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Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization

(centrioles/cytoskeleton/embryogenesis/maternal inheritance/microtubule organizing centers)

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ABSTRACT **Microtubules forming within the mouse egg during fertilization are required for the movements leading to the union of the sperm and egg nuclei (male and female pronuclei, respectively). In the unfertilized oocyte, microtubules are predominantly found in the arrested meiotic spindle. At the time for sperm incorporation, a dozen cytoplasmic asters assemble, often associated with the pronuclei. As the pronuclei move to the egg center, these asters enlarge into a dense array. At the end of first interphase, the dense array disassembles and is replaced by sheaths of microtubules surrounding the adjacent pronuclei. Syngamy (pronuclear fusion) is not observed; rather the adjacent paternal and maternal chromosome sets first meet at metaphase. The mitotic apparatus emerges from these perinuclear microtubules and is barrel-shaped and anastral, reminiscent of plant cell spindles; the sperm centriole does not nucleate mitotic microtubules. After cleavage, monasters extend from each blastomere nucleus. The second division mitotic spindles also have broad poles, though by third and later divisions the spindles are typical for higher animals, with narrow mitotic poles and fusiform shapes. Colcemid, griseofulvin, and nocodazole inhibit the microtubule formation and prevent the movements leading to pronuclear union; the meiotic spindle is disassembled, and the maternal chromosomes are scattered throughout the oocyte cortex. These results indicate that microtubules forming within fertilized mouse oocytes are required for the union of the sperm and egg nuclei and raise questions about the paternal inheritance of centrioles in mammals.**

Fertilization results in the union of the parental genomes, and in most animals a microtubule-containing cytoskeleton forming within the activated egg participates in the motility necessary for the cytoplasmic migrations of the sperm and egg nuclei (reviewed in ref. 1). The participation of the egg microtubules during mammalian fertilization is less well understood, though microtubule inhibitors (2–4) prevent the completion of meiosis, resulting in polyploidy; microtubules have also been found within fertilized mammalian eggs with electron microscopy (5–8) and during oogenesis with immunofluorescence microscopy (9).

To explore the participation of egg cytoplasmic microtubules during mammalian fertilization and early development, we have performed anti-tubulin immunofluorescence and transmission electron microscopy on mouse oocytes and zygotes* throughout fertilization and have studied the effects of microtubule inhibitors. These results indicate that the egg cytoplasmic microtubules, organized by sources other than the sperm centriole, are required during mammalian fertilization.

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MATERIALS AND METHODS

Virgin CD-1 mice (Charles River Breeding Laboratories) were superovulated with 10 international units of pregnant mare serum followed 48 hr later with 10 international units of human chorionic gonadotropin (10) and introduced to experienced males. After mating, fertilized oocytes were collected (11, 12) and maintained at 37°C in 114 mM NaCl/3.2 mM KCl/2 mM $CaCl_2$/0.5 mM $MgCl_2$/2 mM $NaHCO_3$/0.4 mM NaH_2PO_4/5 mM glucose/10 mM sodium lactate/0.1 mM sodium pyruvate/10 mM Hepes/100 units of penicillin G per ml/10 μg of phenol red per ml/2 mg of bovine serum albumin per ml (13). The cumulus and zona were removed with 0.1% hyaluronidase and 0.5% Pronase, respectively.

For anti-tubulin immunofluorescence microscopy the oocytes were affixed to polylysine-coated coverslips (14), extracted in a microtubule-stabilization buffer composed of 25% (vol/vol) glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM 2-mercaptoethanol, and 50 mM imidazole·HCl at pH 6.7, with 2% Triton X-100 for 60 min at 37°C (15) and rinsed with phosphate-buffered saline, and the microtubules were detected with monospecific affinity-purified antibody to porcine brain tubulin (16–18). Second antibody alone (fluorescein-labeled goat anti-rabbit IgG, Miles) did not label oocytes, and the enzymatic removal of the zona and cumulus did not affect the microtubule configurations. Transmission electron microscopy was performed on glutaraldehyde-fixed oocytes processed by conventional methods. The effects of microtubule inhibitors, Colcemid (50 μM), griseofulvin (100 μM), and nocodazole (10 μM) were explored during fertilization *in vitro* (19) and compared with untreated controls.

