A comparison of human spermatozoa immunolabeling features using xenogenic reagents for centrosomal proteins

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Summary

The centrosome is an organelle essential to proper chromosomal migration and normal cell growth. In the human, the centrosome is comprised of two centrioles and the pericentriolar cytosol; its control of embryo cleavage processes derives from its role as a locus for spindle organisation. At fertilisation, it is the human sperm centrosome that is responsible for ordering these processes, as the oocyte appears not to contain working centrosomal structures. Abnormalities in fertilisation or early embryo cleavage could be related to impaired sperm centrosome structure or function in some cases. While potential future treatments of infertility due to a defective centrosome could involve use of a donor centrosome to restore normal cell development, such an approach would depend on accurate localisation of this organelle for subsequent transplantation. To locate centrosomal components in the heads and tails of human spermatozoa, labeling was performed on intact spermatozoa using antibodies of known specificity to highly-conserved centrosomal elements. Following general mapping of immunofluorescent signals, unlabeled sperm were dissected to form head/tail sperm fragments which were then separately tested. Distribution of centrosomal proteins in head and tail fragments was assayed for each separation method. Three reagents were compared: 1) rabbit anti-mitotic spindle protein (anti-MSP) antibody, 2) rabbit polyclonal centriole-specific antibodies, and 3) mouse monoclonal anti-MPM-2 (a centrosome phospoprotein) antibody. Of these, anti-MPM-2 antibody appeared to be the most reliable, labeling centrosomal elements in 63% (n=1,386) of treated spermatozoa. Sequential utilization of n-butylamine to effect head/tail separation followed by anti-MPM-2 antibody labeling was a satisfactory method of centrosome localisation. Microextraction of centrosomes and pericentriolar matrix identified by this method awaits further testing.

Key words: Spermatozoa; Immunohistochemistry; Centrosome; Anti-MPM-2.

Introduction

Proper cytoplasmic microtubule assembly and function are fundamental to normal cellular growth and division, since chromosomal arrangement and distribution at mitosis are directly orchestrated by microtubules. Microtubule kinetics are complex and regulated in most animal cells by a microtubule-organising center known as the centrosome (Vandre and Borisy, 1989). This organelle determines the intrinsic polarity and orientation of microtubule assembly. In the human, the centrosome is comprised of three elements: two perpendicularly-oriented centrioles and the pericentriolar cytosol. Generally, the somatic cell centrosome orders an interphase array of microtubules and replicates at mitosis to nucleate the two poles of the mitotic spindle. During fertilisation, however, special mechanisms exist at the gamete or zygote level to control centrosome inheritance and duplication. Unique centrosome processes at fertilisation are important to avoid centrosomal contributions from both gametes. If centrosomes from both oocyte and spermatozoa were retained and remained functional after fertilisation, the zygote would enter the first mitotic division with two sets of centrosomes and four centrioles. This would result in the generation of abnormal multipolar spindles, aneuploidy, and mosaicism (Sluder *et al.*, 1989]. To avoid this, the centrosome of one gamete is incapacitated upon fertilisation. In most mammals (the mouse being an exceptional case), such inactivation is not random and the functional centrosome is typically derived from the fertilising spermatozoa (Schatten, 1994).

There is now little doubt that in humans the male gamete is the source of the active centrosome at fertilisation (Palermo *et al.*, 1994). Whereas human spermatozoa have distinct centrioles, extensive analysis by transmission electron microscopy (TEM) has shown that human oocytes lack centriolar structures (Sathananthan *et al.*, 1991). The proximal centriole of the sperm is located within the connecting piece near the basal plate of the sperm head, and has the characteristic structure of nine microtubule triplets surrounded by electron dense material flanked by nine cross-striated columns. Initially, the distal centriole gives rise to the axoneme; later during spermiogenesis, this centriole produces dense fibers (Sathananthan, 1991; Sathananthan *et al.*, 1996).

