REVIEW ARTICLE

Microtubule assembly crucial to bovine embryonic development in assisted reproductive technologies

Shinichi HOCHI

Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Nagano, Japan

ABSTRACT

Centrosome integrity and microtubule network are crucial to the events around fertilization, including pronuclear development, migration and fusion, and the first mitotic division. The present review highlights the importance of bull spermatozoal centrosomes to function as a microtubule-organizing center for successful fertilization and the subsequent embryonic development. Spermatozoal centrosomes need to be blended with ooplasmic pericentriolar materials accurately to nucleate and organize the sperm aster. Dysfunction of the spermatozoal centrosomes is associated with fertilization failure, which has been overcome with supplemental stimuli for oocyte activation following intracytoplasmic sperm injection in humans. Even though the spermatozoal centrosomes are functionally intact, abnormal sperm aster formation was frequently observed in vitrified-warmed bovine oocytes, with delayed pronuclear development and migration. Treatment of the post-warm oocytes with Rho-associated coiled-coil kinase inhibitor or α-tocopherol inhibited the incidence of the abnormal aster formation, resulting in higher blastocyst yields following *in vitro* fertilization and culture. Thus, understanding of centrosomal function made it possible to improve the performance of advanced reproductive technologies.

Key words: aster formation, fertilization, microtubule-organizing center, oocyte vitrification, spermatozoal centrosomes.

INTRODUCTION

Many reproductive biotechnologies have been applied to efficient production of domestic species, such as cattle and pigs, with high economic values. Those originally developed in cattle include artificial insemination (AI) and multiple ovulations and embryo transfer (MOET), and successful cryopreservation of spermatozoa (Polge & Rowson 1952) and embryos (Wilmut & Rowson 1973) made these AI and MOET technologies more practical and available for commercial use because estrus synchronization of recipients is no longer necessary. Discovery of 'sperm capacitation' phenomenon (Chang 1959) activated the research field of *in vitro* fertilization (IVF), and IVF-derived calves were first obtained using *in vivo*-matured oocytes (Brackett *et al.* 1982). The IVF technique was successfully combined with *in vitro* maturation (IVM) of immature oocytes retrieved from abattoir-derived ovaries (Shioya *et al.* 1988) and *in vitro* culture (IVC) of the presumptive zygotes (Lu *et al.* 1988). Thus, large numbers of transferable bovine blastocysts can be prepared by the *in vitro* production (IVP) system.

Since Uehara and Yanagimachi (1976) reported that hamster oocytes microinjected with spermatozoa exhibited pronuclear development, the intracytoplasmic sperm injection (ICSI) technique has been used not only as a powerful research tool to study the fertilization process but also as one of the assisted reproductive technologies (ART) in various mammalian species, including rabbits (Iritani & Hosoi 1989), cattle (Goto *et al.* 1990), humans (Palermo *et al.* 1992) and small rodents (Kimura & Yanagimachi 1995; Hirabayashi *et al.* 2002). Despite the current routine use of the ICSI technique in humans to treat male factor infertility, bovine ICSI has not been very successful, probably due to failure of oocyte activation and compromised sperm chromatin remodeling, as well as technical difficulties (Hochi *et al.* 2011).

Further understanding of the mechanism responsible for fertilization and embryonic development would be helpful to efficiently increase the number of transferable bovine blastocysts produced by the IVP or ART system. Centrosome integrity and microtubule network are crucial to the events around fertilization, including the pronuclear development, migration and fusion, and the first mitotic division (Schatten *et al.* 1985). The

present review highlights the importance of spermatozoal centrosomes and microtubule assembly for successful fertilization and subsequent embryonic development in cattle.

CENTROSOMES AND CYTOSKELETONS

In the cellular cytoskeletal system, the microfilaments of actin (7 nm in diameter two-standard helix) are involved in cell shape modifications and movements, the intermediate filaments (10 nm in diameter) relate to mechanical resistance to stress, and the microtubules (25 nm in diameter cylindrical bundle, composed from heterodimers of α- and β-tubulin) form the spindle apparatus (Fuge 1974; Ferreira *et al.* 2009). The microtubules adhere to motor proteins such as dynein, dynactin and kinesin, which bind to molecules (such as enzyme or substrate) and to membranes of the organelles (such as mitochondria) and promote the movement along the microtubules (Steffen *et al.* 1997; Quintyne & Schroer 2002). The motor activity of dynein/dynactin-driven transportation directs to the minus-end, while that of kinesin-driven transportation directs to the plus-end. Thus, the microtubules are more directly involved in the process of organelle movement (Sun & Schatten 2006).