RESULTS

The meiotic spindle of unfertilized oocytes is anastral, barrel-shaped, and attached to the oocyte cortex (Fig. 1 *A* and *B*; ref. 9). After ovulation and frequently at the time for sperm incorporation, about a dozen (mean ± SD: 12.9 ± 3.5) small cytoplasmic asters assemble (Fig. 1*B*); at times these asters are in association with the oocyte cortex.

During sperm incorporation (Fig. 1 *C* and *D*), these asters enlarge and are often found in association with the pronuclei; the meiotic spindle has rotated, with the resultant formation of the second polar body with a midbody of microtubules persisting.

As the pronuclei develop (Fig. 1 *E* and *F*), microtubules are found to fill the entire cytoplasm with a fine latticework

*Since the events during mammalian fertilization overlap with both meiosis and mitosis, and since pronuclear fusion never occurs, the term "oocyte" is used here prior to the pronucleate stage and then "egg" and "zygote" are used interchangeably.

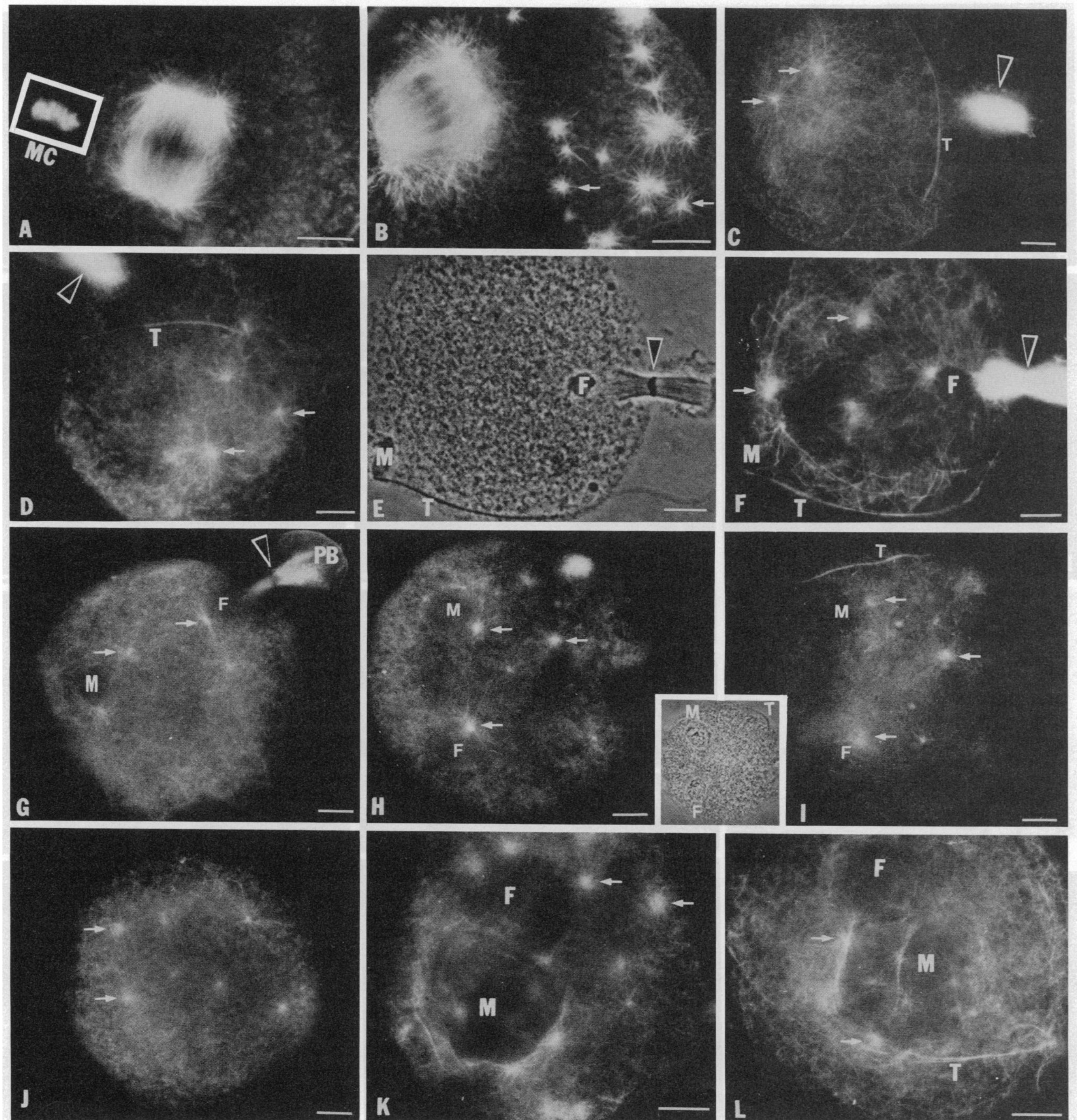

FIG. 1. Microtubules during sperm incorporation and in pronucleate eggs. Unless noted otherwise, photographs are anti-tubulin immunofluorescence. (Bars = 10 μm.) (*A*) Unfertilized oocyte. The meiotic spindle is usually the sole microtubule-containing structure in the unfertilized oocyte; it is barrel-shaped and anastral, with broad meiotic poles, and anchored parallel to the oocyte cortex. (*Inset*) Meiotic chromosomes (MC) detected with DNA fluorescence. (*B*) Cytoplasmic asters in unfertilized oocyte. Several hours after ovulation, about a dozen cytoplasmic asters (arrows) assemble throughout the cytoplasm. (*C* and *D*) Sperm incorporation. At sperm incorporation, the microtubules of the axoneme (T), the meiotic midbody (△), and the cytoplasmic asters are apparent. (*E* and *F*) Early pronucleate eggs, 6 hr after ovulation. *E* is a phase-contrast micrograph; *F* is the same cell, with anti-tubulin immunofluorescence. Microtubules are found in the incorporated axoneme (T), in the midbody of the rotated meiotic spindle (△), and ramifying throughout the cytoplasm as a latticework extending from asters (arrows), some of which are in association with each pronucleus. The asters are not organized by the base of the incorporated sperm axoneme. M, incorporated sperm nucleus; F, female pronucleus. (*G–J*) Pronucleate eggs, 12 hr after ovulation. As the male and female pronuclei form, the cytoplasmic asters enlarge, and a pair associate with the pronuclei (arrows). PB, Polar body nucleus. (*Inset*) Phase-contrast micrograph. (*K* and *L*) Late pronucleate eggs, 18 hr after ovulation. As the pronuclei are moved together to the egg center, a dense array of microtubules forms. This array has focal sites with the pronuclei embedded within its center.

organized by several foci. Some foci are associated with the male and female pronuclei, and others are free in the cytoplasm; the base of the sperm axoneme does not nucleate astral microtubules.

As the pronuclei enlarge and are moved from the surface (Fig. 1 *G–J*), asters are found extending from the sperm and egg nuclei. When the pronuclei reach the egg center, a dense array of microtubules is assembled, with the adjacent but separate pronuclei embedded at the center (Fig. 1 *K* and *L*).

