Structural Heterogeneity of the Axonemes of Respiratory Cilia and Sperm Flagella in Normal Men

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Abstract

The ultrastructure of normal human cilia and flagella was examined and quantitatively assessed to determine the normal variations in the structure of the axoneme. Ciliated respiratory epithelial cells and spermatozoa from 10 normal, nonsmoking males who had normal semen parameters were fixed for electron microscopy. Tannic acid and MgSO_4 were included during fixation to enhance, in particular, axonemal components. In 75 axonemal cross sections per sample, the number of outer doublet and central singlet microtubules, outer and inner dynein arms, and radial spokes were recorded. Statistical analysis of the results showed a marked reduction, from the expected value of nine, in the numbers of inner dynein arms (mean±SE, cilia, 5.31±0.13; sperm, 5.38±0.16) and radial spokes (cilia, 4.95±0.22; sperm, 5.80±0.19). The ideal axoneme with all its structural components was seen in only 0.13% of cilia and 0.80% of sperm tails. Significantly more doublet microtubules (P < 0.05) and less central microtubules (P < 0.01) and radial spokes (P < 0.01) were seen in cilia than in sperm tail axonemes. Between subjects there was little variation in the mean number of a structure seen per axoneme. However, within each sample, the variation was considerably higher, particularly for the inner and outer dynein arms and radial spokes. The doublet microtubules had significantly greater standard deviations in the sperm tails compared with the cilia (P < 0.01), and furthermore, a significantly greater number of sperm tails compared with cilia showed the incorrect number of doublet microtubules (P < 0.02). In one semen sample, with normal semen analysis, 20% of the sperm tails showed incorrect numbers of doublet microtubules, ranging from 12 ± 2 to 5 + 2 compared with only 1.3% in cilia from this subject. This study has demonstrated that the ideal axoneme is rarely seen even in normal samples, probably because of the technical difficulties in resolution and visualization, and stresses the need for thorough documentation of axonemal ultrastructure. This work provides a normal data base for comparison with patients who have chronic respiratory disease and suspected infertility.

Introduction

The complex ultrastructure of the cilia and flagella of many species has been well-described in the past (for reviews, see 1, 2). Many features of the axoneme, including the 9 + 2 organization of microtubules, the outer and inner dynein arms, and the radial spokes, are thought to contribute to its motility. Disorders of the ultrastructural pattern of cilia and flagella disrupt axonemal motility (3, 4). In humans the co-existence of chronic sinobronchial infections and male infertility has in some patients been shown to be associated with immotile or dyskinetic cilia or flagella; this is known as the "Immotile" or "Dyskinetic" cilia syndrome (5-7). This syndrome is generally attributed to either congenital absence of dynein arms, disruption of radial spokes, or translocation of the microtubule doublets (7-9). There are many case reports of other human axonemal abnormalities thought to be associated with abnormal motility. However, too frequently, studies have ascribed less clearly defined defects, such as partial dynein arms (10-14), as the cause of a clinical condition without supplying data on its frequency in the sample and without knowledge of variations in axonemal structure and their frequency in normal tissue samples. In a recent study, Rossman et al. (15) compared microtubule abnormalities in axonemes of cilia from 30 normal subjects and 21 patients with various respiratory diseases. In the normal subjects, 2.6% of cilia had peripheral microtubule abnormalities and 0.6% had central microtubule defects. However, the frequencies of other axonemal abnormalities, such as dynein arms and radial spokes, were not recorded and the data were not compared with sperm flagella.

To date there are no detailed comparisons of cilial and flagellar ultrastructure from normal men, making it difficult to draw conclusions about the axonemal ultrastructure of specimens from patients. This paper presents a controlled, quantitative assessment and comparison of respiratory cilia and sperm flagella from 10 normal males using a new technique of fixation to enhance the ultrastructural detail of the axoneme. We examine the variation in ultrastructure, the types of abnormalities that occur, and their frequency in a large number of axonemes.

Methods

Subjects. Ten normal, nonsmoking males were selected for study using the following criteria: no personal or family history of chronic respiratory illness, no history of upper or lower respiratory tract infection in the 3 mo prior to study, and no personal or family history of genital disease or infertility. They also had normal mucociliary clearance time and semen analysis (see below). The study was approved by the Monash University Standing Committee on Experiments Involving Human Subjects and followed the ethical guidelines of the National Institutes of Health and National Health and Medical Research Council of Australia. Subjects participated only after signing a form of consent.