Since the centrosome (which has also been called the "cell center") figures so prominently in zygote development, competent centrosomal function is necessary to enable fertilisation and normal cell homeostasis. How might centrosomal defects in the immediate post-fertili-

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sation period result in clinical manifestations of human infertility? Some spermatozoa are ineffective in organising aster microtubules essential for coordinating pronuclear apposition and genomic union during fertilization, leading to the speculation that these sperm have centrosome defects (Navara *et al.*, 1995; Simerly *et al.*, 1995; Sutovsky *et al.*, 1996; Navara *et al.*, 1997). When inseminated oocytes were subjected to immunohistochemical imaging techniques, discrete stages of fertilisation arrest were apparent (Asch *et al.*, 1995; Navara *et al.*, 1997). It is possible that immotile or nonprogressively motile spermatozoa contain defective or absent centriolar structures (Sathananthan, 1994), resulting in the observed arrested or impaired fertilisation. Similarly, antisperm antibodies against centrioles may be responsible for mitotic errors in some cases (Van Blerkom, 1996). Although not yet experimentally proven, one theoretical approach to restore proper centrosomal activity might involve microsurgical transplantation of a competent donor centrosome. While specific antibodies to centrosomal components have been isolated by others (Masuda *et al.*, 1992; Kuang *et al.*, 1994; Long *et al.*, 1997), how best to prepare human spermatozoa to localise centrosomes using these reagents has not been determined. This investigation compared three xenogenic immunohistochemical labeling techniques, as well as methods for effecting spermatozoa head/tail separation with a view to using such fragments for experimental organelle transplantation.

Materials and Methods

Pre-labeling preparation of intact spermatozoa

Semen samples obtained from donors of known fertility (n=38) were allowed to liquefy for ~30 min prior to assessment of concentration. Specimens were washed by centrifugation at 500 *g* x 5 min in human tubal fluid (HTF, Irvine Scientific, Santa Ana, California, USA) supplemented with 10% synthetic serum substitute (SSS, Irvine Scientific) (HTF-SSS). The resuspended pellet was layered on a trilaminar discontinuous density gradient (90/70/50%) and centrifuged at 300 *g* x 20 min. To remove any residual silica gel particulate matter, the 90% fraction containing the spermatozoa was washed twice by adding 4 mL HTF-SSS and then centrifuged at 500 *g* x 5 min (Palermo *et al.*, 1995). The final pellet was resuspended in a volume of HTF-SSS sufficient to obtain a final sperm concentration of 70-80 x 10^6/mL. Ten microliters of the final suspension were placed on precleaned slides.

Spermatozoa were fixed by immersing slides in a coplin jar containing 2% paraformaldehyde (Sigma Chemical Co., St. Louis, Missouri, USA) in phosphate-buffered saline (PBS), pH 7.2, for 30 min at room temperature. Slides were washed for 1 h in PBS and then in ethanol 100% at –20°C for 10 min to permeabilise the spermatozoa. To reduce non-specific background fluorescence, slides were immersed for 15 min in a blocking solution of PBS with 0.2% bovine serum albumin (BSA, Sigma Chemical Co.).

Three immunomarkers were evaluated in intact spermatozoa: 1) rabbit anti-mitotic spindle (anti-MSP) antibody, 2) rabbit polyclonal anti-centriole antibodies, and 3) mouse monoclonal mitotic protein Mα (anti-MPM-α) antibody. Specificity for centrosomal structures was confirmed by positive and negative controls for rabbit anti-MSP (Long *et al.*, 1997), rabbit anti-centriole antibody (Connolly and Kalnins, 1978; Turksen *et al.*, 1982), and murine anti-MPM-2 (Masuda *et al.*, 1992, Kuang *et al.*, 1994).

Method 1: spermatozoa labeling by rabbit anti-mitotic spindle (anti-MSP) antibody

Forty µl of anti-MSP antibody (gift from J. Robl, University of Massachusetts-Amherst, USA) at a 1:50 dilution in PBS-BSA were transferred to the sperm slides (n=8) and covered with 22 mm^2 plastic coverslips. After the solution had spread evenly under the coverslips, the edges were marked with a tungsten-carbide pencil to permit identification of the processed area following coverslip removal. Antibody incubation was performed in a humidified chamber at 37 °C for up to 20 h. Unbound antibody was removed by three washings in PBS of 10 min each. Next, 40 µl of FITC conjugated goat anti-rabbit IgG (Sigma Chemical Co.) diluted 1:20 in PBS-BSA was placed on the slide and the area was covered with a plastic coverslip. The slides were incubated in a humidified darkroom at 37 °C for >1 h, and again washed three times in PBS for 10 min after coverslip removal.

