Morphological Abnormalities in the Spermatozoa of Fertile and Infertile Men

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ABSTRACT The morphological analysis of the spermatozoa from fertile and infertile men was performed using light and electron microscopy to clarify the relationship between sperm morphology and fertility. Semen samples obtained from 22 partners of pregnant women were prepared according to the protocol standardized in an international collaborative study. Semen samples from 17 patients with asthenozoospermia or varicocele were collected in a hospital. Abnormalities in the spermatozoa were classified into three types for the tails, two for the midpieces, and six for the heads according to the criteria adapted from WHO guidelines (World Health Organization, 1999: WHO laboratory manual for the examination of human semen and semen–cervical mucus interaction (4th edition)). Approximately 14% of the spermatozoa from the fertile men had abnormal tails at the light microscopic level while approximately 44% had abnormal heads. Most types of abnormalities found in the spermatozoa from the asthenozoospermic and varicocele patients were encountered in those from the fertile men, although the semen from the fertile men contained a higher percentage of normal spermatozoa than that from the patients. These results were also confirmed at the ultrastructural level. Most abnormal cell types are encountered in semen from fertile men, although the incidence of abnormalities is low.

INTRODUCTION Several recent studies have revealed that sperm morphology was the best parameter to predict sperm fertilizing capacity (Kruger et al., 1988; Ombelet et al., 1997; Bonde et al., 1998; Gunalp et al., 2001; Menkveld et al., 2001). However, sperm morphology is not easily related to sperm function, even though morphologically normal spermatozoa are closely related to binding of sperm to the zona pellucida and to the acrosome reaction (Franken et al., 1997). Therefore, it is necessary to examine the morphological characteristics of spermatozoa from fertile men, not only for understanding the mechanisms of fertilization but also for measuring the fertilizing potential of semen.

There have been many studies on sperm morphology using a light microscope; however, there are not many studies using the electron microscope. This is surprising because ultrastructural analyses of spermatozoa from fertile men have been shown to be useful. Ultrastructural analysis has been used for the diagnostic assessment of the infertile condition (Zamboni, 1987, 1992; Carbone et al., 1998; Chemes et al., 1998; Chemes, 2000). Minute structural deforms and multiple dysplasia are clearly visible under the electron microscope (Bisson et al., 1979; Holstein and Schirren, 1979; Holstein and Roosen-Runge, 1981; Chemes et al., 1987; Zamboni, 1987; Holstein et al., 1988; Baccetti et al., 1989; Chemes et al., 1999), and furthermore, tail abnormalities that involve the entire population of spermatozoa have been shown to be associated with the total loss of sperm motility (Afzelius et al., 1975; Baccetti et al., 1979; Williamson et al., 1984; Toyama et al., 1996). On the other hand, little is known about the relationship between the morphological characteristics observed using the light microscope and the electron microscope.

We had an opportunity to obtain semen from fertile men in an international collaborative study that mainly evaluated the sperm concentration (Jørgensen et al., 2001). In the present study, special attention has been paid to the abnormal forms of spermatozoa in order to precisely analyze sperm quality. Abnormal spermatozoa were classified into 11 categories according to the
criteria adapted from WHO guidelines (World Health Organization, 1999) and the percentage and types of abnormal forms of the spermatozoa from the fertile men compared to those of counterpart from asthenozoospermic and varicocele patients. In addition, a similar analysis was conducted at the ultrastructure level.

MATERIALS AND METHODS

Semen Samples

Recruitment of the semen from fertile men was done according to the protocol standardized in an international collaborative study (Jørgensen et al., 2001). Twenty-two fresh semen samples were obtained by masturbation from 22 male partners of pregnant women at Osaka University Hospital or at their homes and delivered to the laboratory within 1 hr of collection during October 2000 to May 2001. The reported duration of sexual abstinence was 2 to 7 days. Seventeen fresh semen samples were obtained by masturbation from 17 patients diagnosed with severe asthenozoospermia or varicocele in the hospital of St. Marianna University, and prepared by the same methods mentioned above. Each individual semen sample is identified with a small letter of the alphabet in Figures 1 and 2.