Collection of tissue and assessment of axonemal function. Ciliated nasal epithelium was collected from the inferior turbinate of each subject using a standard nylon cytology brush (Olympus BC-9C, Olympus Corp., New Hyde Park, NY). This method of sample collection does not require local anaesthesia and does not cause
bleeding (16). Cellular material from the brush was suspended in a
small volume of Dulbecco's Modified Eagles medium containing 1
mM NaHCO₃, 35 mM NaCl, and 10 mM Hepes (pH 7.4, 297 mOsm)
at 37°C. The specimen was analyzed at ×400 magnification and 37°C
using a Nikon Diaphot inverted microscope (Nikon Inc., Garden City,
NY) with attached tissue culture incubator. Ciliary motility was
assessed visually and all specimens had actively beating cilia.

In vivo nasal mucociliary clearance was assessed by the saccharine
transit time (17, 18). All subjects had normal transit times of < 20 min
(Table 1).

Semen samples were collected by masturbation and the percentage
of motile sperm and the motility index (19) determined within 2 h.
Semen samples were regarded as normal and acceptable for further
ultrastructural analysis if > 50% of the sperm were motile, the motility
index was at least twice the percentage motility, and the sperm
concentration > 20 million/ml (Table 1).

Fixation of tissue. Semen samples and ciliated respiratory cells
were fixed in a solution of 2.5% glutaraldehyde, 2.0% paraformaldehyde,
0.02% picric acid, and 2 mM MgSO₄ in 0.1 M cacodylate buffer. Samples
were postfixed in 2% OsO₄ and then dehydrated through a
graded series of alcohols, with the final alcohol containing tannic acid
(se below). The cells were then washed in epoxy-propane and left
overnight in a 1:1 mixture of epoxy-propane and epon-araldite. After
infiltration for 4–6 h in pure epon-araldite, the tissues were embedded
and allowed to polymerize at 60°C.

Improved axonemal staining using tannic acid and magnesium
sulphate. The resolution and assessment of axonemal ultrastructure
is highly dependent upon the staining and contrast achieved during
fixation and processing of the tissue. Many previous reports describing
axonemal abnormalities include electron micrographs of poorly fixed
tissue, making the structural components difficult to analyse and the
diagnosis of the tissue questionable.

Tannic acid has been used to resolve individual microtubular
protofilaments (20), whereas MgSO₄ has been shown to improve the
visibility of dynein arms (21, 22). By altering the concentration of
MgSO₄ and tannic acid it has been possible to find a combination of
these two compounds which dramatically enhances the fixation, staining,
and resolution of axonemal ultrastructure, in particular the dynein
arms and radial spokes (Fig. 1). The optimum concentrations are 2
mM MgSO₄ in the primary fixative and 0.1% (for flagella) or 1.0% (for
cilia) tannic acid in the final alcohol dehydration step. Higher concen-
trations of tannic acid were found to be unsuitable because the
increased electron density produced poorer definition between micro-
tubule structures and dynein arms.

Tissue sectioning. Thin sections showing gold-to-silver interference
patterns were cut with a Reichert OM U3 ultramicrotome (C. Reichert
A. C., Vienna, Austria), mounted on 150-mesh copper grids, and
stained in uranyl acetate and lead citrate. Sections were examined
using a Jeol 100S electron microscope (Jeol Ltd., Tokyo, Japan) at
60 kV.