Sperm nuclei were counterstained with 15 µl of 4', 6-diamino-2-phenylindole (DAPI) in antifade solution (0.5 mg/ml, Vysis, Downers Grove, Illinois, USA) and covered with a 22 mm^2 cover glass. Spermatozoa were assessed at 1,000 X with an epifluorescence microscope (Olympus B Max 60, New Brunswick, New Jersey, USA) equipped with a DAPI filter (Olympus U-C83360). A triple band-pass filter (Olympus U-C83103) allowed the observation of sperm nuclei in blue, together with red or green labeling, while single band-pass filters for FITC (Olympus U-C83490) were used to identify centrosomes. A total of 100 spermatozoa were assessed on each slide.

Method 2: spermatozoa labeling by rabbit polyclonal anti-centriole antibodies

Preparation of sperm was as described above. However, spermatozoa from additional study subjects (n=8) were incubated in 40 µl of rabbit centriole-specific antibodies (gift from V. Kalnins, University of Toronto, Toronto, Canada) at a 1:40 dilution in PBS-BSA (Connolly and Kalnins, 1978). FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) diluted 1:20 in PBS-BSA was used as the secondary antibody; 100 spermatozoa were assessed on each of the eight treated slides.

Method 3: speramatozoa labeling by anti-MPM-2 antibody

Samples of spermatozoa (n=22) were treated with anti-MPM-2 antibodies (M3514, Dako Corporation, Carpinteria, California, USA) following sperm preparation as detailed above. The antibody solution was combined with PBS-BSA in a 1:80 dilution, and the secondary antibody was FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co.) diluted 1:20 in PBS-BSA (Vandre *et al.*, 1984; Pinto-Correi *et al.*, 1994). For each slide prepared, 100 spermatozoa were evaluated via fluorescence microscopy.

Following comparisons of these three labeling strategies in intact cells, whole spermatozoa were dissected by one of the following methods and the resulting head and tail fragments were subjected to further staining.

Sperm head/tail dissection techniques

Method 1: sonication

Separation of sperm heads from tails was achieved in samples (n=5) by ultrasonic treatment with the Microson XL-2007 apparatus (Heat Systems Inc., Farmingdale, New York, USA). Following sperm pellet dilution in HTF-SSS, the resul-

ting suspensions were sonicated at a frequency of 23 kHz for 1 min at 4 °C. Power output during all treatment was 2 watts. To avoid contact between the sonication probe and specimens, a cup horn accessory with a high-intensity water bath was used. Following sonication, the semen samples were thinly layered on microslides and labeled with mouse monoclonal antibody against the centrosome phosphoprotein MPM-2 as previously described for intact spermatozoa. Efficacy of spermatozoa head-tail separation was assessed by phase-contrast microscopy on >100 cells/sample.

Method 2: mechanical (glass micropipette)

Spermatozoa from donors (n=14) were dissected with a microinjection pipette, the preparation of which has been previously reported (Palermo *et al.*, 1995). Briefly, spermatozoa were positioned perpendicular to the tip of the pipette which compressed the midpiece of the tail. This section of the sperm was rolled over the bottom of the petri dish and the head was popped off from the tail. Spermatozoa fragments obtained by this technique were placed on microslides and labeled with mouse monoclonal antibody against the centrosome phosphoprotein MPM-2, as for intact spermatozoa. Slides were fixed horizontally (rather than in a coplin jar) to reduce cellular loss.

Method 3: chemical (n-butylamine)

Chemical dissection of spermatozoa was performed according to the technique of Young and Cooper (1983), with slight modifications. Briefly, after addition of buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.005M EDTA, pH 7.2), semen samples (n=19) were centrifuged twice at 1,800 *g* for 5 min in conic tubes (Falcon 2095, Becton Dickinson and Co. Lincoln Park, New Jersey, USA). Sperm suspensions (50-150 x 10^6/ml) were then exposed to *n*-butylamine (Sigma Chemical Co.) at a concentration of 75 µl per one million spermatozoa. To enhance cell dissection, spermatozoa were vortexed for 1 min (Maxi-Mix I vortex, Thermolyne, Dubuque, Iowa, USA) at maximum setting. The *n*-butylamine was removed by double centrifugation in the buffer medium at 1,800 *g* for 5 min. Following evaluation of head-tail separation by phase-contrast microscopy, samples were placed on microslides and labeled with mouse monoclonal antibodies against the centrosome phosphoprotein MPM-2, as outlined above. For each sample assayed, >100 spermatozoa were randomly selected for examination.