![Figure 1](image.png)

**Fig. 1.** Morphological characteristics of spermatozoa obtained from 22 fertile men at the light microscopic level. Histogram shows percentages of spermatozoa morphologically classified by the tails (A), midpieces (B), and heads (C). A small letter of the alphabet indicates each individual semen sample. The variform tails are arranged into an ascending order of normal shape (A).
In order to examine the morphological characteristics of the actively motile spermatozoa, motile spermatozoa were obtained by swim-up procedures after being selectively concentrated by continuous-step density gradient centrifugation (Kaneko et al., 1987). Sperm suspensions were obtained by mixing 2.0-ml semen from patients with 2.0-ml Hanks’ solution containing 1.0 mg/ml BSA; these were gently placed on a 5-ml layer of 90% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) dissolved in Hanks’ solution in a 15-ml polystyrene tube, and the sperm suspensions and the upper half of the 90% Percoll were stirred by two or three strokes of a plastic stick (10 cm long, 5 mm wide, and 1 mm thick) with the last 5 mm of the tip bent at a right angle in order to form a linear density gradient column. The column was then centrifuged at 800 g for 30 min so that mature sperm were sedimented at the bottom of the gradient. The supernatant was aspirated, and 2.0 ml of fresh Hanks’ solution was layered onto the loosely packed sperm pellet. Leaving the tube in an upright position for about 45 min at 37°C to allow swimming up of the actively motile spermatozoa, the upper part of the sperm

Fig. 2. Morphological characteristics of spermatozoa obtained from 17 patients at the light microscopic level. Histogram shows percentages of spermatozoa morphologically classified by the tails (A), midpieces (B), and heads (C). A small letter of the alphabet indicates each individual semen sample. The variform tails are arranged into an ascending order of normal shape (A).
suspension was carefully collected and transferred to a 1.5-ml Eppendorf tube.

**Light Microscopy**

Approximately 100 spermatozoa from each semen sample were evaluated using a Nikon Optiphot microscope for the tail, midpiece, and head, according to the WHO guidelines (World Health Organization, 1999) with a slight modification in the classification of abnormal spermatozoa; the spermatozoa were classified into one of 14 categories: normal and three abnormalities for the tail (coiled, short, and bent), normal and two abnormalities for the midpiece (cytoplasmic droplet and thin), and normal and six abnormalities for the head (small, amorphous, large, tapered, pyriform, and double), and the percentages of each category were then calculated.

**Electron Microscopy**

The well-mixed and liquefied semen was fixed in the solution containing 2.5% glutaraldehyde and 0.2 M cacodylate buffer for a few days at 4°C, given a 1-hr wash in the same buffer, and postfixed with 1% OsO₄ in the same buffer for 1 hr at room temperature. After being dehydrated through a graded series of ethanol solutions at room temperature, the samples were embedded in Quetol 812. Thin sections were cut on a Sorvall ultramicrotome MT-2, doubly stained with uranyl acetate and lead citrate, and observed using a JEOL 1200 EX II electron microscope at an 80 kV accelerating voltage. To assess the whole spermatozoon by electron microscopy, negatively stained spermatozoa were examined: a small aliquot of semen fixed in 2.5% glutaraldehyde was deposited onto carbon-film-coated copper grids and rinsed twice with deionized water. After being negatively stained with a drop of 1% uranyl acetate, the grids were fully drained and allowed to dry.

**Statistical Analysis**

The Pearson correlation coefficient (r) was used to examine the relationship between the percentages of the spermatozoa classified into different categories.

**RESULTS**

**Light Microscopic Analysis**

The percentage of normal and abnormal spermatozoa in each semen sample from the 22 fertile men is shown in Figure 1. The percentage of spermatozoa with morphologically normal tails ranged from 59.8% to 97.1% (Fig. 1A), and the average was 85.6% (Table 1). The frequency of malformations varied from sample to sample. In three types of tail defects, the mean percentage of coiled and short tails were similar, but the mean percentage of bent tails was rather low (Table 1). When the variform tails are arranged into an ascending order of normal shape, several important features were noticed. Semen samples that contained a high percentage of spermatozoa with normal tails had a negligible percentage of spermatozoa with coiled tails (a and b in Fig. 1A). The percentage of spermatozoa with normal tails decreased as the percentage of spermatozoa with coiled tails increased (Fig. 1A). Thus, a significant and negative correlation observed between the percentage of spermatozoa with normal tails and that of spermatozoa with coiled tails (r = -0.93) was expected. On the other hand, there was no significant correlation between the percentages of spermatozoa having the normal, short, or bent tails. The percentage of spermatozoa with normal midpieces varied from 26.5% to 95.0% (Fig. 1B), and the average was 73.4% (Table 1). These values were much

