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Sperm mitochondrial DNA depletion in men with asthenospermia

Shu-Huei Kao, Ph.D.,[a,b] *Hsiang-Tai Chao, M.D., Ph.D.,*[c] *Hwan-Wun Liu, M.D., Ph.D.,*[d] *Tien-Lin Liao, B.S.,*[b] *and Yau-Huei Wei, Ph.D.*[a]

National Yang-Ming University, Taipei Medical University, Taipei Veterans General Hospital, and National Defense Medical Center, Taipei, Taiwan

Objective: To determine the content of sperm mitochondrial DNA (mtDNA) in patients with asthenospermia or with poor sperm motility.

Design: Analysis of the content of mtDNA as the ratio between the amount of mtDNA and nuclear DNA by using a new concurrent polymerase chain reaction.

Setting: University hospital infertility center.

Patient(s): Eighty-six men who sought infertility therapy.

Intervention(s): Moving characteristics of sperm were examined with a computer-assisted semen analyzer.

Main Outcome Measure(s): Sperm samples were classified into two groups, one with asthenospermia and the other with normal moving characteristics. The content of mtDNA in sperm was determined by polymerase chain reaction. We analyzed the mitochondrial mass by MitoTracker Green staining and analyzed DNA content with propidium iodide staining by flow cytometry.

Result(s): A decrease in sperm mtDNA content was detected in patients with asthenospermia or with poor sperm motility (<20% motility). The relative mtDNA contents in the asthenospermia and normal groups were 7.2 ± 1.3 (mean ± SD, n = 23) and 74.1 ± 2.0 (n = 29), respectively. Lower intensities of propidium iodide staining were detected in patients with asthenospermia or poor motility, but there was no significant difference in MitoTracker Green staining between the sperm with different motility.

Conclusion(s): We suggest that mtDNA content may serve as a useful indicator of sperm quality and that mtDNA depletion may play an important role in the pathophysiology of some male infertility. (Fertil Steril® 2004;82:66–73. ©2004 by American Society for Reproductive Medicine.)

Key Words: Depletion, infertility, mitochondrial DNA, motility, sperm

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Reprint requests: Yau-Huei Wei, Ph.D., Department of Biochemistry, School of Life Science, National Yang-Ming University, No. 155, Li-Nong Street, Sec. 2, Shih-Pai Taipei 112, Taiwan (FAX: 886-2-28264843; E-mail: joeman@ym.edu.tw).

[a] Department of Biochemistry, National Yang-Ming University.

[b] Graduate Institute of Biomedical Technology, Taipei Medical University.

[c] Department of Obstetrics and Gynecology, Taipei Veterans General Hospital.

[d] Department of Biology and Anatomy, National Defense Medical Center.

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The mitochondria in the sperm midpiece are the energy generator for mammalian sperm. Sperm require a sufficient supply of adenosine triphosphate from mitochondrial oxidative phosphorylation for normal function (1, 2). In somatic cells, mitochondrial respiratory chain function depends on the coordinated gene expressions of both the mitochondrial and nuclear genomes (3, 4). The somatic mtDNA mutations have been proposed to be a major contributory factor for aging and age-related degenerative diseases (5). In addition to the qualitative change of mtDNA, quantitative alteration caused by a decrease of mtDNA copy number, that is, mtDNA depletion, has also been found in patients with infantile death (6), spinal muscular atrophy (7), fatal hepatic failure (8), and cardiomyopathy (9).

It is known that each mature mammalian sperm contains approximately 50–75 mitochondria and one copy of mtDNA in each mitochondrion in midpiece (10). During spermatogenesis, the sperm mitochondria undergo drastic morphological changes and subcellular reorganization (11). A reduction in the number of mitochondria and mitochondrial genome was also demonstrated in the maturation of mouse sperm (12). To maintain a suitable number of mitochondria in sperm, the mtDNA replication and subcellular organization must be accurately controlled in the process of spermatogenesis.

In previous studies, we have demonstrated multiple deletions of mtDNA in human sperm with low motility scores (13, 14). Here we report our recent finding that the relative content of mtDNA is significantly decreased in sperm from male patients with asthenospermia

or with poor sperm motility. To detect the extremely low amount of mtDNA in some sperm, we developed a concurrent polymerase chain reaction (PCR) technique to determine the mtDNA content of sperm. Furthermore, we applied flow cytometry to determine the DNA content and mitochondrial mass in sperm. In addition, we employed the electron microscopic method to visualize the assembly and morphology of sperm mitochondria. The results led us to suggest that depletion of mtDNA is one of the factors contributing to the pathophysiology of some men with asthenospermia or with poor sperm motility.

