Highly degenerated distal centrioles in rhesus and human spermatozoa

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In humans and other mammals except rodents, the spermatozoa contribute the proximal centriole during fertilization. The inheritance of the distal centriole is not yet fully clear. In the present work, the distal centrioles of rhesus and human spermatozoa have been studied by transmission electron microscopy. The round and elongating rhesus spermatids possess both proximal and distal centrioles. The distal centriole extends posteriorly as an axoneme while the proximal centriole produces a microtubular adjunct. Ejaculated rhesus and human spermatozoa have intact proximal centrioles, but the distal centrioles have degenerated. The central pair of microtubules of the axoneme extends continuously into the distal centriolar region up to the sperm head. Serial transverse and longitudinal sections of the sperm neck region reveal few scattered microtubule duplexes or triplets in the distal centriolar region. The loss of the centriolar microtubules is more extensive on theventral side of the neck region, the side where the proximal centriole resides. The distal centriole degenerates caudally from the rostral area. Immunogold electron microscopy with anti-β-tubulin antibody showed that the distal centriolar regions possess 50% fewer gold particles than the proximal centrioles, indicating a significant loss of centriolar microtubules in the distal centriolar region. The A-tubules of the remaining triplets are filled with a dense material, as observed in the axoneme. Thus, rhesus and human spermatozoa introduce only proximal centrioles intact, whereas the distal centrioles are mostly disorganized in the mature spermatozoa.

Key words: centriolar adjunct/centrosome/distal centriole/fertilization/proximal centriole

Introduction

Mammalian fertilization comprises a cascade of cellular processes, eventually resulting in the union of male and female gametes and the achievement of cytoplasmic conditions crucial for successful cleavage. The centrosome plays an important role in these events. The centrosome of the spermatozoon organizes the sperm aster, which brings the parental genomes in close apposition during fertilization (Schatten, 1982). Centrosomes form bipolar spindles that ensure equal distribution of the genomes to daughter cells during cleavages. Due to such vital roles, the zygote must inherit a correct number of centrosomes and exert tight control over their replication. Improper centrosomal inheritance is one of the major causes of fertilization failure and abnormal embryonic development (Simerly et al., 1995; Palermo et al., 1997).

A typical animal cell centrosome comprises two components: a pair of orthogonally oriented centrioles (Vorobjev and Nadezhdina, 1987) and a variety of fibrous proteins associated with the pericentriolar region (Kimble and Kuriyama, 1992). Several lines of evidence indicate that most of the zygotic centrosomal proteins are contributed by the oocyte cytoplasm (Schatten, 1994). However, it is not fully clear how the centrioles originate in the zygote. In the sea urchin, the spermatozoon introduces two centrioles that duplicate before each cleavage and are distributed to the daughter cells following cell division (Paweletz et al., 1987a,b). That rodent spermatozoa totally lack centrioles (Woolley and Fawcett, 1973; Manandhar et al., 1998) precludes the generality that centrioles are inherited paternally in these species. Hence, centrioles are not necessary components of the zygotic centrosome in rats and mice. Non-rodent mammalian spermatozoa introduce the proximal centriole into the oocyte at the time of insemination (Crozet, 1990; Sathananthan et al., 1996; Sutovsky et al., 1996); the inheritance of the distal centriole has not been shown. The present study shows that ejaculated rhesus and human spermatozoa do not possess intact distal centrioles. More than half of the distal centriolar microtubules are disorganized, and the remaining exhibit highly aberrant ultrastructure.