Axonemal ultrastructural analysis. The number of outer doublet
microtubules, central singlet microtubules, outer dynein arms, inner
dynein arms, and radial spokes were counted in 75 axonemal cross
sections in matched samples of normal human sperm tails and cilia.
This number was determined by the summation average method (23),
in which a progressive mean is calculated until further observations
do not significantly alter the mean. In the case of the radial spokes,
which had the highest variation (see Results), examining 75 axonemes
was sufficient to stabilize the mean, making further observations

<table>
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<th>Parameter</th>
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<th>Range</th>
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<td>Age (yr)</td>
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<tr>
<td>Sperm/milliliter ejaculate (×10⁶)</td>
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<td>Motile sperm/ejaculate (%)</td>
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<td>Motility index</td>
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<td>146–239</td>
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<tr>
<td>Mucociliary clearance (min)</td>
<td>12.3±5.1</td>
<td>5–20</td>
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**Table 1. Average Age, Semen Parameters, and Mucociliary Clearance for 10 Normal Subjects**

**Figure 1.** Enhancement of axonemal ultrastructure with tannic acid
and MgSO₄. (a) A typical cilium seen in routinely fixed tissue. Some
dynein arms (arrows) and radial spokes (arrowheads) can be seen but
most are difficult to distinguish. (b) A representative outer dynein
arm (large arrow), inner dynein arm (small arrow), and radial spoke
(arrowhead) are shown in this cilium fixed with tannic acid and
MgSO₄. This technique enhances resolution of axonemal compo-
nents and greatly increases the number of axonemes suitable for a
comprehensive ultrastructural assessment.
unnecessary. Compound cilia or flagella, i.e., more than one axoneme within a membrane, were also documented. The tissue was methodically scanned and all axonemes that were in perfect transverse section were assessed. To find 75 flagella it was necessary to scan at least two grids of sections. The 75 cilia came from not less than 15 respiratory cells from at least two grids. The total number of structures was averaged to give a mean frequency of each individual structural component per axoneme. All ultrastructural assessments were made by one observer.

To minimize any possibility of observer bias in these assessments, a single blind procedure was used and cilia samples from patients with respiratory disease and low motility semen samples were interspersed with the volunteer cilia and sperm samples. The scoring technique has been shown to be highly reproducible with an intra-observer variation of 0.43, 0.54, and 1.44% for the outer dynein arms, inner dynein arms, and radial spokes, respectively, and an inter-observer variation of 0.43, 2.7, and 1.9% for the same three components.

To be counted the dynein arms had to show a definite hook projection from the doublet microtubule. Nexin linkages, which join adjacent microtubule doublets, are difficult to visualize in human axonemes and were not assessed in this study. To avoid confusion between the nexin linkages and inner dynein arms, structures that ran in a direct line between adjacent microtubule doublets were not counted. Radial spokes had to be continuous from the doublet microtubules to the sheath surrounding the central pair. Examples of how the axonemal structures were identified and defined are presented in Fig. 2. Clearly both of these axonemes have nine doublet microtubules and two central microtubules. The cilium in Fig. 2a has nine outer dynein arms but the inner dynein arms and radial spokes associated with doublets 6 and 7 are not clearly visible. The other inner dynein arms and radial spokes are visible and would be counted. The sperm tail in Fig. 2b has outer dynein arms on all except doublet 4, and is missing the inner dynein arm on doublet 5 and the radial spoke associated with doublet 6.

In axonemes where an incorrect number of doublet microtubules existed, e.g., 10 + 2, 6 + 2, etc., the number of outer and inner dynein arms and radial spokes observed were adjusted to a fraction of 9. This prevented the abnormality of microtubules carrying over to bias the number of dynein arms and radial spokes. For example, consider an axoneme with six doublet microtubules (instead of the expected nine) in which only four outer dynein arms are visible. The number of outer dynein arms would be converted to 4/6 × 9 = 6 outer dynein arms, i.e., the number of dynein arms that would be expected if nine doublets were present. This conversion procedure would also be used to correct for numbers of inner dynein arms and radial spokes in such an axoneme.

At the distal tip of an axoneme the microtubular pattern becomes 9 + 0. In order not to be documented as abnormal, transverse sections from this region of the axoneme were not assessed.

Statistical analysis. Because both cilia and sperm tails were observed from each subject, the data were paired before statistical comparisons. Each SD resulted from 75 observations, and so, a paired Student's t test was a quick and approximate method of calculating significance levels. A more precise test, the −2 ln λ method, confirmed all statistical comparisons. Because the SEs were very low and the variation within each sample was considerably higher (see Results), the data were also analysed after being pooled.