<table>
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<th>Characteristics</th>
<th>Fertile (%)</th>
<th>Patient (%)</th>
<th>Patient/fertile</th>
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<tr>
<td>Tail</td>
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<td>48.2 ± 23.6</td>
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<tr>
<td>Coiled</td>
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<td>Short</td>
<td>6.1 ± 4.5</td>
<td>25.1 ± 23.7</td>
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<tr>
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<tr>
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<td></td>
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<tr>
<td>Cytoplasmic droplet</td>
<td>15.1 ± 9.7</td>
<td>23.8 ± 14.0</td>
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<td>Thin</td>
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<td>40.8 ± 24.3</td>
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<td>Double</td>
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<td>1.8 ± 1.6</td>
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*Mean ± SD. These were calculated from the percentages shown in Figures 1 and 2.*
lower than those of spermatozoa with the normal tails. There was no significant correlation between the percentages of spermatozoa having the normal, cytoplasmic droplet, or thin midpieces. The percentage of spermatozoa with normal heads was rather low compared with that of spermatozoa with normal tails or midpieces; the percentage of spermatozoa with normal heads ranged from 36.3 to 80.2% (Fig. 1C) and the average was 56.5% (Table 1). No significant correlation was found between either the percentages of spermatozoa with three types of midpieces nor seven types of heads. Furthermore, no significant correlation was found between the percentages of spermatozoa with normal tails, midpieces, or heads. From the mean percentages of spermatozoa with normal tails (85.6%), midpieces (73.4%), or heads (56.5%), the percentage of spermatozoa having a totally normal shape in the fertile men was calculated (35.5%). Most of each individual semen sample had all types of abnormalities (Fig. 1), even though it was obtained from a fertile man.

The percentage of normal and abnormal spermatozoa in each semen sample from the 17 patients with asthenozoospermia or varicocele is shown in Figure 2. The percentage of spermatozoa with normal tails continuously decreased when the semen samples were arranged in descending order of its percentage (Fig. 2A). The percentage of spermatozoa having morphologically normal tails at the light microscopic level ranged from 4.8 to 83.3%, and the average was 48.2%, a value roughly half that of the fertile men (Table 1). For the three types of tail defects, the mean percentages of the coiled or the short tails were similar, but the mean percentage of the bent tails was rather low (Table 1). The percentage of spermatozoa with normal midpieces varied from 5.4 to 66.0% (Fig. 2B), and the average was 35.4% (Table 1). The percentage of spermatozoa with normal heads was low compared with that of spermatozoa having normal tails or midpieces; the percentage of spermatozoa having normal heads ranged from 1.9 to 41.0% (Fig. 2C), and the average was 22.8%, a value that was almost half that of the fertile men (Table 1). From the mean percentages of spermatozoa with normal tails (48.2%), midpieces (35.4%), or heads (22.8%), the percentage of spermatozoa having a totally normal shape in the asthenozoospermic patients was calculated (3.9%). Even with the high degree of correlation (r = 0.88) observed between the percentages of spermatozoa having the normal midpieces or normal heads, no significant correlation was found between the percentages of spermatozoa having the other normal types. Furthermore, there was no significant correlation between the percentages of spermatozoa having different types of abnormalities. Most types of abnormalities were found in each semen sample of the patients as well as in each semen sample of the fertile men.

Abnormalities, especially the short and coiled tails, thin midpieces, and double heads, dramatically increased in the semen samples from the patients (Table 1). This confirms the doctor's diagnosis of asthenozoospermia; namely, poor sperm motility, which was primarily caused by morphological defects of the flagellum and mitochondria (Wilton et al., 1992; Chemes et al., 1998).

**Electron Microscopic Analysis of the Spermatozoa From Fertile Men**

The examination of spermatozoa under the electron microscope not only revealed a more accurate definition of morphological defects but also detected abnormalities not resolved using the light microscope.