Pronuclear fusion is never observed, and at the end of first

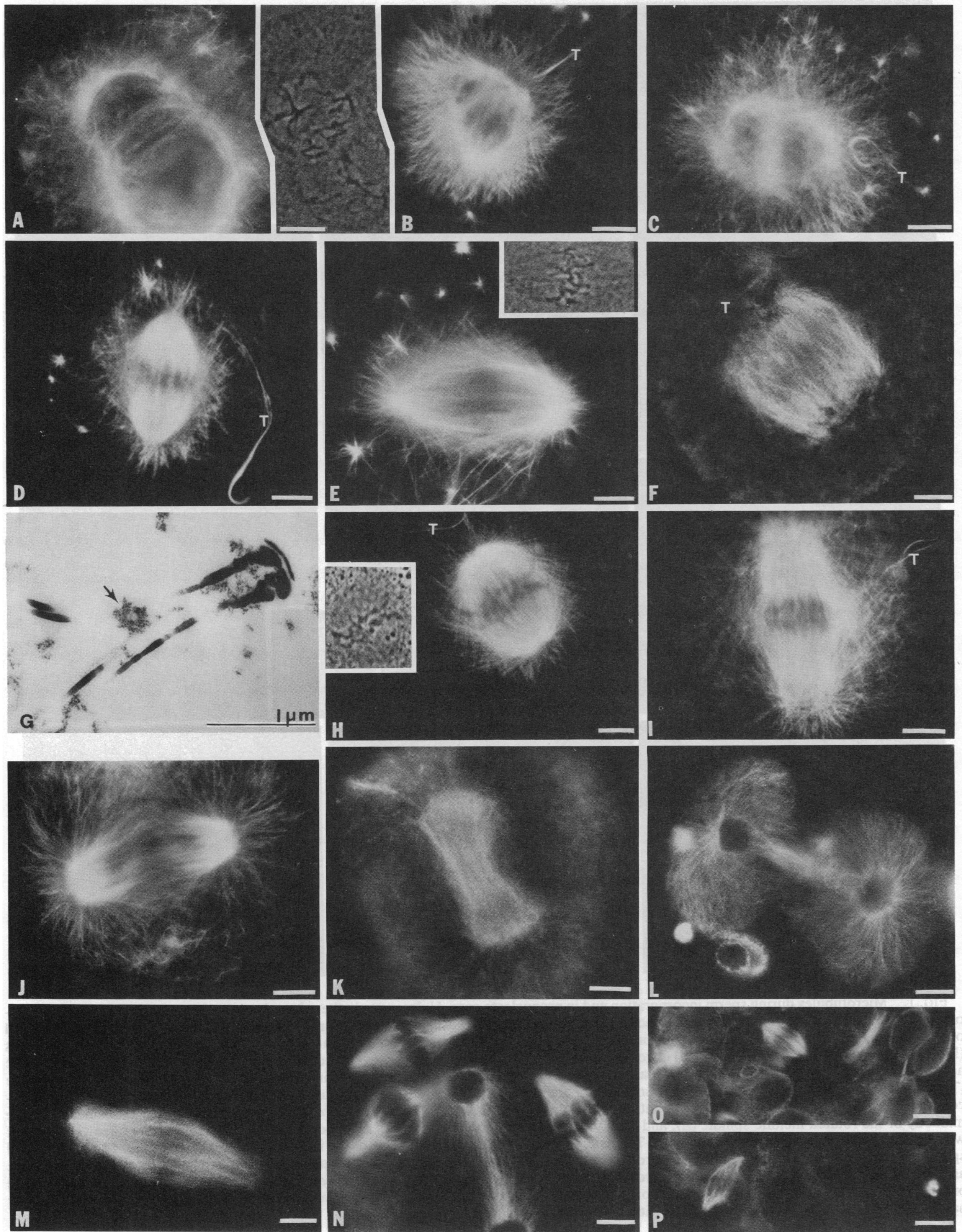

FIG. 2. Mitosis and early development: First division to blastocyst. (Bars = 10 μm, except *G*.) (*A*) At the end of first interphase, 16 hr after ovulation, the cytoplasmic microtubules disassemble from the interior and are replaced by sheaths of microtubules surrounding the adjacent, but still separate, pronuclei. (*Inset*) Same field, phase-contrast micrograph of chromosomes condensing within the adjacent pronuclei. *Insets* in *E* and *H* have the same relationship to their immunofluorescence micrographs. (*B* and *C*) Prophase. The paternal and maternal chromosome

interphase, the array disassembles from the interior and is replaced by perinuclear shells of microtubules surrounding the still adjacent but separate pronuclei (Fig. 2A).