MATERIALS AND METHODS

Semen Collection and Assessment of Sperm Motility Characteristics

We collected 86 semen samples from 46 healthy donors who had normal semen characteristics and from 40 subfertile or infertile patients at the Department of Obstetrics and Gynecology, Taipei Veterans General Hospital, Taipei, Taiwan. Before collection of the semen, consent was obtained from each of the donors for analysis of motility characteristics and possible molecular defects of sperm. All of the samples had been obtained by masturbation after 3–4 days' abstinence. After the semen had been liquefied, moving characteristics of sperm were examined with a computer-assisted semen analyzer (CASA; HTM-2000 motility analyzer; Hamilton Thorne Research Inc., Danvers, MA). Leukospermia and viscous semen were excluded from this study. A 10-μL aliquot of semen was placed in a Makler chamber (Sefi-Medical Instrument Inc., Haifa, Israel), and the motility characteristics were immediately assessed by the CASA system as described elsewhere (14).

Ficoll-Paque Fractionation

To avoid contamination of sperm by the other types of cells such as lymphocytes and epithelial cells, we removed the contaminant cells by Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) separation before DNA extraction and flow cytometric analysis.

Sperm DNA Extraction

Before DNA extraction, an aliquot of 5–8 $\times$ 10^7 sperm was treated with osmotic shock (15). Sperm were incubated in 15 mL of 50 mM Tris-HCl buffer (pH 6.8) at 8°C for 20 minutes to lyse the contaminated cells. Sperm cells, which were resistant to this treatment, were then subjected to DNA extraction according to the method described elsewhere (14). After digestion at 56°C for 2 hours in 1.5 mL of lysis buffer, the sperm lysate was extracted once each with phenol, phenol-chloroform, and chloroform, respectively. The aqueous layers were pooled and precipitated with isopropanol (1:1, vol/vol) and a one-tenth volume of 3 M sodium acetate (pH 5.6) and were incubated at −20°C overnight. The DNA precipitate was washed with 75% ethanol (vol/vol) and dried by speed vacuum. The sperm DNA was finally dissolved in 10 mM Tris-HCl buffer, pH 8.5.

Oligonucleotide Primer Synthesis

The primers with desired DNA sequences were chemically synthesized by Roche Molecular System, Inc. (Branchburg, NJ). The nucleotide sequence of BAu is 5′-ATGTTTGAGACCTTCAACAC-3′, and that of BAd is 5′-CATCTCTTGCACGAAGTCGA-3′. The nucleotide sequences of L1 and H1 for the ND1 gene are 5′-ACATACCCATGGCCAACC TC-3′ and 5′-AATGATGGCTAGGGTGACTT-3′, respectively.

Concurrent PCR

The relative content of mtDNA in human sperm was determined by a hot-start concurrent PCR technique, which was developed to measure the amount of mtDNA relative to nuclear DNA. The desired segment was amplified from about 20 ng of each DNA sample in a 50-μL reaction mixture containing 200 μM of each dNTP, 0.2 μM of primers L1 and H1, 0.6 μM of primer BAu and BAd, 1 unit of Taq DNA polymerase (Perkin-Elmer Life Science, Inc., Boston, MA), 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.3). Polymerase chain reaction was performed for 25 cycles in a DNA thermal cycler (Model 9600, Perkin-Elmer). A suitable amount of α-[^{33}P]-dATP (0.5 μCi) was added to the reaction mixture at the last cycle to label the PCR products.

Quantification of PCR Products

The PCR products were separated by electrophoresis on a 2.25% agarose gel at 100 V for 1 hour. The DNA bands were then blotted onto a piece of Hybond-N^+ membrane. A Kodak roentgenogram film was exposed to the blotted membrane for 1 to 2 days. Polymerase chain reaction products of two different sizes, 315 base pairs (bp) and 450 bp, were visualized on the film. The relative intensity of the two DNA bands was quantified by use of a PhosphorImager (Model Strom 840; Molecular Dynamics, Sunnyvale, CA). The content of mtDNA relative to nuclear DNA was calculated as follows: Relative content of mtDNA = (area of the *ND*1 band/area of the *β-actin* band) $\times$ (315/450).