Materials and methods

Three samples each of rhesus testis and spermatozoa were obtained from three different individuals with proven fertility. Two samples of human spermatozoa were provided by two different fertile anonymous donors. The testicular tissues and spermatozoa were fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer for 1 h. After glutaraldehyde fixation, the testicular tissues and the sperm pellets were postfixed with 1% OsO4, dehydrated in ethanol series, perfused with propylene oxide and embedded with EMbed 812 (Electron Microscopy Sciences, Fort Washington, PA, USA) following a standard protocol. Semithin sections were cut with glass knives, stained with a mixture of 1% Aure B and 1% methylene blue and studied under a bright field microscope to select seminiferous tubules with desirable stages of spermiogenesis. Ultrathin sections were cut with a diamond knife, stained with 1.5% aqueous uranyl acetate and Reynolds’ lead citrate and studied under a Phillips 300 TEM. The negatives were scanned with a Umax Power Look 2000 flatbed scanner, processed with Adobe Photoshop 4.0 and printed with a Sony UP-D8800 dye sublimation printer.
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Figure 1. Centriolar complex of rhesus spermatids. (A) A round spermatid showing an orthogonally arranged centriolar duplex in the cytoplasm. The distal centriole produces an axoneme which extends into the intercellular space. Inset, centriolar duplex of the spermatid appearing in the adjacent section. (B) A longitudinal section of an elongating spermatid passing through the centrioles and the axoneme. The centriolar complex is attached to the thickened process of the nuclear envelope, called the implantation fossa (arrowheads). The spermatid has manchette microtubules (arrows) emanating from the perinuclear ring below the acrosome. (C) A section passing longitudinally through the proximal centriole and the adjunct of an elongating spermatid. The centriolar microtubules are continuous with the adjunct microtubules without any transitional region. The inner circumference of the adjunct has deposition of a dense material which shows certain periodicity (small arrows). (D) A section passing transversely through the adjunct approximately at the plane indicated by a line in section C. The microtubular triplets of the adjunct display (9 + 0) arrangement as that of a typical centriole, however the C-tubules (c) have an open edge which joins the A-tubule (a) of the preceding triplet. Externally, the adjunct is surrounded by a flocculent fibrous material. Axn = axoneme; Acr = acrosome; Dc = distal centriole; Pc = proximal centriole; M = mitochondria; Man = manchette; N = nucleus; Sc = striated columns. Bars = 0.2 µm.

For immunogold labelling, the three rhesus sperm samples, as mentioned above, were fixed with a mixture of 2% formaldehyde and 0.25% glutaraldehyde, washed and treated with 1 mg/ml NaBH₄ for 30 min, rapidly dehydrated with 50 and 70% ethanol, perfused with LR-White:ethanol (1:1) for 1 h and embedded in LR-White (London Resin Co., Ltd, UK). Polymerization was carried out under UV light at 4°C for 24 h. The thin sections were collected on pioloform-coated nickel grids that were sequentially incubated for 1 h each with 10% normal goat serum, anti-β-tubulin antibody (E7; Hybridoma Bank, Iowa City, IA, USA) and colloidal gold-conjugated antimouse IgG (12 nm gold; Jackson ImmunoResearch Inc., West Grove, PA, USA) followed by postfixation with 2.5% glutaraldehyde (20 min) and staining with uranyl acetate (10 min). Control immunogold labelling was done by following the same protocol, except replacing the E7 antibody by mouse preimmune serum (Sigma, St Louis, MO, USA).

Results

Rhesus spermatids have elaborate centriolar complexes

Rhesus round and elongating spermatids possessed two orthogonally oriented centrioles (Figure 1A, B). The distal centriole produced an axoneme that extended into the intercellular space. The proximal centriole of elongating spermatids developed a microtubular adjunct. The adjunct had a structure similar to that of a centriole, and was formed by the extension of the centriolar microtubules (Figure 1C). Their A- and B-tubules
Figure 2. Serial sections of a rhesus sperm neck region passing longitudinally through the distal and the proximal centrioles. The proximal centriole (Pc) has intact microtubular structure and is encased within a dense material (C–E). The lower end of the distal centriole has been marked with arrows (B–D). The distal centriolar microtubules are mostly deformed or lost in section B. They appear remarkably thicker and darker than the axonemal microtubules (C, D). The ventral side of the distal centriolar region marked by the presence of the proximal centriole is fully degenerated in section D. The striated columns are visibly attached to the outer aspect of the outer dense fibres (arrow in E). Axn = axoneme; N = nucleus; M = mitochondria; Sc = striated column; Odf = outer dense fibre. Bar = 0.25 µm.

displayed complete rings in cross sections but the C-tubule showed an open edge that joined the A-tubule of the preceding triplet (Figure 1D). Dense material was deposited on the inner surface of the adjunct cylinder (Figure 1C). Externally, the adjunct was surrounded by floculent fibrillar material (Figure 1D) resembling pericentriolar centrosomal proteins of the somatic cells (Kimble and Kuriyama, 1992). One of the remarkable features of elongating spermatids was the presence of dense bundles of microtubules, called the manchette, around the nucleus (Figure 1B). These microtubules extended in close proximity to the adjunct (Figure 1C), but the dense fibrillar material of the adjunct was not involved in the manchette microtubule nucleation (Figure 1C)

