing. These techniques make it possible to produce mammalian offspring without any contribution from a sperm centrosome. Thus, we are forced to ask the question, "Is sperm centrosomal function crucial for the completion of fertilization?"

Commonalities and Differences in Micorotubule Organization during Rabbit ICSI in the Presence or Absence of a Sperm Centrosome

Rabbit are lagomorphs, unique animals in which centrosomal inheritance during fertilization is a blend of paternal and maternal factors. Centrosomes were considered to follow a paternal pattern of inheritance, due to the presence of a monoastral sperm aster during fertilization [31, 32]. An isolated sperm head lacking a midpiece failed to nucleate sperm asters in rabbit eggs [33]. These reports support the concept that a functioning paternal centrosome is necessary for rabbit fertilization. During *in vivo* rabbit fertilization, microtubules are organized into a radial aster from the sperm head; later, cytoplasmic microtubules are organized around the male and female pronuclei without a distinct nucleation site [9]. Microtubule distribution at the late pronuclear stage, however, is more similar to that described for the mouse [24] than that described for humans. In parthenogenetically activated rabbit eggs, microtubule arrays organized around the single female pronucleus [9], *de novo* formation of centrioles was observed at the morula or early blastocyst stages, but were not observed in the first cell cycle of fertilization [34]. These reports indicate the possibility of a maternal contribution to fertilization.

Taken together, these observations suggest the possibility of biparental centrosomal contribution during rabbit fertilization, which contrasts with previous reports of a strictly paternal inheritance pattern [31, 32]. The specific roles of the paternal and maternal centrosomes, however, remain unclear in species with biparental inheritance.

Morita *et al.* tested a model of paternal centrosome dysfunction to identify the role of the sperm centrosome in rabbit fertilization [35]. The sperm centrosome was removed from the sperm nucleus by sonication. In this model of paternal centrosomal dysfunction, ICSI with an isolated sperm head was performed using a Piezo-driven pipette (Piezo-ICSI, Primtech, Tukuba, Japan) [36, 37]. As a control, rabbit Piezo-ICSI using intact rabbit sperm was also evaluated. To assess the relative contributions of paternal and maternal centrosomes during rabbit fertilization, microtubule organization and early embryonal development in rabbit zygotes following Piezo-ICSI with and without sperm centrosomes were compared. In Piezo-ICSI using intact sperm, the observed microtubule organization and chromatin configuration were similar to that observed in *in vivo* fertilization [9]. In the centrosomal dysfunction model, no aster formation could be observed in oocytes following injection with an isolated sperm head. Microtubule organization between male and female pronuclei was observed without a distinct nucleation site. In contrast, sperm aster formation was readily observed in eggs subjected to Piezo-ICSI with intact sperm. At the late pronuclear stage, ICSI with an isolated sperm head produced a similar microtubule organization as that seen in late pronuclear stage eggs after intact sperm injection. The first mitotic spindle was organized in eggs following ICSI with either an isolated sperm head or an intact sperm head. These results suggest that the maternal centrosome fulfilled the paternal sperm centrosomal function in its absence, as microtubule organization without a clear nucleation site

was not observed in oocytes following ICSI with an intact sperm. In rabbit oocytes, the function of the paternally-derived MTOC can be replaced by the maternal cytoplasmic centrosome, suggesting that normal fertilization in rabbits can progress in the absence of a sperm-derived centrosome.

How can normal fertilization proceed without a sperm-derived centrosome? Factors endogenous to the oocyte, including γ-tubulin or a self-organizing system of microtubules, may be able to compensate for the loss. γ-Tubulin is present in microtubule organization centers that are not associated with a morphological centriole [38]. During rabbit fertilization, the sperm centriole could attract oocyte γ-tubulin. The disappearance of γ-tubulin during spermiogenesis indicates that sperm aster nucleation is dependent on oocyte γ-tubulin [39]. Following ICSI without a sperm-derived centrosome, microtubule organization in the egg appears to be promoted by oocyte-derived γ-tubulin. In an independent experimental model, after a portion of the cytoplasm of a fish pigment cell was removed by a needle, new centrioles lacking a MTOC were observed in the detached cell [40]. Although this experiment did not utilize a gamete cell, the result indicates the possibility of a self-organizing system of microtubules. These results support the idea that the maternal cytoplasm contributes to the first cell cycle in rabbits.