Typical centrioles are cylinder-shaped structures composed from nine symmetrically oriented microtubular triplets (500-nm in length, 200-nm in diameter). Standard centrosome is composed from a pair of the centrioles surrounded by pericentriolar materials (PCMs; more than 100 different types of proteins), such as γ-tubulin, centrin and pericentrin (Fig. 1). Their shapes and activities change dynamically among different stages of the cell cycle, and the centrosome structure is highly variable among different cell types and organisms. A spermatozoon of most mammalian species (except for rodents; referred later) has a pair of distinct centriolar structures, such as the proximal centriole located within the connecting piece under the sperm head and the distal centriole organized vertically to dispersedthe proximal counterpart and aligned with the sperm tail (Sathananthan *et al.* 1996). After gametogenesis, spermatozoon is left with centrioles but has dispersedlost most of the PCMs. In contrast, unfertilized oocyte has lost centrioles that can serve as a focal center for centrosomal aggregation, but has retained PCMs that are dispersed throughout the ooplasm (Schatten 1994). In pronuclear-stage zygotes as well as cycling cells, the centrosomes duplicate before entry into mitosis, split and then form the poles of bipolar spindles during the prometaphase (Vorobjev & Nadezhdina 1987; Crozet *et al.* 2000). Because an interphase network of microtubules and the mitotic bipolar spindle are nucleated from the centrosome, the centrosome is considered as the microtubule-organizing center (MTOC) (Glover *et al.* 1993).

The microtubule nucleating activity of MTOCs requires the presence of γ-tubulin. The γ-tubulin ring complexes, composed from γ-tubulin and other PCMs, are permanently associated with the centrosome core structure and nucleate microtubules throughout the cell cycle. Unfertilized oocytes contain γ-tubulin that is recruited to the sperm centrosome to nucleate increasing numbers of microtubules as the sperm aster grows. Accurate recruitment of γ-tubulin is important, as recruitment of insufficient amounts of γ-tubulin will result in aster formation abnormalities and decreased developmental potential. Sperm aster formation and size has been analyzed during bovine fertilization and correlated to *in vitro* embryonic development to the blastocyst stage (Navara *et al.* 1996). A bull-dependent variation was also shown in the degree of sperm-derived centrosome and aster organization (Navara *et al.* 1996) that affects male fertility and early development in humans (Rawe *et al.* 2002).
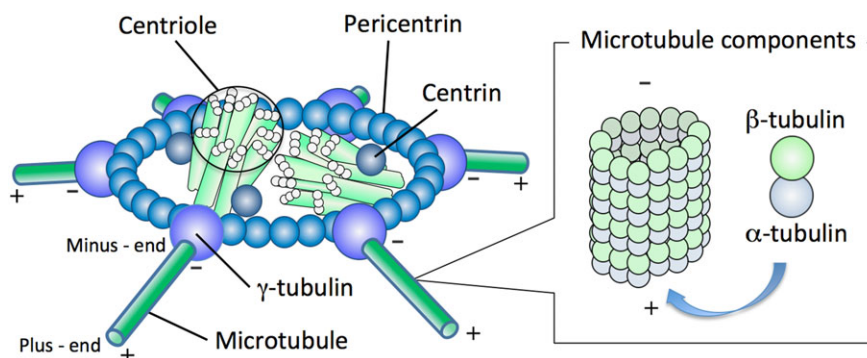

Figure 1 Centrosome structure. The standard centrosome is composed from a pair of centrioles, surrounded by pericentriolar materials such as pericentrin (ring structure), centrin and γ-tubulin in either fibrous or amorphous form. The molecules of γ-tubulin, access points for microtubules (25-nm in diameter cylindrical bundle, composed from heterodimers of α- and β-tubulin) are organized for aster formation by the conformational change through reduction of disulfide bonds in sperm centrosome and the recruitment of γ-tubulin molecules present in the ooplasm. During aster formation, microtubules anchored with their minus-ends are polymerized with recruiting α- and β-tubulin molecules toward the distal plus-end.