To precisely examine the entire shape of the spermatozoa, negatively stained spermatozoa were observed using an electron microscope (Fig. 3). Various types of abnormalities were seen at a higher magnification, i.e., coiled, short, and double tails for tail defects; thin and thick midpieces and a cytoplasmic droplet for midpiece defects; and amorphous, round, small, and tapered heads for head defects. A bent neck abnormality (spermatozoa were bent sharply at the neck) was also detected. In the cross sections of the posterior part of the principal piece, some axoneme defects such as some loss of doublet microtubules, double axonemes, an excess doublet microtubule outside the fibrous sheath, and missing central pairs were recognized (Fig. 4A). There was another type of tail defect in a cross section of the principal piece (Fig. 4B): one of the central pair microtubules was absent, both extra microtubules and doublet microtubules were present peripherally to the axoneme, and a fibrous sheath was abnormally arranged. In the cross section of the midpiece (Fig. 4C), three outer dense fibers accompanied by doublet microtubules were missing, while the mitochondria were normal. The tail defect observed was that the axoneme was bent at the end of the midpiece (Fig. 4D). The midpiece defect observed was a long and irregular mitochondrial sheath (Fig. 4E). The axoneme and fibrous sheath were disordered at the posterior region of the mitochondrial sheath. There were some vacuoles in a cytoplasmic droplet. Several head defects such as amorphous, round, and vacuolated heads (the nucleus including a large vacuole with a membranous structure) were detected (Fig. 5). Acrosome dysplasia was characterized as frilly, wavy, and fragile. Some spermatozoa had a large cytoplasmic residue containing a clump of mitochondria, large vacuoles, and cross sections of flagella.

A severely abnormal spermatozoon in Figure 6A showed a bent neck that had a dislocated implantation fossa, no mitochondrial sheath in the midpiece, a disordered fibrous sheath in the principal piece, and a large vacuole, included some membranous structure in the nucleus; however, the acrosome over the nucleus was normal. Another example of severely abnormal spermatozoa showed one flagellum surrounding three amorphous nuclei linked together by one acrosome (Fig. 6B). The neck components were composed of the basal body and segmented columns, but the basal plate did not contact the base of the nucleus. Figure 6C shows a typical example of the cratered head covered by a crater acrosome. An example of decapitated spermatozoa is shown in Figure 6D. The acrosome was separated at
the tip of the nucleus. Figure 6E shows an amorphous nucleus with three vacuoles. The wavy acrosome covered the anterior part of the nucleus. The posterior nucleus and anterior midpiece were surrounded by a large amount of residual cytoplasm. These severely abnormal spermatozoa were due to the combinations of acrosome, nucleus, and tail defects.

Electron Microscopic Analysis of the Spermatozoa From Patients

Various types of abnormalities were observed in the spermatozoa from the asthenozoospermic or varicocele patients. Although most of these defects were also detected in the semen samples from the fertile men, the percentage of abnormal spermatozoa from the patients was higher than that of counterpart from the fertile men. To examine the entire shapes of the spermatozoa from the patients, negatively stained spermatozoa were observed using an electron microscope (Fig. 7). There were various forms of sperm heads classified as pyriform, amorphous, and tapered head, as observed in the semen sample from the fertile men. There were also various defects, i.e., a spermatozoon with a slightly bent neck and thick midpiece, a cytoplasmic droplet at the midpiece, and a coiled tail. Thin sections of the head showed amorphous, small, tapered, and vacuolated heads (Fig. 8). The immature and amorphous nucleus included a vacuole with a membranous structure and was surrounded by a large cytoplasmic residue. Acrosome dysplasia was characterized as thin, wavy, and fragile. There was a decapitated spermatozoon of which the head was covered by an acrosome surrounded by a large cytoplasmic residuum, together with a clump of mitochondria, large vacuoles, and cross sections of flagella.

Electron Microscopic Analysis of the Spermatozoa Selected by Swim-Up Procedures

As mentioned above, there was no significant correlation between the percentages of spermatozoa having normal tails or normal heads at the light microscopic level. To confirm this result at the ultrastructure level, we examined the ultrastructure of the actively motile spermatozoa selected by the swim-up procedures. The spermatozoa obtained by the swim-up procedures had perfect tails. The percentage of abnormal axonemes of the spermatozoa from the patients was 54.9 ± 11.3% (mean ± SD obtained from 557 spermatozoa of eight semen samples) while that of counterpart of spermatozoa selected by swimming-up procedures was 2.8 ± 3.5% (mean of 111 spermatozoa of three semen samples). Therefore, motile spermatozoa were free from abnormality in the tails, although they had various abnormalities in the heads, for example, vacuolated and small heads (Fig. 9).