Results

The ultrastructural analysis showed that there was considerable deviation from the expected theoretical values of nine outer dynein arms, inner dynein arms, and radial spokes (Fig. 3). Significantly more microtubule doublets (P < 0.05) and significantly less central pair microtubules (P < 0.01) and radial spokes (P < 0.01) were seen in cilia than in sperm flagella. No significant difference was found between cilia and sperm flagella for the outer or inner dynein arms. The results were similar irrespective of whether the data were pooled or analysed as means from individual samples.

Although the mean values for each sample were very similar, and consequently, the SE values were low (Fig. 3), the
The ideal 9 + 2 axoneme in which all dynein arms and radial spokes were visible was seen in only 0.13% of cilia and 0.8% of sperm tails. The micrographs presented in Fig. 5 demonstrate some of the axonemes seen throughout the samples. Abnormalities of the 9 + 2 organization of microtubules were rare in cilia existing in only 4.1% of axonemes examined. They were more common in sperm at 9.9% (P < 0.02). This data includes one particular semen sample in which 20% of the flagella demonstrated an inappropriate number of microtubules ranging from 12 + 2 to 5 + 2. When the results were averaged for the 75 flagella observed in the sample above, the means were not significantly different from normal at 8.89 for the doublet microtubules and 2.05 for the central singlet microtubules. This semen sample had normal functional parameters of 62% motile sperm with a motility index of 157, and in the corresponding sample of cilia, only 1.3% of axonemes showed abnormal number of microtubule doublets. Even when this sample was excluded, there were still significantly more axonemes with microtubule abnormalities in the sperm compared with the cilia (P < 0.02). Abnormal numbers of central pair microtubules were found in 1.3% of cilia and 2.4% of flagella (P < 0.05). Compound axonemes were rare, occurring in only 0.13% of cilia and 0.4% of sperm tails.

**Discussion**

This study provides a detailed analysis of axonemal ultrastructure in sperm and cilia from normal, nonsmoking men. It has shown the deviation from theoretical values and a comparison of cilia and flagella from the same subjects.

The demonstration of a reduction in the number of outer and inner dynein arms and radial spokes in nondiseased tissue is important and may be attributed to two factors: (a) the

![Graph showing mean frequency per axoneme](image)

**Figure 3.** Mean±SE number of structures per axoneme (unpooled data). The number of inner dynein arms and radial spokes seen per axoneme was considerably less than the expected value of nine. Significantly more outer doublets (P < 0.05) and less central pairs (P < 0.01) and radial spokes (P < 0.01) were counted in the cilia compared with the sperm tails. *, P < 0.05; **, P < 0.01.

![Graph showing standard deviation](image)

**Figure 4.** SDs of structures in individual samples, so that each of the 10 dots in a column represents the SD of that structure in one subject. **P < 0.01. The variation between subjects of the mean frequencies of structures per axoneme was low (from Fig. 3), but within subjects the SDs were large, particularly for the outer and inner dynein arms and radial spokes. There was significantly greater SD in the outer doublets of the sperm tails compared with the cilia.
Figure 5. Some of the abnormal axonemes observed in normal tissue. 
(a) 8 + 2 Cilium with no well-defined dynein arms or radial spokes. 
(b) 9 + 5 Cilium showing disrupted circular arrangement of doublet microtubules. Some dynein arms (arrows) and radial spokes (arrowheads) are present. (c) 13 + 2 Sperm axoneme, with its surrounding fibrous sheath (fs). (d) Sperm flagellum with two extra pairs of central singlet microtubules (arrows).

technical difficulties in preserving and visualizing such small components, and (b) the full complement of these structures rarely occurs. It is impossible to isolate the contribution of either of these factors but it is likely that the technical problem of fixation and resolution of the tissue plays the major role. This has been noted previously in cilia from 10 “normal” subjects (22) when only 31.3% of the expected total number of dynein arms could be observed. This was considerably less than the number we recorded and could be because four of the subjects in the other study had chronic sinusitis and one had allergic rhinitis. The difficulty in visualizing dynein arms must be considered when diseased tissues are described as having, for example, “partial dynein arms” (10–14). Such rather obscure terminology should be used with caution and only after the examination of many axonemes.