MICROTUBULE ASSEMBLY DURING FERTILIZATION

Once oocytes arrested at prometaphase-I resume the first meiotic division with stimulation by gonadotrophins, the nuclear envelope (germinal vesicle: GV) is disintegrated, allowing the nuclear materials to mix dispersedinto the ooplasm. Some alterations also occur in organelles such as mitochondria, cytoskeleton and cortical granules, along with disruption of oocyte-cumulus gap junctions. The GV-breakdown oocytes reach through metaphase-I, anaphase-I and telophase-I to metaphase-II (M-II) stage when ovulation takes place *in vivo*. Then, the oocytes become arrested again at the M-II stage till they are fertilized. During the oocyte maturation, microtubule asters appear close to the condensed chromatin in bovine oocytes (Albertini 1992; Li *et al.* 2005). The microtubules are nucleated at both poles anchoring the chromosomes (Schatten & Sun 2009). The lack of centrioles in the M-II spindle poles has been documented in many mammalian species, including mice, humans and cattle (Hertig & Adams 1967; Szollosi *et al.* 1972; Manandhar *et al.* 2005). The perinuclear MTOC stability at the meiotic poles is crucial for maintaining spindle integrity to ensure accurate microtubule-chromosome attachment and chromosome segregation. Dysfunction of the meiotic acentriolar MTOC is associated with inaccurate chromosomal segregation that may relate to female factor infertility and developmental abnormalities.

During fertilization, the centrosome brought into the M-II stage oocyte by spermatozoon plays a crucial role in assembly of the microtubule network (sperm aster, Fig. 2A) that brings both male and female pronuclei to the center of the newly formed zygote. Motor proteins, dynactin and dynein, are located on the surface of female pronucleus and male pronucleus in bovine zygotes, respectively (Payne *et al.* 2003). This phenomenon has been reported in many mammalian species, including humans (Simerly *et al.* 1995), rhesus monkeys (Hewitson *et al.* 1996), rabbits (Pinto-Correia *et al.* 1994) and cattle (Navara *et al.* 1994). Following centrosomal duplication, the microtubules are nucleated from paternal centrosomes at both poles anchoring the chromosomes during the first mitotic cleavage (Chen *et al.* 2003; Schatten & Sun 2009). For successful fertilization, sperm and oocyte centrosomal components need to be blended accurately to form a functional centrosome that can nucleate and organize sperm aster. Abnormalities of the spindle / MTOC function / sperm aster have been shown to directly correlate with the loss of developmental competence after IVF or ICSI, because they are crucial for migration and fusion of the pronuclei and formation of the first mitotic spindles (Schatten *et al.* 1985). The centrosome further serves as a unique signaling platform to recruit and distribute regulatory components and enzymes for cell cycle-specific and adaptive regulations. Impaired MTOC function as a possible cause of male factor infertility will be referred later.
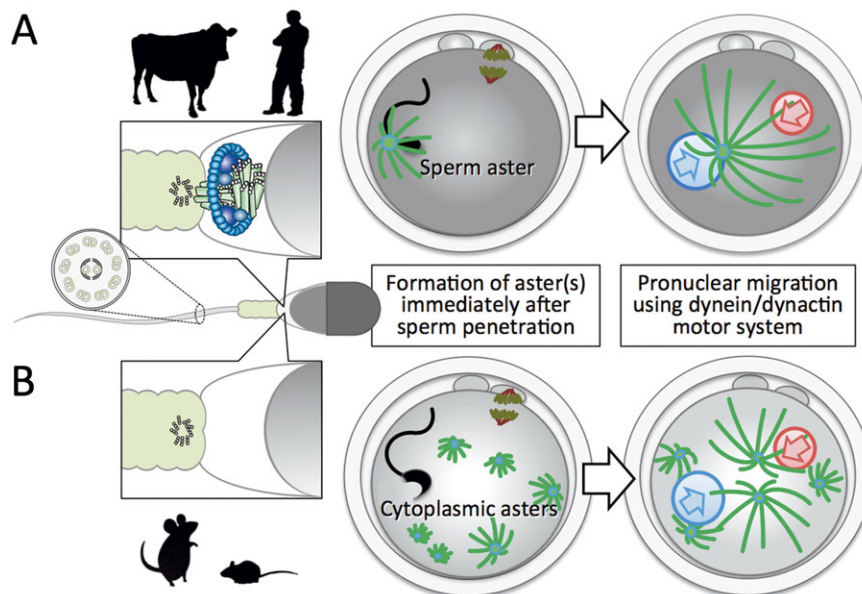
Figure 2 Species-specific organization of microtubule-organizing center (MTOC). (A) In many mammalian species, including primates and large domestic animals, spermatozoal centrosomes are brought into oocytes during fertilization and function as MTOC to form a monopolar array of microtubules, sperm aster. The well-developed microtubule network is essential for pronuclear migration to mix the male and female genomes using the dynein/dynactin motor system. (B) In rodents, paternal centrosomes degenerate during spermiogenesis while basal body ('9 triplets + 0' arrangement) for axoneme ('9 doublets + 2 singlets' arrangement) remains intact. Microtubules assemble with pericentriolar materials scattered in ooplasm during the pronuclear stage, and acentriolar cytoplasmic multiple asters can function as MTOCs.