Data analysis

Statistical analysis of fluorescent centrosome signals was by the two-tail χ^2 test, performed using a computerised data program (Statsoft, Tulsa, Oklahoma, USA). A p<0.05 was considered significant for all comparisons.

Results

Centrosomal protein labeling: intact spermatozoa

Work in several mammalian species previously identified sera (Connolly and Kalnins, 1978) and antibodies (Turksen *et al.*, 1982; Masuda *et al.*, 1992; Kuang *et al.*, 1994; Long *et al.*, 1997) specifically staining centrioles in somatic cells, and these results were validated. When one of these reagents, rabbit anti-MSP antibody, was used against human spermatozoa, a diffuse fluorescence pattern covering the entire sperm tail was observed. Although the greatest intensity originated from the proximal flagella, this preparation failed to show fluorescence perpendicular to the tail near the nucleus (Fig. 1).

Treatment of intact spermatozoa with rabbit polyclonal centriole-specific antibodies resulted in diminished signal strength from the distal tail but relatively brighter midpiece fluorescence (Fig. 2) in 43.0% of cells. When whole sperm were labeled with mouse monoclonal anti-MPM-2, maximal signal intensity was confined to the extreme proximal flagella (Fig. 3) in 63.0% (n=1,386) of assayed cells. Comparison of the latter two treatments in a total of 3,000 spermatozoa (Table 1) showed anti-MPM-2 to be more localised (i.e., restriction of signal to proximal flagella) than rabbit polyclonal centriole-specific antibody (63.0% vs. 43.0%, p<0.05).

Based on these results in intact human spermatozoa, anti-MPM-2 antibody was chosen for use with sperm dissected by one of three head/tail separation techniques.

Centrosomal protein labeling: dissected spermatozoa

Positive and negative controls for antibody specificity were reviewed and/or replicated prior to human spermatozoa immunostaining as described above. Analysis of sonicated spermatozoa specimens (n=5) revealed variable disruption of the sperm flagellum, and fluorescent analysis of the resulting fragments was not possible. In preparations (n=14) containing 64 spermatozoa heads obtained by glass micropipette separation, 57.8% (n=37) showed single or double labeling, while the remaining 27 sperm heads displayed no immunofluorescent signal. Of 27 spermatozoa tails produced by this method, 62.9% (n=17) demonstrated centriole labeling.

Table 2. — *Localisation of centrosome immunofluorescence in dissected head/tail sperm fractions following murine anti-MPM-2 antibody treatment.*

Separation method	Samples	Immunofluorescent signal (+) heads/total heads [%]	(+) tails/total tails [%]
Sonication	5	—[a]	—
Glass micropipette	14	37/64 [57.8]	17/27 [63.0]
n-butylamine	7	165/6,525 [2.5][b]	99/114 [86.8][b]

[a]discrete head/tail fragments were not uniformly obtained after sonication treatment

[b]p=0.0001, by Chi-square analysis.

Table 1. — *Immunohistochemical labeling results in intact human spermatozoa*

Antibody	Samples	Spermatozoa analysed	No. sperm with (+) immunofluorescent labeling (%)	Labeling pattern
Rabbit anti-MSP[a]	8	800	800 (100)	Diffuse/non-specific
Rabbit anti-centriole	8	800	344 (43)	Diffuse/non-specific
Murine anti-MPM-2[b]	22	2,200	1,386 (63)	Localised