Because of the wide variation in the number of dynein arms and radial spokes seen in different axonemes within individual tissue samples, this study has also demonstrated the necessity for a quantitative assessment of a large number of axonemes to characterize the entire sample. This can be made much simpler and less tedious by enhancing ultrastructural components using the fixation techniques described in this paper. The increase in contrast achieved by the inclusion of tannic acid and MgSO₄ in fixation procedures greatly increases the number of axonemes which are suitable for counting. The results of statistical analyses between cilia and flagella
were similar whether the means of individual samples were compared or the data were pooled. In both cases there were significantly more microtubule doublets and less central pair microtubules and radial spokes per axoneme in the cilia than in the sperm. It has been previously suggested that radial spokes are more readily visualized in sperm tails (24, 25). It is difficult to postulate why this is so, but these differences should be recognized and considered when drawing conclusions about axonemal ultrastructure. A significantly greater number of sperm tail axonemes had an abnormal number of microtubule doublets compared with the cilia (P < 0.02). This was observed in 8:10 of the semen samples and is a further demonstration of the morphological heterogeneity of human sperm in comparison with other species (26, 27). When abnormalities in the microtubule doublets were seen in a semen sample, the number of doublets observed was not consistently higher or lower than the expected number of nine. In other words both 13 + 2 and 4 + 2 might be present in the semen sample. This suggests that the assembly of that particular axoneme may be defective and that the abnormality is not due to either a general excess or deficiency of microtubules. The possibility was considered that the higher frequency of assembly variations in sperm tail axonemes may be caused by a difference in cell turn-over rates between the seminiferous and nasal epithelia. However, at present, there is insufficient evidence to substantiate this suggestion.

When considering cilia only, our results compare well with other studies of ultrastructure in normal tissue. Fox et al. (22) observed that 4% of cilia did not have the 9 + 2 microtubular arrangement and Rossman et al. (15) found 1.7% of cilia with an abnormal number of doublet microtubules and 0.6% with abnormal number of central pair microtubules. We found 4.1 and 1.3%, respectively. Clearly these variations in the number of doublet and central pair microtubules are caused by morphogenetic factors affecting the assembly of microtubules during development of the axoneme rather than problems of visualizing these structures with the fixation and staining procedures described.

One notable feature of this study is the demonstration of a relatively large proportion of axonemes with abnormal numbers of doublet microtubules in a semen sample displaying normal parameters of 62% motile sperm and a motility index of 157. It might be expected that the 20% of flagella which are abnormal are confined to the 38% of the sperm which are immotile. If the axonemal ultrastructure of such a sample had only been qualitatively assessed, it would certainly be described as abnormal. Interestingly, in the nasal biopsy of one healthy subject, Fox et al. (22) found that 20% of cilia had abnormalities in the 9 + 2 microtubular organization. Our data clearly show that a simple “eyeball” observation of a few axonemes is not adequate and that a comprehensive structural assessment must be combined with functional studies. As indicated previously the subject from our study showed no such defects in his respiratory cilia. This, in itself, is an important observation particularly when considering studies that describe normal cilia and abnormal flagella or vice versa (25, 28). This confirms other reports that structural defects of axonemes are not necessarily distributed throughout all cell types and may be confined to a particular location within the body.

In some studies on axonemal ultrastructure (e.g., 24, 29), the Markham rotation technique (30) has been used to demonstrate the presence or absence of dynein arms and radial spokes. The procedure involves rotating the axoneme through 40 degrees a total of nine times to superimpose the microtubules, dynein arms, and radial spokes before assessment. For example, when only eight outer dynein arms are present, this method will incorrectly show nine outer dynein arms. Since our study has shown that the full complement of structures can rarely be visualized, the rotation technique is not appropriate.

This study has clearly demonstrated that variations in axonemal ultrastructure occur frequently in normal functional tissue. Despite our attempts to maximize conditions for visualizing the various axonemal components, the ideal axoneme with its full complement of dynein arms and radial spokes is rarely seen. We stress that assessment of axonemal ultrastructure must be quantitative and well-documented, and that false conclusions could easily be drawn if only a few axonemes are qualitatively observed and the motility parameters of the tissue not recorded. The data provide not only a structural base but also functional parameters for both the respiratory epithelium and sperm tail. Such data now provide a definitive base line for future studies of the role of ciliary and sperm axonemal disorders in such diseases as bronchiectasis and infertility.

Acknowledgments

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References