DISCUSSION

Morphological analysis of the spermatozoa from the 22 fertile men and the 17 asthenozoospermic or varicocele patients with the light microscope revealed that both semen samples exhibited almost the same types of abnormalities although the percentage of abnormal spermatozoa from the fertile men was much lower than that from the patients; namely, the most important difference between the spermatozoa of the fertile men and the patients is not type but the incidence of
Fig. 4. Electron micrographs of tail and midpiece defects of spermatozoa from the fertile men. A: Transverse sections of posterior part of the principal piece. Arrows show four types of axoneme defects: missing doublets (md), double axonemes (da), excess doublets or microtubules (ed), and missing central-pair microtubules (ms). B: Transverse sections of the principal piece. One of the central-pair microtubules is absent while excess doublets or microtubules are present in the residual cytoplasm. C: Transverse sections of the midpiece. Three of the outer dense fibers and of peripheral doublets are missing. D: The tail defect is associated with the bent axoneme (ba) at the end of the midpiece. E: The midpiece defects are associated with a long and irregular mitochondrial sheath and a cytoplasmic droplet (cm). Bar = 0.1 μm in A, B, and C and bar = 0.5 μm in D and E.
abnormalities. The percentage of normal spermatozoa from the fertile men (36%) in the present study is similar to 30% from 78 fertile men (Haidl and Schill, 1993) and passes the criteria of normal morphology (>30%) according to the WHO guidelines (World Health Organization, 1992).

There are not many reports on the ultrastructural abnormalities of spermatozoa from fertile men, although there are various types of reports on the ultrastructural defects of spermatozoa from infertile men, such as round-headed (Renieri, 1974; Aughey and Orr, 1978; Holstein et al., 1988; Lalonde et al., 1988), large head (Escalier, 1983), tapered head (Portuondo et al., 1983), decapitated head (Perotti et al., 1981; Toyama et al., 1995; Rawe et al., 2002), double heads (Holstein and Schirren, 1979), cratered head (Baccetti et al., 1989), coiled tail (Renieri, 1974), and short tail (Baccetti et al., 1975; Toyama et al., 1996). Comparing the abnormal forms of the spermatozoa from the fertile men with those from the patients at the ultrastructural level, we could not find a significant difference in the types of sperm abnormalities between the fertile men and the patients, as shown at the light microscopic level. Of course, we cannot rule out the possibility that the spermatozoa from a man suffering from a rare disease have different types of abnormalities.

As seen in Figure 1, the percentage of the malformations always varied from sample to sample. Furthermore, there was no significant correlation between the percentages of abnormal spermatozoa having defects in different parts of the spermatozoa. These findings suggest that the abnormalities observed in the present study are not due to specific alterations of the spermiogenesis and spermatogenesis of congenital origin, but many abnormalities observed were classified in the genetic origin (Chemes, 2000; Baccetti et al., 2001). The cause of these genotypic sperm defects is intriguing, but more work is required for a better understanding of this problem.

The spermatozoa selected by the swim-up procedures did not have abnormal tails, while they had abnormal midpieces or heads both at the light microscopic and the ultrastructural levels. This suggests that the abnormality in the tails is independent of that in the midpieces or heads. This result sweeps away some suspicion of ICSI treatment (intracytoplasmic sperm injection; injection of an immotile spermatozoon into the egg using a fine micropipette) because midpieces or heads of the immotile spermatozoa are not always dysfunctional.