Establishment of the Standard Curve for Determination of mtDNA Content

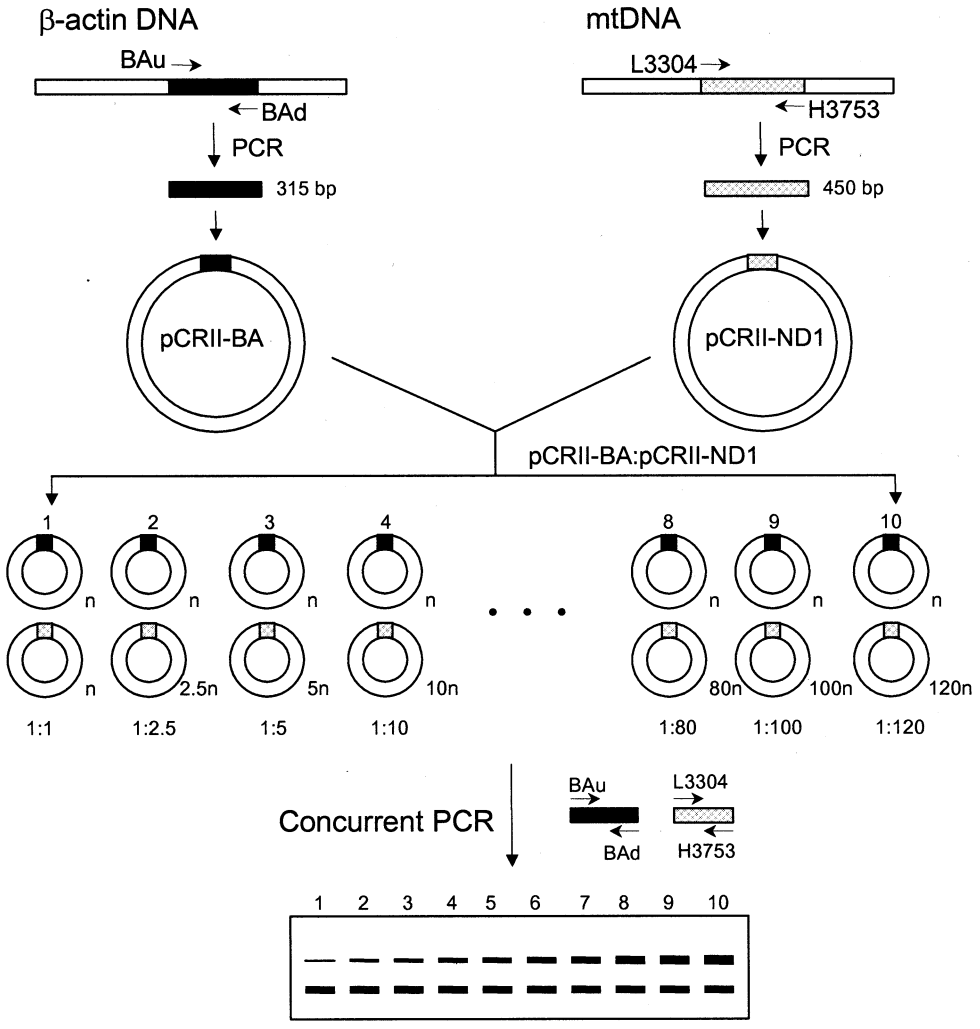
The PCR products of the *ND*1 and *β-actin* genes were cloned by cohesive ligation to a PCRII vector (Invitrogen Co., NV Leek, the Netherlands). The DNA mixture was composed of different molar ratios of the plasmid DNA containing the gene fragment of *ND*1 and that containing *β-actin* DNA fragment. By using the primer pairs L1–H1 and BAu–BAd, the concurrent PCR was performed for each of the plasmid DNA mixtures as we have described earlier in this article. The construction of the plasmids and establishment of the standard curve are schematically illustrated in Figures 1 and 2.