On the other hand, the paternal inheritance of MTOC does not occur in rodents such as the mouse (Schatten *et al.* 1985) and the rat (Woolley & Fawcett 1973) and the microtubule network developed from multiple cytoplasmic asters, instead of a single sperm aster, is involved in the migration and fusion of pronuclei (Fig. 2B). The rodent spermatozoa lack centrioles and majority of PCMs after the spermiogenesis (Woolley & Fawcett 1973; Manandhar *et al.* 1998). Thus, the pattern of centrosome inheritance during fertilization is dependent on the animal species.

CHEMICAL TREATMENT IN BOVINE ICSI

In addition to studying the fundamental aspects around fertilization or to overcome male factor infertility, the homologous ICSI technique is used for offspring production with spermatozoa freeze-dried and rehydrated (Wakayama & Yanagimachi 1998), retrieved from testicular tissues (Silber *et al.* 1995) or exposed to foreign DNA (Perry *et al.* 1998; Hirabayashi & Hochi 2010). However, the ICSI procedure may disturb some post-fertilization events essential for early development, including sperm-induced oocyte activation, epigenetic remodeling of paternal genomes, and microtubule organization for pronuclear fusion (Yoshizawa *et al.* 2010; Hara *et al.* 2011). In cattle, the ICSI technique is applicable to achieve the best utilization of genetically superior bulls with low sperm concentration and/or low motility in their ejaculate, or suboptimal reaction with the *in vitro* capacitation process required for IVF protocol (Ushijima & Nakane 2006).

Pretreatment of bull spermatozoa with a reducing agent dithiothreitol (DTT) induced a partial decondensation of the sperm nucleus in the ICSI oocytes (Rho *et al.* 1998), probably due to the stabilized and condensed structure of sperm nucleus by disulfide bonds of protamine, a specific nuclear protein in the sperm (Calvin & Bedford 1971) which has been modified. The DTT treatment also improved the proportion of oocytes with a sperm aster-derived microtubule network on bovine ICSI (our unpublished results). The DTT not only destabilizes nuclear packaging in the sperm head, but also organizes γ-tubulin in the sperm centrosome, from which microtubules are nucleated. The conformational change induced by reducing the disulfide bonds would facilitate the γ-tubulin to access the microtubule components present in the ooplasm.