[a]anti-mitotic splindle protein; [b]anti-mitotic protein M-2

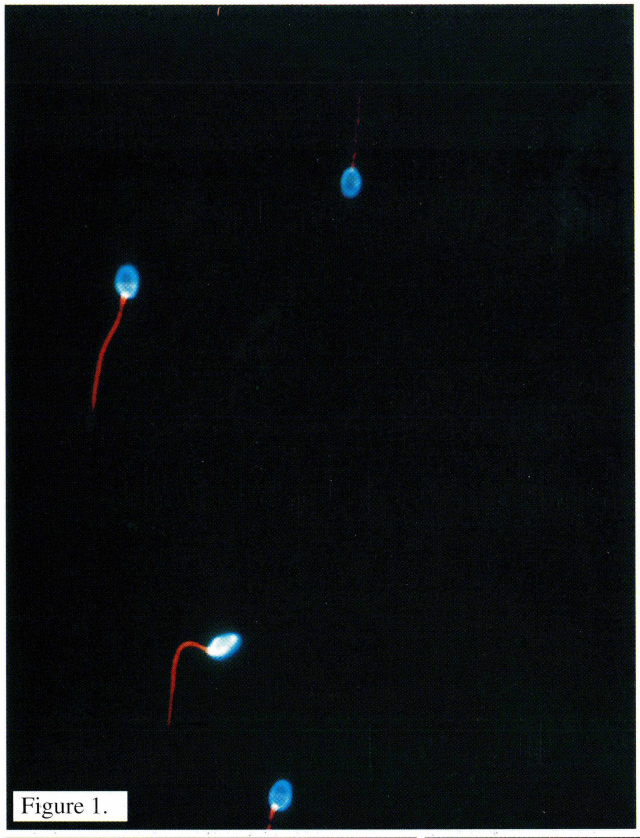
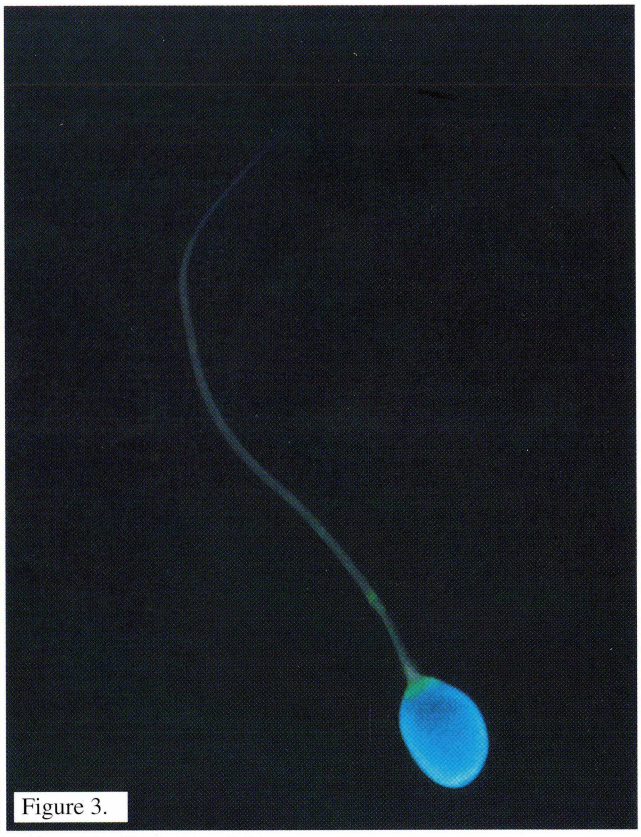