At prophase the chromosomes condense separately, and an irregular sphere of microtubules assembles within a monaster (Fig. 2 B and C). A spindle begins to emerge, and by metaphase it typically appears barrel-shaped and anastral (Fig. 2 D–F). At times, the poles are narrow during the initial formation of the spindle (Fig. 2D). The sperm axoneme is usually not associated with the broad mitotic poles. In a metaphase egg, the sperm axoneme with its implantation fossa and an embedded centriole is found at a cytoplasmic region devoid of microtubules (Fig. 2G); numerous microtubules are observed in the spindle region.

At anaphase the spindle lengthens and, at times, sparse astral microtubules appear (Fig. 2 H and I). Interzonal microtubules form at telophase (Fig. 2J), and the spindle retains its broad poles. At first cleavage (Fig. 2K), the interzonal microtubules aggregate into a midbody and a new cytoplasmic array forms, extending from the blastomere nuclei to the cell surfaces. This cytoplasmic array develops into monasters after cleavage with a persisting midbody (Fig. 2L).

Second mitosis is characterized by an anastral mitotic spindle with broad poles (Fig. 2M). By third division (Fig. 2N), and in morulae (Fig. 2O) and blastocysts (Fig. 2P), the spindles are fusiform with well-focused mitotic poles.

Colcemid (50 μM), griseofulvin (100 μM), and nocodazole (10 μM) prevent the microtubule assembly and disrupt the meiotic spindle (Fig. 3A). Sperm incorporation is unaffected (Fig. 3 B and C). However, the male pronucleus does not develop and the maternal chromosomes scatter along the oocyte cortex (Fig. 3C). Interactions between the meiotic chromosomes and cortical actin (20) and the involvement of microtubules during nuclear lamin acquisition by the pronuclei (21) have been recently reported. A compilation of the microtubule containing arrays and the associated nuclear migrations is presented in Fig. 4.