Rhesus and human mature spermatozoa have degenerated distal centriole

Rhesus and human mature spermatozoa possess proximal centrioles (Zamboni and Stefanini, 1971; Sathananthan et al., 1991, 1996; Sutovsky et al., 1996; Figures 2C–E, 3). Some of the ejaculated human spermatozoa displayed an intact microtubular adjunct extending from the proximal centriole (Figure 3B). Though immature rhesus spermatozoa possessed prominent adjuncts, the ejaculated spermatozoa did not retain them (Figure 2). The present study was focused mainly on the mature sperm distal centrioles. We have analysed 11 complete serial longitudinal sections and nine complete serial transverse sections of rhesus sperm distal centriolar region. The spermatozoa for analysis were randomly chosen from the three samples. The numbers of human spermatozoa analysed were 10 and six respectively, in longitudinal and transverse serial sections. In addition, numerous random TS, LS and oblique sections were also studied.

The distal centriolar vault was surrounded by outer dense fibres. The striated columns were attached to the outer surface of the outer dense fibres in the rostral region of the centriole (Figure 2E). Longitudinal sections of the rhesus and human sperm neck region reveal highly degenerated distal centrioles (Figures 2, 3A). In a study of serial longitudinal sections of rhesus spermatozoa, intact microtubules were observed in some planes of sections while in other planes, centriolar microtubules were absent or disorganized (Figure 2B–D). Human spermatozoa also revealed similar features in serial longitudinal sections (data not shown). Apparently, disintegration of the centriolar microtubules takes place from the rostral
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In some sections the microtubular triplets seemed to be deformed or collapsed (Figure 5E–G). The central microtubular duplex of the axoneme extended anteriorly and traversed throughout the entire distal centriolar region up to the proximal centriolar vault (Figures 4, 5).

Half of the microtubules of the distal centrioles are lost in rhesus spermatozoa

Monoclonal anti-β-tubulin antibody (E7) labelled the various microtubular structures of rhesus spermatids in immunofluorescent preparations (data not shown). Immunogold labelling of β-tubulin of the distal and proximal centrioles of rhesus mature spermatozoa was investigated to estimate the extent of microtubule loss in the distal centrioles. The number of gold particles on the distal and proximal centrioles was counted in 200 spermatozoa that were randomly selected on the sections cut from the three samples as mentioned in Materials and methods. Because the distal centriole was continuous with the axoneme, an unbiased estimation of gold particles on the distal centriole was ensured by mounting a micrometer in the viewing binocular microscope and counting the gold particles lying within the same length as the proximal centriole. Furthermore, in transverse sections, the distal centrioles could not be identified unambiguously without investigating their consecutive serial sections. Therefore, they were not included in the study.

The study showed that anti-β-tubulin antibody labels the microtubules of the distal and proximal centrioles and the axoneme (Figure 6). The gold particles were absent in those regions of the distal centriole where microtubules were disintegrated. There were twice as many gold particles on the proximal centriole as there were on the distal centriole (Table I). This signified that tentatively half of the microtubules of the distal centrioles were lost, as compared to those of the proximal centriole. The control preparations treated with preimmune serum instead of E7 antibody did not show gold particles on the microtubular structures.