Flow Cytometric Analysis

An aliquot of 1 $\times$ 10^6 sperm were gently stained for mitochondria in 0.4 mL of phosphate-buffered saline (PBS) with 0.5

FIGURE 1

Construction of plasmid and the concurrent PCR system for the determination of mtDNA content. A plasmid PCRII-BA was constructed to encompass the sequences of the *β-actin* gene from nucleotide position (np) 940 to np 1,254 (315 bp), and the other plasmid PCRII-ND1 was constructed to encompass the sequences of the *ND*1 gene from np 3,304 to np 3,753 (450 bp). The molar ratio between the plasmid PCRII-BA and the PCRII-ND1 was set at 1:1, 1:2.5, 1:5, 1:10, 1:20, 1:40, 1:60, 1:80, 1:100, and 1:120 (*β-actin*: *ND*1), respectively. By using both primer pairs BAu-BAd and L1-H1, the concurrent PCR was then performed for each of the plasmid DNA mixtures, as described in Materials and Methods.



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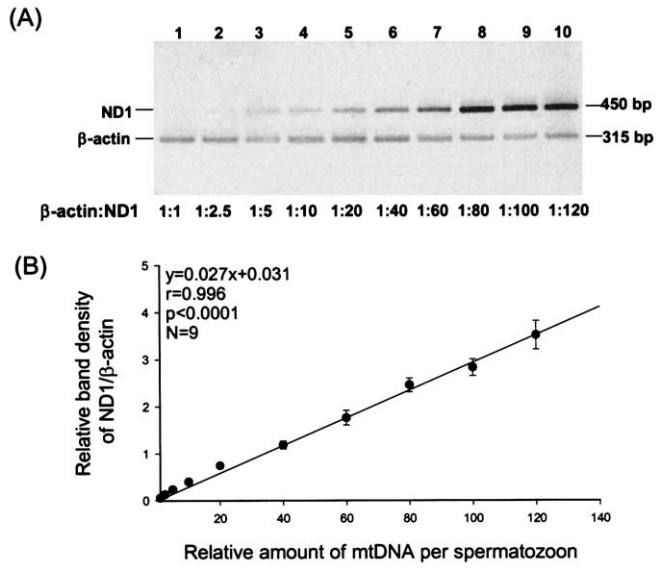
μmol MitoTracker Green (Molecular Probes, Eugene, OR) (16) for 30 minutes at room temperature. After washing with PBS, sperm were fixed in 2% paraformaldehyde in PBS for 1 hour at room temperature. Another aliquot of sperm was stained with propidium iodide (PI) in 0.4 mL of PBS containing 50 μg/mL of PI, 0.1% sodium citrate, and 0.1% Triton X-100 and was analyzed after 20 minutes of incubation at room temperature (17). After staining, sperm were washed with PBS and subjected to analysis with the FACScan (Becton Dickson, San Jose, CA), equipped with a single 488-nm argon laser. A minimum of 30,000 sperm per sample was analyzed. Debris was gated out based on light-scattering measurements. Data were acquired in list mode, and relative proportions of sperm within different areas of fluorescence profiles were quantified with the LYSYS II software program (Becton Dickinson and Company, Franklin Lakes, NJ).

Sperm Head Separation

The sperm were fractionated into two portions; one is the intact nuclear fraction, and the other, the tails, mitochondria, or perinuclear theca. The intact nuclear fraction was subjected to PI staining. Sperm were collected and then sus-

FIGURE 2

Establishment of the standard curve for determination of sperm mtDNA content. (**A**) The PCR products of the DNA mixtures composed of differential molar ratios of the two plasmid DNAs (*β-actin*: *ND1*) are shown in *lanes 1* to *10*. (**B**) The band intensity of the PCR products was plotted against the molar ratio between the two plasmid DNAs. There was a strong positive correlation between the content of mtDNA and band intensity ($r = 0.99$, n = 9). This demonstrates the validity of the method.



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pended in 2 mL of nuclear isolation medium (NIM; 121.6 mM KCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1% polyvinyl alcohol, 10 mM ethylenediaminetetraacetic acid, 0.5 mM phenylmethylsulfonyl fluoride) supplemented with an ionic detergent, 0.5% mixed alkyltrimethylammonium bromide (ATAB), and 2 mM dithiothreitol. The pH was adjusted to 8.2 by 1 N KOH. This suspension was used after 1 or 2 weeks of storage at 4°C. Alkyltrimethylammonium bromide separates the sperm head from the tail (18, 19). Sperm were suspended in this NIM medium for 15 minutes at room temperature and were processed by centrifuge at 700 $\times$ *g* for 5 minutes. The sperm pellet was washed in 10 mL of NIM without ATAB or dithiothreitol and were processed by centrifuge again. The pellet was suspended in NIM, and sperm heads were used for PI staining and cell sorting.