Unlike the IVF-derived bovine oocytes, the majority of bovine ICSI oocytes did not show normal pattern of calcium oscillations, resulting in the failure of oocyte activation (Malcuit *et al.* 2006), even though sperm-borne oocyte activating factor (SOAF), possibly phospholipase C-ζ (Fissore *et al.* 1995; Wu *et al.* 2001), was mechanically incorporated into the oocytes. To overcome this problem, bovine ICSI oocytes have been treated to induce intracellular calcium spike with a direct current pulse(s) (Hwang *et al.* 2000), calcium ionophore (Keskintepe & Brackett 2000), ionomycin (Rho *et al.* 1998; Galli *et al.* 2003; Oikawa *et al.* 2005; Abdalla *et al.* 2009) or ethanol (Horiuchi *et al.* 2002; Oikawa *et al.* 2005; Abdalla *et al.* 2009). Because these stimuli do not induce long-lasting oscillations, they have been combined with other chemicals, such as cycloheximide (Galli *et al.* 2003) or 6-dimethylaminopurine (Rho *et al.* 1998; Oikawa *et al.* 2005) that interfere with either the re-synthesis or the activation of the metaphase-promoting factor (MPF), respectively. In our laboratory, the best blastocyst yield from bovine ICSI oocytes (30% vs. 40% in IVF control group) is achieved by a supplemental activation treatment composed from 5 µmol/L ionomycin for 5 min (immediately after ICSI) plus 7% ethanol for 10 min (4 h after the ICSI), without additional chemicals for MPF inactivation (Abdalla *et al.* 2009).

ASSESSMENT OF CENTROSOMAL DYSFUNCTION

Interspecies assays with rodent oocytes have been used to evaluate the capacitation status via IVF (Hanada & Chang 1972) and the SOAF activity via ICSI (Rybouchkin *et al.* 1995). However, such rodent oocytes are not valid for assessing the functional integrity of non-rodent spermatozoal centrosomes, because the cytoplasmic asters play a crucial role in pronuclear development, migration and fusion in the rodent zygote (Schatten *et al.* 1985). In fact, normal human spermatozoa did not organize the sperm aster in hamster oocytes (Hewitson *et al.* 1997). Therefore, heterologous ICSI using oocytes from rabbits (Terada *et al.* 2002, 2004) or cattle (Nakamura *et al.* 2001; Terada *et al.* 2002) has been proposed to assess the dysfunction of human spermatozoal centrosomes in relation to male infertility. Bovine assay system is more convenient to achieve, because preparation of IVM oocytes is relatively easy as the abattoir-derived ovaries are available, and is used especially for clinical application in humans.

The proportion of bovine oocytes with human sperm aster formation is independent from semen characteristics and pronuclear formation rate on clinical IVF, but correlates with embryonic cleavage and clinical pregnancy (Yoshimoto-Kakoi *et al.* 2008). Spermatozoa from globozoospermia and dysplasia of the fibrous sheath (DFS) patients have centrosomal dysfunction, as shown by the decreased aster formation rates in bovine oocytes (Nakamura *et al.* 2002; Rawe *et al.* 2002). The low aster formation rate in globozoospermia (16% vs. 68% in normal sperm) doubled to 32% when supplemental oocyte activation treatment was given with ethanol to the post-ICSI bovine oocytes. An immunofluorescent study showed that expression of centrin is observed in only 2% of DFS patient sperm mid-piece (Nakamura *et al.* 2005). The intrinsic function of spermatozoal

centrosomes can be restored by a combined treatment of human dead sperm with DTT and of heterologous ICSI oocytes with paclitaxel (Taxol[TM]), which can act as a cytoskeletal stabilizer by enhancing microtubule polymerization; the expected effect is not found in DFS patients (Nakamura *et al.* 2005). Pre-ICSI treatment of human oocytes with calcium ionophore resulted in a successful pregnancy for a couple with sperm centrosomal dysfunction (Terada *et al.* 2009), and such a supplemental treatment for oocyte activation is used to increase the clinical pregnancy rate. A novel challenging approach may be to replace dysfunctional centrosomes with functional donor sperm centrosomes (Mitchison & Kirschner 1984).