Discussion

Rhesus and human immature spermatozoa possesses two centrioles, proximal and distal (DeKretser, 1969). Although the proximal centrioles remain intact in ejaculated spermatozoa (Zamboni and Stefanini, 1971; Sathananthan et al., 1991, 1996; Sutovsky et al., 1996) the distal centrioles undergo various levels of degeneration in different animal spermatozoa (Fawcett, 1965; Fawcett and Ito, 1965; Gordon, 1972). The distal centriole loss is more prominent in rodent species such as guinea pig, chinchilla, squirrel, hamster etc. (Fawcett and Phillips, 1969). Prior to this study, however, it was unconfirmed whether the distal centrioles undergo degeneration in primate spermatozoa. Earlier electron microscopic studies pursued on random sections showed loss of some microtubular triplets in the distal centriolar regions (Holstein and Roosen-Runge, 1981). However, the possibility that the absence of centriolar microtubules in isolated sections could simply be caused by their oblique orientation and the planes of section missing them was considered (Zamboni and Stefanini, 1971). The
Figure 4. Serial sections of a rhesus sperm neck region passing transversely through the distal centriole. The microtubular triplets have been numbered according to the standard method (Phillips, 1974). Sections A and B have passed obliquely through the proximal centriole (Pc). Sections C and D show the gap region above the distal centriole. Sections E and F are the rostral region of the distal centriole in which most of the microtubular triplets have been degenerated. In section E, a duplex of microtubules is visible at the seventh position while the microtubules at the eighth position are mostly degenerated (arrows). The other microtubules are completely lost. Section F shows sixth and seventh triplets (arrows). Sections G and H show the middle portion of the distal centriole. In section G, the first to third and seventh to eighth microtubular triplets are intact while the ninth triplet is missing. The fourth to sixth triplets are deformed or collapsed. In section H, the sixth to eighth triplets are intact and the others are degenerated. One microtubule of the triplet is visible at the fifth position. Section I shows the caudal region of the distal centriole in which duplex microtubules are visible at the first and fifth to seventh positions. The other microtubules are degenerated or deformed. Section J shows the transitional zone between the centriole and the axoneme. Microtubular structure is not discernible at this level. Section K passes through the axoneme showing the microtubular duplexes. In sections F–K, A-tubules of the triplets or the duplexes appear dark because their lumen is filled with electron-dense material. The central duplex of the axoneme extends throughout the entire length of the distal centriole (arrowheads in C, K). M = mitochondria; Odf = outer dense fibre. Bar = 0.25 μm.

Table 1. Colloidal gold particle distribution on sections of rhesus sperm proximal and distal centrioles labelled with anti-β-tubulin antibody

<table>
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<tr>
<th>Proximal centriole</th>
<th>Distal centriole</th>
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<tr>
<td></td>
<td>TS</td>
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<tr>
<td>TS* (n)</td>
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<tr>
<td>21.4 ± 9.4 (73)</td>
<td>25.5 ± 13 (88)</td>
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<tr>
<td>11.1 ± 7.6 (111)</td>
<td>10.8 ± 10 (89)</td>
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*Transverse section; †Longitudinal section.
Values in parentheses are means ± SD.

The present electron microscopic study of serial sections and immunogold labelling has conclusively shown that the distal centriole systematically degenerates in rhesus and human spermatozoa. Though the loss of the centriolar microtubules is not complete, it is more remarkable on the ventral side and the rostral portion of the centrioles. The remnants of the degenerated distal centrioles show other abnormal features such as the presence of central duplex and A-tubules filled with a dense material. These features are reminiscent of an axoneme, raising a fundamental question of whether this axonemal-like structure might revert back to a centriole and reconstruct a zygotic centrosome during fertilization.
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**Figure 5.** Serial transverse sections of human sperm distal centriole. The microtubule triplets have been numbered according to the standard method (Phillips, 1974). Sections A and B show the gap region between the proximal and distal centrioles. The microtubules of the proximal centriole are visible in section A (arrows). Sections C and D pass through the rostral portion of the centriole. In section C, the fifth to seventh triplets are visible while the first, second and ninth ones are lost. The third and fourth triplets show partial disintegration (arrows). The eighth triplet is cut obliquely in this and subsequent sections. In section D microtubules at the first, third and fourth to fifth locations are recognizable. The other microtubules are disorganized. Sections E–G show the middle and caudal regions of the centriole. The triplets at the first, second and ninth locations are recognizable while others appear deformed or collapsed. Section H passes through the axoneme region. The central microtubular duplex of the axoneme (arrowheads in A, H) extends upward, reaching the base of the head. The A-tubules of the centriolar microtubule triplets show deposition of a dense material (arrowheads in C, E). M/H11005 mitochondria; Odf/H11005 outer dense fibre. Bar = 0.25 µm.

**Figure 6.** Comparative β-tubulin immunogold labelling of the proximal (Pc) and the distal centrioles of rhesus spermatozoa. The lower end of the distal centriole has been marked with white arrows. The proximal centriole of this spermatozoon shows 65 gold particles while the distal centriole has only 16. The distal centriolar region appears mostly empty. M = mitochondria; N = nucleus; Pc = proximal centriole. Bar = 0.25 µm.