Electron-Microscopic Examination of Sperm Mitochondria

The sperm were washed and fixed for 30 minutes in the Karnovsky's solution containing 4% formaldehyde, 2% glutaraldehyde, 2 mM $CaCl_2$, and 0.1 M cacodylate buffer, pH 7.2. Excess fixative solution was removed by washing three times with PBS. After fixation in 1% OsO_4 for 15 minutes and washing with PBS three times, sperm were dehydrated through a series of graded ethanol solution (from 70% to 99.9% ethanol), and then embedded in Spurr's resin (ERL-4206 embedding mixture; Merck Co., Darmstadt, Germany). Thin sections were cut with a Reichert Ultracut II ultramicrotome (Leica, Inc., IL) with a Diatome diamond knife (Diatome U.S., Hatfield, PA). After being mounted on precleaned and uncoated copper grids, thin sections were further stained with uranyl acetate and lead citrate. The ultrastructure of sperm was examined with a Zeiss 900 transmission electron microscope.

Statistical Analysis

Analysis of variance was employed to determine the significance of the correlation between the relative content of mtDNA and the score of each of the sperm motility parameters. A P value of $<.05$ is considered significant.

RESULTS

Establishment of the Standard Curve for Determination of Sperm mtDNA Content

We amplified 450-bp and 315-bp PCR products from the mtDNA and nuclear DNA of sperm, respectively. The ratios between the DNA band intensities of the two PCR products were 0.054 ± 0.013 (mean ± SD, n = 9), 0.127 ± 0.020, 0.232 ± 0.031, 0.397 ± 0.017, 0.743 ± 0.031, 1.181 ± 0.086, 1.762 ± 0.157, 2.458 ± 0.146, 2.831 ± 0.178, and 3.518 ± 0.302 of the plasmid DNA mixture with *ND1* to *β*-actin gene ratios of 1:1, 2.5:1, 5:1, 10:1, 20:1, 40:1, 60:1, 80:1, 100:1, and 120:1, respectively (Fig. 2A). There was a strong positive correlation between the relative ratio of the PCR products and the relative content of mtDNA ($r = 0.99$, n = 9; Fig. 2B). On the basis of this calibration curve, we were able to determine the relative content of mtDNA in sperm with different motility scores.

Correlations Between the Content of mtDNA and Motility Parameters

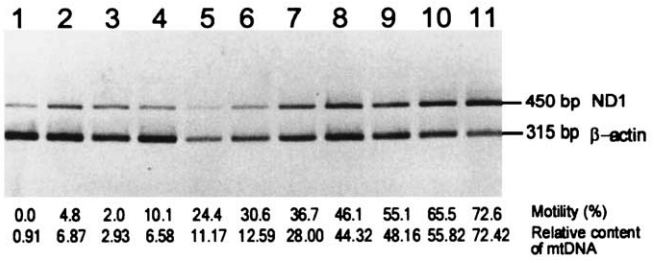
We examined the relative contents of mtDNA of the sperm with different motility. As shown in Figure 3, the relative contents of mtDNA were determined to be 0.91, 6.87, 2.93, 6.58, 11.17, 12.59, 28.00, 44.32, 48.16, 55.82, and 72.42 for the sperm with motility scores of 0.0, 4.8%, 2.0%, 10.1%, 24.4%, 30.6%, 36.7%, 46.1%, 55.1%, 65.5%, and 72.6%, respectively. The relative mtDNA contents in the asthenospermia or sperm with poor motility (<20%) and in sperm of healthy men were 7.2 ± 1.3 (mean ± SD, n = 23) and 74.1 ± 2.0 (n = 29), respectively. Moreover, the extremely low contents of mtDNA were found in 6 of 13 patients with asthenospermia, whose relative content of mtDNA in sperm were approximately one to eight.

Mitochondrial Mass and DNA Content in Sperm

After PI staining and flow cytometric analysis, the average median of total cellular DNA was 117.90 ± 14.94 (n = 23) for the normal samples, and that was 48.87 ± 9.66 (n =

FIGURE 3

Determination of mtDNA content of human sperm by concurrent PCR. The relative contents of mtDNA were calculated for the sperm from individuals with the different motility scores in *lanes 1* to *11*, respectively.