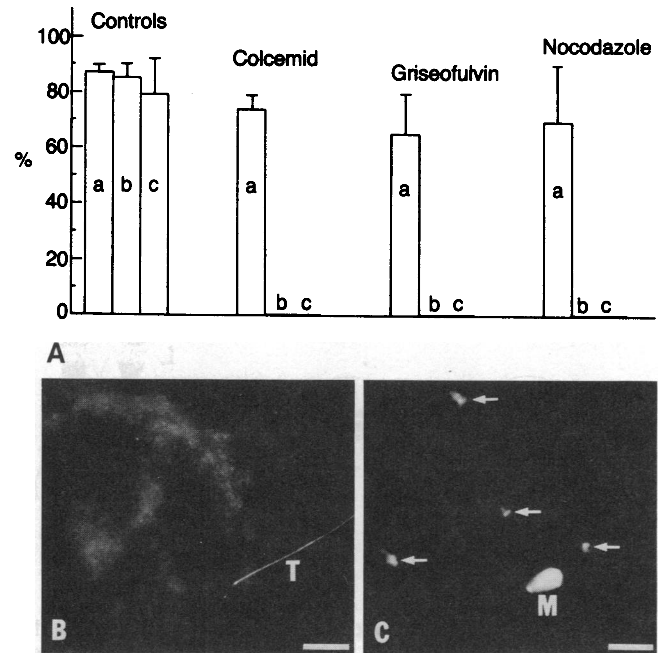

FIG. 3. Effects of microtubule inhibitors. (A) Colcemid, griseofulvin, and nocodazole prevent pronuclear formation (b bars) and the movements leading to pronuclear centration (c bars). Sperm incorporation (a bars) is not significantly inhibited. Ordinate is percent of cells examined; bars are mean +SD for the indicated processes. (B and C) Colcemid disassembles the meiotic spindle and prevents the movements leading to the union of the male and female pronuclei. Sperm incorporation is unaffected, and only the microtubules of the incorporated sperm axoneme (T) are found. The sperm nucleus (M) does not decondense in Colcemid, and the maternal meiotic chromosomes are scattered along the oocyte cortex (arrows). (B, Anti-tubulin; C, Hoechst 33258 stain; bars = 10 μm.)

DISCUSSION

Microtubules are found to play a crucial role during mammalian fertilization. These microtubules probably interact with the perinuclear actin found in pronucleate eggs (22), since either cytochalasin (22) or latrunculin (23) will prevent pronuclear apposition. This study supports the hypothesis predicting the cyclical appearance of microtubules (24) and raises questions about the active microtubule organizing centers and the paternal inheritance of centrioles in mammals.

Szöllösi et al. (ref. 25; reviewed in refs. 26 and 27) demonstrate the absence of centrioles in meiotic mouse oocytes, though the sperm centriolar complex has been found during fertilization (28). Calarco-Gillam et al. (29), using autoimmune sera to pericentriolar material, show that meiotic oocytes and mitotic eggs have broad centrosomes that aggregate into foci by fifth division. The centrosomes during mammalian fertilization are probably of maternal origin, unlike those in other cases of fertilization in animals (reviewed in ref. 1), because the asters appear before sperm incorporation and are independent of the sperm axoneme position; indeed, B. Maro, S. K. Howlett and M. Webb (personal communication) have found about 16 foci in unfertilized oocytes in addition to the meiotic poles. Parthenogenesis in mammals (reviewed in ref. 30) must require maternal microtubule organizing centers. Though the mammalian centriole, expected to appear after the third division cycle, may be maternally inherited, it is premature to exclude a paternal participation.

The recent hypothesis of Mazia (31) suggests that "flexible centrosomes" may be present in many forms, each sort nucleating a particular array of microtubules. Plant cells typically have broad centrosomes (32–37), as does the mouse zygote (ref. 29; Fig. 2 F–K), though in both cases narrow poles may be observed initially (refs. 32–37; Fig. 2D). Centrosomes in sea urchin eggs, which have punctate poles at metaphase, also broaden during the centrosome cycle (38,

sets are still separated by the perinuclear microtubules within a monaster. (C) A spindle begins to emerge as the parental chromosomes meet. The sperm axoneme (T) is apparent. (D–G) Metaphase, 18 hr after ovulation. The metaphase spindle is typically barrel-shaped and anastral, with relatively broad mitotic poles. It sometimes has focused poles, as in D, which appear to broaden during mitosis. (G) Electron micrograph of sperm axoneme and centriole complex in a metaphase egg. Though numerous parallel microtubules are found in the spindle region, microtubules are not observed near the incorporated sperm axoneme, with its centriole (arrow) and implantation fossa. (H and I) Anaphase. The spindle lengthens, and sparse microtubules extend from the broad poles towards the cell surface. (J) Telophase. Interzonal microtubules develop, and a few microtubules extend from the wide poles towards the cell surface. (K) Cleavage. The interzonal microtubules bundle into a midbody. (L) Second interphase. The daughter nuclei are positioned at the blastomere cell centers within monasters extending from the nuclear surfaces. A midbody persists, and the second polar body remains attached at the left. (M) Second mitosis, 36 hr after ovulation. At metaphase the spindle still has broad mitotic poles. (N) Third mitosis, 44 hr after ovulation. Fusiform spindles with well-focused mitotic poles are observed at third division. (O) Morula, 64 hr after ovulation. (P) Blastocyst, 80 hr after ovulation. Typical fusiform mitotic spindles are detected at fourth (O) and fifth (P) divisions.