Microtubule Organization during Mammalian Parthenogenesis: Cleavage in the Absence of the Sperm Centrosome

Parthenogenesis is an extraordinary process, in which the activated oocyte initiates full development in the absence of a genetic contribution from a male, resulting in a sexually mature adult. Parthenogenesis is observed in many insects, crustaceans, rotifers, and reptiles, but is not naturally seen in mammals. Artificial parthenogenesis, however, can be induced in mammals by artificial activation of the egg. In mammals, almost all parthenogenetic embryos die at the early stages of development. Kono *et al.*, however, reported that parthenogenetic mice generated from reconstructed oocytes containing two haploid copies of the maternal genome developed to adulthood [30].

In humans [42], rhesus monkeys [43], cows [44], and many other mammals [27], the egg loses its centrosome during oogenesis, but the sperm reintroduces a centrosome at fertilization to function as the MTOC. The sperm aster plays functions in pronuclear migration and positioning, a process that requires cytoplasmic dynein, a microtubule-based motor protein, and its cofactor dynactin [27, 45, 46]. Multiple studies have focused on the mechanisms governing microtubule organization, centrosome behavior, and pronuclear migration during fertilization. In parthenogenesis, however, the mechanisms of microtubule organization and pronuclear positioning in the absence of a sperm centrosome has not been elucidated.

Fertilization in rodents, unlike that in other mammals, relies on maternal centrosomes. After the sperm enters the rodent oocyte, the cytoplasm fills with microtubules in a disarrayed pattern, which ultimately move the pronuclei into close apposition at the cell center [23]. The microtubule and chromatin dynamics of rodent parthenogenesis are very similar to those observed during normal fertilization, with the exception that there is no male contribution. Hewitson *et al.* reported that cytoplasmic microtubules are first detected at the telophase-II stage in hamster parthenogenesis ane then enlarge throughout the cytoplasm, causing the female pronucleus, which is surrounded by disordered microtubules, to move towards a more central position [25].

While the paternal inheritance of functional centrosomes has been suggested during fertilization of non-rodent mammals, in some mammals, such as cows [47, 48], rabbits [33], pigs [49], and marsupials [50], parthenotes exhibit disarrayed microtubules in the cytoplasm shortly after artificial activation. These results suggest that mammalian oocytes have the competence to form functional centrosomes in the absence of any contribution from sperm.

Morito *et al.* [51] reported that maternal centrosomes in bovine parthenotes promoted cleavage without a sperm centrosome, as observed by imaging of microtubule organization, pronuclear position, and the distribution of γ-tubulin by immunocytochemistry and conventional epifluorescence microscopy. In bovine parthenotes treated with paclitaxel, cytoplasmic microtubule asters became organized shortly after chemical activation, with the microtubules radiating dynamically toward the female pronucleus. The patterns of microtubule localization correlated well with pronuclear movement to the cell center. Microtubules aggregated at regions of high γ-tubulin concentrations, although γ-tubulin did not localize into spots until the first interphase of bovine parthenogenesis. These findings indicate that γ-tubulin serves as the maternal centrosome, promoting cytoplasmic microtubule organization to move the female pronucleus to the cell

center. Thus, the maternal centrosome may serve as a functional centrosome in the absence of a sperm contribution, although this structure is less competent at microtubule organization in comparison to centrosomes including sperm centrosomal components.

Mammalian parthenotes can develop to late pre-implantation stages, and even in rare cases, to adulthood, although normal genomic imprinting requires a biparental nuclear contribution to reach full term and birth. Although the mechanisms governing microtubule organization and pronuclear positioning during mammal parthenogenesis have not been well examined, the observation of cytoplasmic microtubules during parthenogenesis in the absence of a sperm centrosome suggests that maternal centrosomes can organize microtubules. These centrosomes are likely responsible for pronuclear movement in parthenogenesis.

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