The distal centrioles show a higher tendency towards degeneration than the proximal ones in mice. They are completely lost before the spermatozoa leave the testis, whereas the proximal centrioles degenerate much later, only during the epididymal stage (Manandhar et al., 1998). Consistently, rhesus and human spermatozoa exhibit preferential disintegration of the distal centriole. The reason behind the selective degeneration of the distal centriole is not fully clear. In G0 stage culture cells, procilia are produced by the mother centrioles, which are one cell generation older than the daughter centrioles (Vorobjev and Chentsov, 1982). Considering a homology between the sperm axoneme and procilium, the distal centriole bearing the axoneme could be the ‘mother’ (an older centriole) and thus prone to disintegration. However, it is evident that the distal centriolar axis crosses the proximal centriole (Figures 1, 2). This spatial relationship suggests that the distal centriole could be equivalent to a daughter centriole (Lange and Gull, 1996).

Considering the fact that the centriole is the structural core of the centrosome in most animal cells, centriolar disintegration reflects the culminating event of centrosome reduction. A typical example of centrosome reduction is provided by mouse spermiogenesis during which the centrosome loses its microtubule nucleating function, β-tubulin and centrin before the centrioles degenerate (Manandhar et al., 1998, 1999). A recent observation of β-tubulin loss from the pericentriolar lattice during monkey and human spermiogenesis (Fouquet et al., 1998) suggests that the mode of centrosome reduction in these species is basically similar to that of a mouse. Moreover, some human mature spermatozoa display residual β-tubulin on the proximal centriole but not on the distal centriole (Fouquet et al., 1998), signifying a higher extent of disintegration of the latter.

The normality of the centriolar apparatus in progenies is
thought to be ensured by inheritance of two centrioles from the spermatozoon, their replication before each cleavage and propagation to the daughter cells. This generalized conclusion is derived mainly from studies carried out on sea urchin fertilization (Paweletz et al., 1987a,b). A simple extrapolation of the sea urchin model may not be valid in higher animal fertilization. As stated earlier, in murine species, centrioles are not contributed by the spermatozoon, but regenerate de novo in late morula stage (Szollosi et al., 1972). Centrioles form spontaneously in rabbit oocytes even in the absence of fertilization (Szollosi and Ozil, 1991), a fact which shows that oocytes have all the components for centriole assembly and are able to form centrioles spontaneously after an appropriate stimulation. During fertilization, the spermatozoon introduces an intact proximal centriole which replicates to form one centriolar duplex and localizes at one of the spindle poles of the first zygotic division (Crozet, 1990; Sathananthan et al., 1996). The present work shows that the sperm distal centriole is highly degenerated. The oocytes might replenish the lost microtubular triplets of this centriole and replicate it to create the second centriolar duplex. Alternatively, the second pair of centrioles might be formed spontaneously, without the participation of the distal centriole. This viewpoint has been supported by some electron microscopic studies. The spindle pole of the first cleavage metaphase is broad (Crozet, 1990; Wu et al., 1996), which is very similar to the acenstriolar spindle pole of the oocyte (Szollosi et al., 1972). An extensive survey of human zygoites by electron microscopy has failed to find centriolar duplexes in all spindle poles during the first cleavage (Sathananthan et al., 1996). In a similar study of sheep fertilization, inheritance and replication of the proximal centriole has been observed (LeGuen and Crozet, 1989; Crozet, 1990). Thus, it is probable that only the proximal centriole is replicated before the first cleavage and the second pair is regenerated during the later cleavage cycle. A systematic study of the spindle polar regions of the first zygotic cleavage with serial sectioning is necessary to make a definite conclusion.

The sperm tail–head attachment is mediated by a connecting piece comprising the mitochondrial sheath, striated columns and the centriolar complex. The function of the tail is to deliver the nucleus into the oocyte by propelling the spermatozoon through the female genital tract and penetrating the zona pellucida. Once these functions are accomplished, the sperm tail detaches from the head and eventually degrades in the later embryonic stages. One of the intriguing questions in this process is how the sperm tail detaches from the head. The present observation of the degenerated distal centriole signifies that there would be a structural discontinuity between the head and the tail after removal of the mitochondrial sheath and the striated columns during fertilization, causing detachment of the tail.

The lack of standard distal centrioles in rhesus and human spermatozoa does not interfere with their ability to form a zygotic centrosome during fertilization and bipolar spindle poles during cleavages. Moreover, the observation of distal centriole disintegration in non-rodent mammalian spermatozoa suggests that centrosome reduction and centriole loss could be a ubiquitous phenomenon, taking place in all mammalian spermatozoa to varying extents, the mouse spermatozoon representing the highest state, with complete loss of both centrioles and centrosomal proteins.

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References


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