Figure 1.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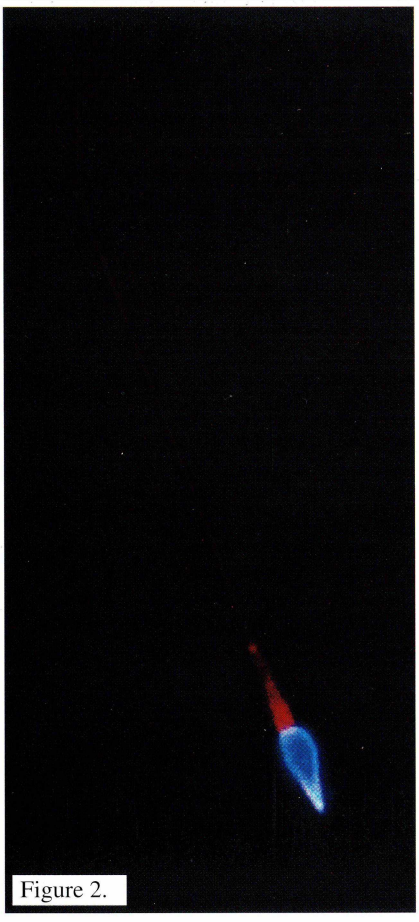
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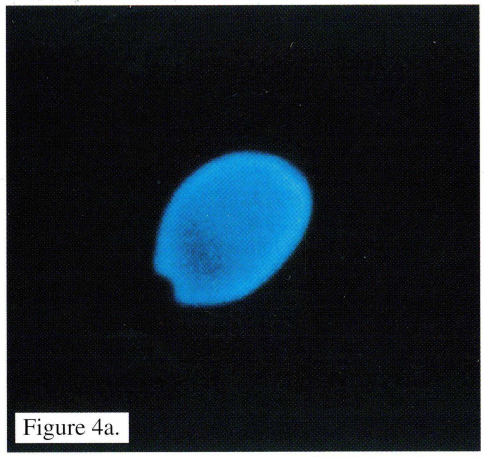
Figure 4a.

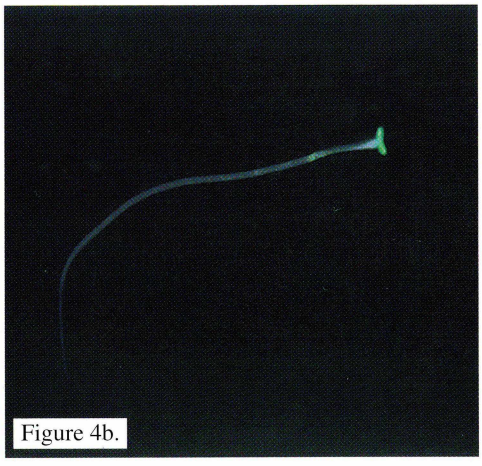
Figure 4b.

Figure 1. — Whole spermatozoa (four) after incubation with rabbit anti-mitotic spindle protein (anti-MSP) antibody, demonstrating a non-specific red fluorescence pattern throughout the tail. 400x.

Figure 2. — Treatment of intact spermatozoon with rabbit polyclonal centriole-specific antibodies, associated with regional immunofluorescent signaling from the entire midpiece. 1000x.

Figure 3. — Immunohistochemical staining of whole spermatozoon with murine monoclonal anti-MPM-2 antibody. Fluorescent signaling is restricted to head/tail junction. 1000x.

Figure 4a. (enlarged). Unattached sperm head (derived from *n*-butylamine dissection) reacted with murine monoclonal anti-MPM-2 antibody. No immunoflorescent signal is observed, corresponding to absence of centriolar elements. Note the small (~1μ) basal arcuate defect at the former site of tail attachment. 1000x (original magnification).

Figure 4b. Isolated sperm flagella (separated by *n*-butylamine treatment) stained with murine monoclonal anti-MPM-2 antibody. The strong arcuate signal is maximal at the margin of the free sperm tail and is complimentary to the defect seen in 4a (above). 1000x.

When intact sperm samples (n=7) were treated with n-butylamine, the mean head/tail separation efficiency was 78% (range 75-81%). The resulting fragments were treated with anti-MPM-2 antibody, and 99 of 114 (86.8%) isolated spermatozoa tail segments demonstrated positive centrosomal signaling. In contrast, after evaluation of a similar number of sperm heads separated by this method, only two sperm heads showed immunofluorescent evidence of a centrosome. To characterise the relationship between the sperm head fragment and centrosome signal more completely, a large number (n=6,639) of n-butylamine derived spermatozoa heads were subjected to further analysis. Positive centrosomal signals were found in 165 (2.5%) of head fragments, consistent with the earlier experimental findings.

Discussion

Localisation of centrosome components responsible for aster and spindle organisation, critical to all cell fission processes, has long been a goal of cell biologists. Indeed, well before the intricate "dynamic instability" hypothesis of microtubule kinetics was developed, it was suspected that some organelle must be charged with the assembly of cytoskeletal members. Throughout the animal kingdom, this role is typically played by the centrosome. Given the ubiquitous need for orderly chromosomal deployment at meiosis, it was not surprising that immunological investigations of various species found protein components of centrosomes to be highly conserved.