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16) for the sperm in the patient with asthenospermia or with poor motility. Left shift of histogram was found in the groups with poor motility (Fig. 4A). Significant differences in the average medians of cellular DNA were detected between the sperm with different motility by linear regression analysis ($r = 0.62$, $P<.005$).

By contrast, the ATAB-treated sperm were fractionated into the intact nuclear fraction and the tails, mitochondria, and perinuclear theca fraction. No left shift of histogram was seen in the PI-stained nuclear fraction. The average median of nuclear DNA was 48.05 ± 3.34 (n = 9) in the intact nuclear fraction of the ATAB-treated sperm with different motility (Fig. 4B). The decrease of total DNA content may be due to the depletion of sperm mtDNA, not a result of the paucity of nuclear DNA. We then used MitoTracker Green staining to visualize mitochondria and measured mitochondrial mass. The average median of mitochondrial mass was 483.94 ± 98.42 (n = 17) for sperm in the patients with asthenospermia or with poor motility and was 492.22 ± 184.68 for the normal subjects (n = 15, Fig. 4C). The differences were considered not significant because the *P* value was >.05 ($r = 0.10$, $P>.1$).

Ultrastructure of Sperm

Ultrastructure of human sperm from six patients with normospermia (n = 2) and asthenospermia (n = 4) were examined with a transmission electron microscope. We examined thousands of sperm for each individual. The subcellular structure and organelles, including cell membranes, acrosome, nucleus, mitochondria, and axoneme of the sperm, were essentially normal in all these patients. No apparent abnormalities were observed in subcellular organization and overall morphology of mitochondria in midpiece of sperm (Fig. 5).

DISCUSSION

The maturation of spermatogonia to a spermatozoon capable of fertilization involves the rearrangement of mitochondria and development of a functional tail. Adenosine triphosphate generated from the mitochondria is delivered to the axoneme and is used for flagellar propulsion. Recently, Ruiz-Pesini et al. (20) used drugs that specifically inhibit the activity of mitochondrial respiratory enzyme complexes to demonstrate their progressive impairment of sperm motility and ultimate blockade of flagellar movement. Their observation underscores the importance of mitochondrial function in sperm motility.