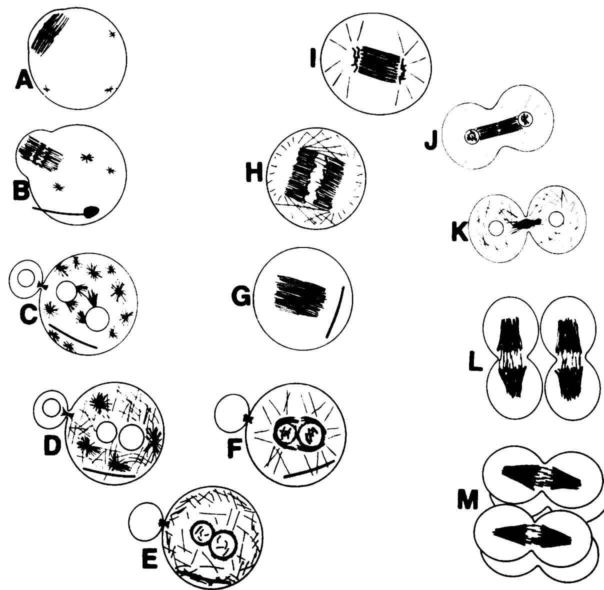

FIG. 4. Microtubule configurations during fertilization in the mouse. (Approximately ×200.) Microtubules are present in the meiotic spindle of the unfertilized oocyte (*A*); the spindle is anastral and attached to the oocyte surface. At the time for sperm incorporation, maternally nucleated cytoplasmic asters assemble (*A*, *B*). During sperm incorporation (*B*), the microtubules of the sperm axoneme are apparent, and as the pronuclei develop (*C*), the meiotic spindle rotates, the cytoplasmic asters enlarge, and a pair is associated with the pronuclei. Meiosis is completed, and the second polar body is formed; a midbody of microtubules persists. When the pronuclei are moved towards the egg center (*D*), a dense array of microtubules assembles. At the conclusion of first interphase (*E*), the array disassembles, and the adjacent, but separate, pronuclei are invested with individual sheaths of microtubules. At prophase (*F*), the spindle emerges from the perinuclear microtubules. At metaphase (*G*), a barrel-shaped, anastral spindle develops with broad mitotic poles independent of the incorporated sperm axoneme. At anaphase (*H*) the spindle elongates and a sparse aster develops. At telophase (*I*) interzonal microtubules appear, and during cleavage (*J* and *K*) interphase cytoplasmic arrays form as monasters positioning each daughter nucleus at the blastomere cell center. At second division (*L*) the poles, though broad, are somewhat narrower, and during third and later divisions (*M*), the mitotic spindle is fusiform with well-focused mitotic poles.

39). As the poles separate around the nuclear surface during interphase, the centrosomes appear to be arc-shaped (D. Mazia and N. Paweletz, personal communication) like those in mouse blastomeres (Fig. 2*L*). While the pronucleate egg has dispersed centrosomes that form a disorganized array, mouse fertilization follows the sequence of centrosomal broadening and separation but on a cycle asynchronous with the typical one contemporaneous with chromosome separation.

In summary, the migrations leading to the union of the sperm and egg nuclei at the mouse egg center require the formation of cytoplasmic microtubules, as in other animal systems. Inhibition of formation of these microtubules prevents this movement, and surprisingly, prevents the normal decondensation of the incorporated sperm nucleus and meiotic chromosomes. Microtubules in the mouse egg during fertilization appear to be required for the proper separation and alignment of the maternal meiotic chromosomes and for the movements of the male and female pronuclei from the cortex into close apposition at the egg center. In contrast to those in most other animals, however, the microtubules are nucleated by numerous maternal sources rather than as a single monaster organized by the incorporated sperm centriole, and centrioles may not be paternally inherited in this mammal.

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