Modern biochemical and immunological advances have enabled the identification of the centrosome and its constituent proteins with a high degree of accuracy (Kimble and Kuriyama, 1992). Accordingly, this investigation compared different approaches to identify centrosomal proteins using xenogenic antibodies with high specificity to these elements.

The three immunohistochemical reagents compared in this study were selected because of their proven affinity for centrosomal components, desirable safety profiles, and ready commercial availability. While such features should facilitate replication of these preliminary results by other researchers, additional centrosomal immunolocalisation techniques may also warrant future study. In particular, anti-pericentrin or anti-gamma tubulin could offer improved centrosome identification, although at present their restricted availability precludes widespread laboratory assessment of their respective specificities.

Experimental detection of the sperm centrosome is of interest since this organelle may be a cause of fertilisation arrest or impairment (Van Blerkom, 1996). Preliminary studies of human sperm centrioles have shown that immotile or poorly motile spermatozoa contain aberrant centrioles more often than spermatozoa with normal motility (Sathananthan, 1994). In vasectomised men who have undergone vasovasostomy, the production of anticentriolar antibodies may contribute to centriole dysfunction and subsequent fertilisation derangement (Van Blerkom, 1996). Zygotes derived from such sperm are typically unable to complete normal syngamy or to generate viable embryos. It may be that a therapeutic approach using donor centrosomes could rectify these early developmental errors (Schatten, 1995; Palermo *et al.*, 1997). As this study shows, further refinements in sperm tail/head isolation techniques, as well as improvements in methods for identification of the centrosome in the resulting fragments will be required for this strategy to succeed.

Aster formation after the injection of mature human oocytes by isolated sperm flagellae (separated by sonication) has been reported by others (Van Blerkom and Davis, 1995). Although our study used a different sonication protocol than that described by Van Blerkom and Davis (1995), incomplete head-tail separation was a feature of both methods. Indeed, our experience with sonically-treated human sperm generally produced a varied and unpredictable disruption of the spermatozoa, resulting in tail fragments of non-uniform length.

Following head/tail separation by micropipette, immunofluorescent labeling showed centrosomes in only about half of sperm tails. This result militates against the use of micropipette sperm separation, as the exact site of dissection is subject only to limited operator control. Breakage site precision is important in influencing which fragment will ultimately contain the centrosome (Tucker *et al.*, 1996).

In this study, the primary amine n-butylamine was chosen because of its known ability to cleave sperm heads and tails in several mammalian species (Young and Cooper, 1983; Bedford and Hoskins, 1990). As this solvent disrupts bonds linking the inner and outer membranes of the nuclear envelope above the basal plate, it was anticipated that the tails so isolated would include intact centrioles. N-butylamine produced reliable head/tail separation in approximately 80% of treated spermatozoa. Immunolabeling with anti-MPM-2 antibody identified the presence of conserved centrosomal proteins in almost 90% of spermatozoa tails derived from n-butylamine treatment. Additionally, our data suggest that MSP is present in outer dense fibres of human sperm, consistent with observations reported by others (Long *et al.*, 1997).

If further research regarding centrosome localisation confirms the results reported here, a number of other applications for centrosomal therapeutics might emerge. For example, immature germ cells obtained from men with spermatogenetic arrest and azoopsermia often represent the only source of gametes to be cultured to attain paternity. The competency of the centrosome in such cells is not well characterised, however (Fischel *et al.*, 1996). Injection of such immature spermatozoa into an oocyte could be augmented by the provision of a donor centrosome (as carried in a properly prepared sperm tail) in an attempt to supply a functional centrosome.

Early fertilsation research could also be assisted by judicious experimental use of transferred centrosomes (Kuretake *et al.*, 1996; Moomjy *et al.*, 1996). Although elucidation of the specific technical requirements of microsurgical centrosomal transplantation will demand extensive experimentation, the current study offers suitable preliminary approaches to two fundamental challenges to successful organelle therapy: sperm dissection and centrosomal identification. Meticulous assessment of the technique for sper-

matozoa dissection, centriolar retention, and associated reproductive outcomes will be needed before organelle transplantation becomes a therapeutic reality.

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