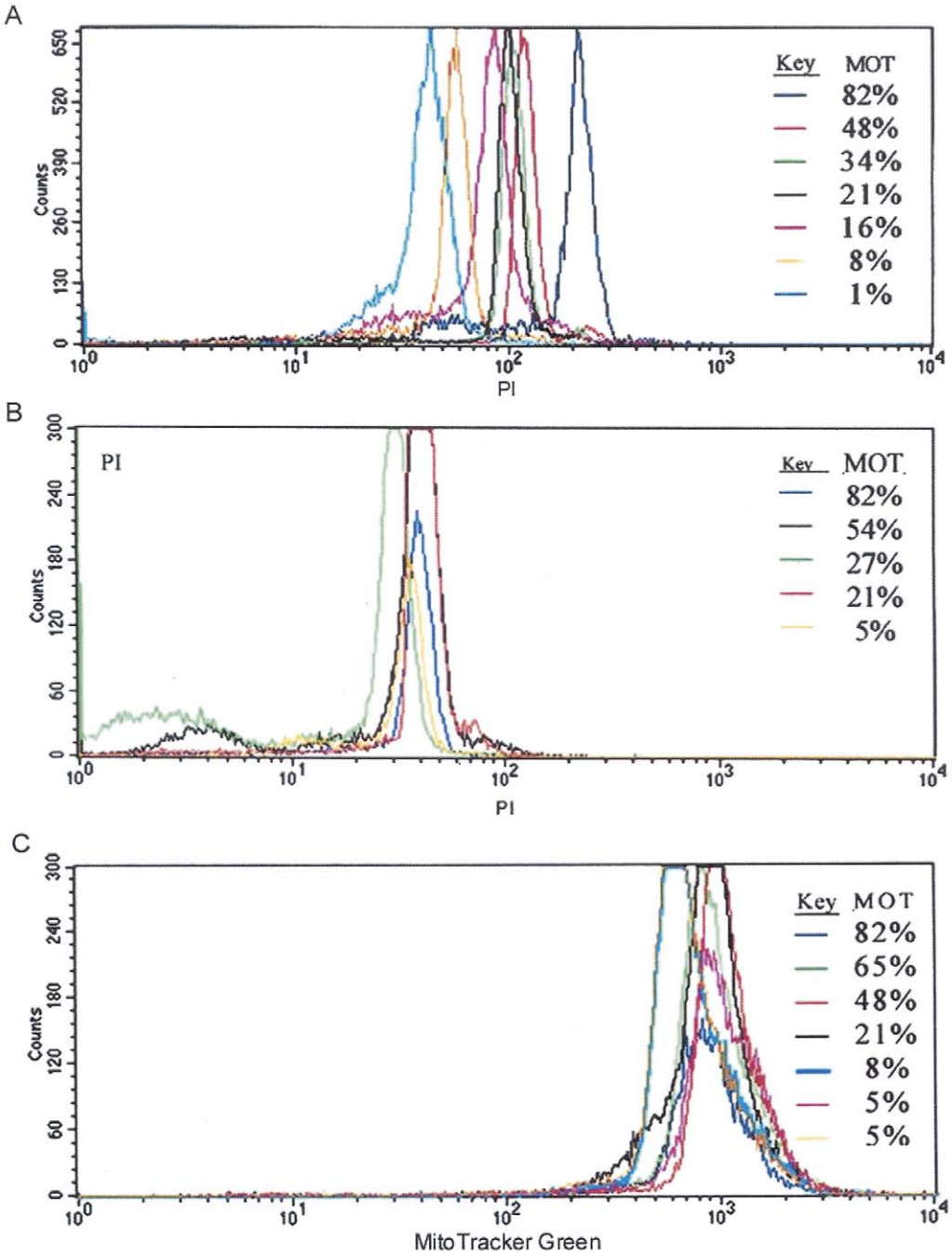
It has been shown that the paternal mitochondria and mtDNA do enter into the ovum but are eliminated rapidly by dilution factor or unknown mechanisms during the early zygotic cell divisions (21, 22). To prevent paternal transmission of modified or mutated mtDNA molecules, the sperm mtDNA must be reduced to a small number during spermatogenesis but still maintain a suitable copy number to provide sufficient oxidative phosphorylation capacity for sperm to swim. The change of sperm mtDNA copy number is a very important event throughout spermatogenesis, fertilization, and embryogenesis. Recently, it was demonstrated that the expression of mitochondrial transcription factor is downregulated in the male germ cells during spermatogenesis in the human and that sperm mtDNA copy number is subsequently reduced (23, 24).

It is known that each mature mammalian sperm contains approximately 50–75 mitochondria and one copy of mtDNA (10). Recently, the mtDNA copy number per spermatozoon was reported to be 75 (12) or 10 to 100 (25), or 10.1 (26) and 1,300 (2) or 700 (27) copies, respectively, for the mouse and human. The variable mtDNA contents might be due to differential preparation of sperm DNA and analytical methods. As discussed by Man-Panloup (26), one explanation for these discordant results may lie in the use of different methods to quantify the mitochondrial genome. Indeed, Southern blot may lead to cross-hybridization with the large number of mitochondrial pseudogenes (286 pseudogenes) recently found in the nucleus (28). These nuclear mitochondrial pseudogenes share high levels of similarity with those in mtDNA and might have been erroneously recognized by the wide-ranging mtDNA probes such as those used in Southern blotting. Another explanation for the discrepancy of the variable content of mtDNA might lie in the presence of contaminated cells (mainly immature sperm and leukocytes) in the sperm preparations. Contamination by only a few of these cells, which might alter mtDNA contents, could easily lead to an overestimation of mtDNA content in sperm.

In this study, we applied Ficoll-Paque and osmotic shock to remove the contaminated leukocytes, immature sperm cells, and other cells before DNA extraction. We found that the relative content of mtDNA was 74.1 ± 2.0 for sperm with normal motility scores of 51%–90%. Moreover, we

FIGURE 4

Determination of DNA content and mitochondrial mass of human sperm by flow cytometry. (**A**) By staining with PI to check DNA content of total sperm, left shift of histogram was revealed in the sperm of a patient with asthenospermia or with poor motility. *Dark blue line,* 82%; *orange line,* 48%; *green line,* 34%; *black line,* 21%; *pink line,* 16%; *yellow line,* 8%; *bright blue line,* 1%. (**B**) The intact nuclear fraction after ATAB treatment was subjected to PI staining. No significant difference in average nuclear DNA content was detected between the spermatozoa with different motility. *Blue line,* 82%; *black line,* 54%; *green line,* 27%; *red line,* 21%; *yellow line,* 5%. (**C**) By staining with MitoTracker Green to analyze sperm mitochondrial mass, the medians were localized in the nearby area in the sperm with different motility. No significant left shift was revealed. *Dark blue line,* 82%; *green line,* 65%; *orange line,* 48%; *black line,* 21%; *bright blue line,* 8%; *pink line,* 5%; *yellow line,* 5%.