As seen in Figure 3, negative staining electron microscopy was a useful evaluation of the entire spermatozoa and led to a more accurate definition of the nature and intensity of the morphological defects, although the preparation technique for the specimens has the potential to create artifacts. Clear images of the head defects (duplicated, large, small, and deformed head), similar to those observed by scanning electron microscopy in fertile men (Fujita et al., 1970), were observed.
Various abnormalities such as axoneme defects and vacuolated heads that are not usually detected with a light microscope were found in the spermatozoa from the fertile men as well as from the asthenozoospermic patients, using the electron microscope. This suggests that an abnormality at the light microscopic level is independent of that observed at the ultrastructural level. In order to identify the baseline abnormalities in fertile men, the percentage of deformed tails evaluated with a light microscope was compared with that of deformed axonemes detected with an electron microscope (Hunter and Kretzer, 1986); the percentage of spermatozoa with abnormal tails was 12.5% at the light microscope level and 24.4% at the ultrastructural level. The former value is similar to ours (14.4%). We did not accurately determine the percentage of abnormal tails at the ultrastructural level, but approximately 58.3% (n = 24) of the cross-sections of sperm tails had some

Fig. 6. Electron micrographs of severely abnormal spermatozoa from the fertile men. A: A bent neck spermatozoon has no mitochondrial sheath at the midpiece. A vacuole in the nucleus contains some membranous structure. B: Three amorphous nuclei (n) are surrounded by a flagellum. The basal plate (bp) is separated from the nuclei. C: Cratered head defects. D: A decapitated spermatozoon. E: An amorphous head embedded in a large cytoplasm. Bar = 0.5 μm.
defects. Thus, the percentage of sperm tails without any defects neither at the light microscopic nor the ultrastructural levels is 35.7% because the abnormality at the light microscopic level is independent of that at the ultrastructural level. By similar estimation of the percentages of abnormal midpieces and heads, the percentage of morphologically normal spermatozoa may be at most 10%, a value close to that evaluated according to the strict criteria (Ombelet et al., 1997; Menkveld et al., 2001). Taking into account all these results, it is necessary to evaluate the morphological characteristics of spermatozoa not only at the light microscopic level but also at the ultrastructural level to clarify the relationship between sperm morphology and sperm function.

Generally there are several vacuoles in the human sperm head. The functional significance of these vacuoles is not known, even though the presence of a large vacuole, single or multiple, empty or filled with membranous materials, affects fertility (Evensen et al., 1978; Zamboni, 1987; Chemes, 2000; Bartoov et al., 2002). The selection of spermatozoa by Percoll gradient centrifugation (Oshio et al., 1987) or micromanipulative techniques (Ishijima et al., 1986; Ishijima, 1995) is probably effective for successful in vitro fertilization.

Morphological analysis of the spermatozoa from the 22 fertile men with a light microscope revealed that the proportion of normal tails of spermatozoa from the fertile men was much higher than that of normal heads, that is, approximately 86% of the spermatozoa had morphologically normal tails, while 73 and 57% of the

Fig. 7. Electron micrograph of negatively stained spermatozoa from the patients. There are various types of malformed cells: a coiled tail (ct) is shown by an arrow; a cytoplasmic droplet (cm); and a thick midpiece (tkm) are indicated by arrowheads; and three head defects (amorphous heads, ah; pyriform heads, ph; and tapered heads, th) and a bent neck spermatozoon (bn) are also observed. Bar = 2 μm.

Fig. 8. Electron micrograph of abnormal spermatozoa from the patients. There are amorphous (ah), small (sh), tapered (th), and vacuolated (vh) heads. Acrosome defects (fragile acrosome, faa; thin acrosome, ta; and wavy acrosome, wa) and a decapitated spermatozoon (dc) are detected (arrows). Bar = 2 μm.
spermatozoa had normal midpieces and heads, respectively. This suggests that the fertilizing ability of the 22 fertile men is involved in the high percentage of normal tails of spermatozoa; in other words, fertilizing ability of the spermatozoa from the fertile men in the present study correlates with a high percentage of sperm motility.

The high correlation between the percentages of spermatozoa having normal tails or coiled tails suggests that an increase in the tail defects of spermatozoa from the fertile men is due to an increase in the coiled tails (Fig. 1A). This is partly true for the spermatozoa from the asthenozoospermic patients because an increase in the coiled tails is also obvious in the spermatozoa from these patients, although other defects such as short tails, thin midpieces, and double heads also increase their frequency in the spermatozoa from the patients. We cannot adequately explain the high correlation between the frequency of the patient’s spermatozoa with normal midpieces or normal heads. Because the patients were diagnosed with severe asthenozoospermia, the mechanism forming the head and midpiece may be influenced similarly.

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SPERM ABNORMALITIES IN FERTILE MEN