HYPER-RESCUE OF CRYOPRESERVED OOCYTES

Successful cryopreservation of spermatozoa and embryos made bovine AI and MOET technologies more practical for commercial use. Thereafter, the bovine IVP system became a more or less routine technique with frozen semen and vitrified blastocysts. In contrast, cryopreservation of bovine oocytes, which can be combined with ART technologies such as ICSI, somatic cell nuclear transplantation or germinal vesicle transplantation, is less successful (Hwang & Hochi 2014). Low fertilization rate of cryopreserved oocytes were reported to be associated with chilling and freezing injuries, including zona hardening due to premature release of cortical granules (Carroll *et al.* 1990; Fuku *et al.* 1995) and spindle disorganization and loss or clumping of microtubules (Aman & Parks 1994). In addition, we propose a third hypothesis for cryodamage of bovine oocytes, in which multiple aster formation frequently observed in vitrified-warmed oocytes following IVF is related to loss of ooplasmic function responsible for normal microtubule assembly (Hara *et al.* 2012). These multiple asters differ from cytoplasmic asters as seen in rodents, and pronuclear development and migration in such post-warm bovine oocytes are significantly delayed.

Vitrification protocol, characterized by an extremely high cooling rate, has been well adapted to oocytes with various types of cryodevices such as open-pulled straws (Vajta *et al.* 1998) or Cryotop (Kuwayama *et al.* 2005). Oocytes from domestic species are rich in cytoplasmic lipid droplets that can serve as energy sources but are increased to chilling sensitivity during cryopreservation. Supplementation of L-carnitine to IVM medium of bovine oocytes has been reported to reduce the amount of cytoplasmic lipid droplets and improve the cryotolerance of the oocytes at the blastocyst yield of 34% (Chankitisakul *et al.* 2013), but it is still controversial whether the positive effect of L-carnitine is reproducible (Phongnimitr *et al.* 2013). Supplementation of L-carnitine to IVC medium reduced the lipid content in IVF-derived bovine embryos and increased cryotolerance

and developmental competence (Takahashi *et al.* 2013). Stabilizing cytoskeletal systems during vitrification may be beneficial to improve oocyte cryotolerance. Treatment of bovine IVM oocytes with paclitaxel (Morató *et al.* 2008) prior to vitrification improved their revivability, but the positive effect of paclitaxel on blastocyst yield is very limited.

The incidence of multiple aster formation, a possible cause for low developmental potential of vitrified-warmed bovine oocytes as described above, can be inhibited by a short-term culture of the post-warm oocytes in the presence of Rho-associated coiled-coil kinase (ROCK) inhibitor (Y-27632), with an improved blastocyst yield of 21% after vitrification (Hwang *et al.* 2013). ROCK can regulate cellular growth, adhesion, migration, metabolism and apoptosis through controlling the actin-cytoskeletal assembly and cell contraction (Riento & Ridley 2003). Because the ROCK regulates microtubule acetylation via phosphorylation of the tubulin polymerization-promoting protein 1, inhibition of ROCK activity resulted in increased cellular microtubule acetylation (Schofield *et al.* 2012). Use of an antioxidant α-tocopherol (vitamin E) during the recovery culture also rescued the post-warm bovine oocytes to give the maximum blastocyst yield at 37% (Yashiro *et al.* 2015). Interestingly, another antioxidant ascorbic acid (vitamin C) was not effective in post-warm oocyte rescue. α-tocopherol also inhibited the formation of multiple asters in the post-warm oocytes, although the action mechanism is unknown. Thus, chemical treatment of bovine oocytes before or after vitrification protocol enables increases in their revivability to 20–40% when evaluated with blastocyst yield.

CONCLUSIONS

Sperm centrosomal integrity and microtubule network are crucial to post-fertilization events, including pronuclear development, migration and fusion, and the first mitotic division. Spermatozoal centrosomes need to be blended with ooplasmic PCMs accurately to act as MTOC and organize the sperm aster. Dysfunction of human spermatozoal centrosomes can be assessed by interspecies ICSI using bovine oocytes, and supplemental stimuli for oocyte activation (e.g. ethanol and ionomycin) and/or for microtubule stabilization (e.g. DTT and paclitaxel) may improve fertilization outcome and the subsequent embryonic development. Abnormal incidence of sperm aster formation in vitrified-warmed bovine oocytes can be inhibited by treatment either with ROCK inhibitor or α-tocopherol, resulting in higher blastocyst yields. Thus, understanding of centrosomal function contributed to improve ART performance.

ACKNOWLEDGMENTS

his careful reading and comments and Mr. Kazuya Tashima (Shinshu University, Nagano, Japan) for his excellent contribution in preparing illustrations.

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