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found extremely low content of mtDNA in 6 of 13 patients with asthenospermia. The relative contents of mtDNA in sperm of these patients were between one and eight, which are usually 70–80 in normal sperm. The reduction in sperm motility could potentially arise from defects in the mitochondrial respiratory function caused by mtDNA depletion.

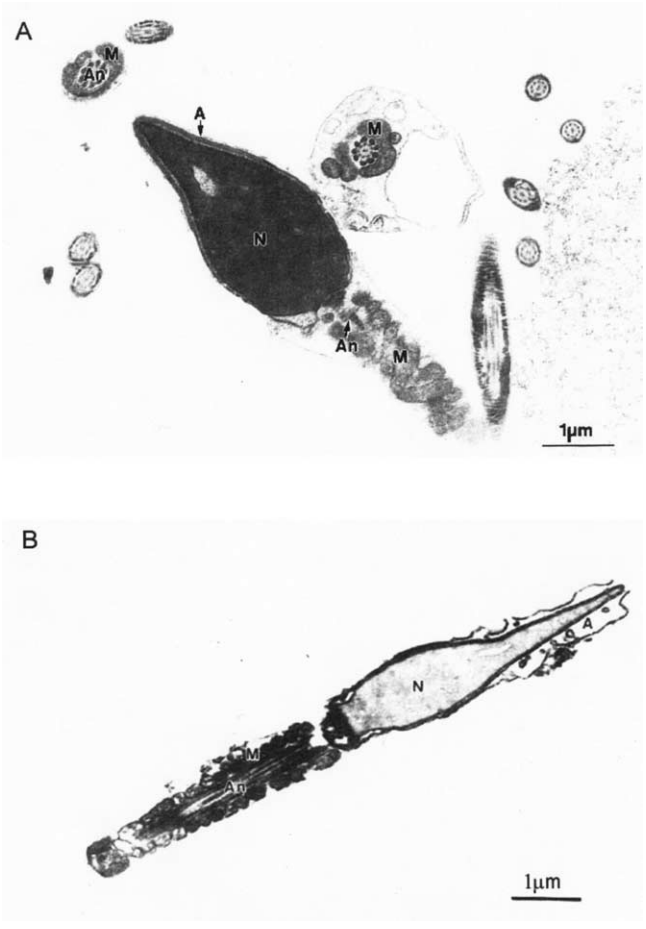
Recently, Díez-Sánchez and colleagues (27) examined the mtDNA content of progressive motile sperm by slot-blot hybridization. Approximately 700 to 1,200 copies of mtDNA were present in the progressively motile sperm and round spermatid cell, respectively. The mtDNA copy number was far from the reported mtDNA contents of mammalian sperm. The high mtDNA copy number might be due to the calculation according to the mtDNA content of the 143B cell line. In the report of May-Panloup et al (26), the mtDNA copy numbers were also much higher in sperm collected from the 40% density gradient layers compared with those collected from the 100% layers. They found that the sperm with poor motility had higher mtDNA content than did sperm with good motility in the same individuals. In this study, mtDNA depletion was found in some men with asthenozospermia or poor motility. There may be different factors involved in mtDNA reorganization during spermatogenesis. On the basis of these findings, we postulate that the mtDNA content could be a valuable biomarker of sperm quality. A defective process of spermatogenesis could generate dysfunctional sperm.

Depletion of mtDNA may arise spontaneously under the genetic pressure during spermatogenesis through defective replication of mtDNA resulting from poor communication between the nuclear and mitochondrial genomes. Although the mechanism of mtDNA depletion is unclear, it has been proposed that mtDNA depletion is caused by mutations in the mitochondrial transcription factors or in proteins involved in mitochondrial replication machinery.

Moreover, the mitochondrial number per se is still maintained within a normal range in the cells with increased or reduced mtDNA copy number in the sperm of infertile patients (29, 30). This indicates that mtDNA depletion is not caused by a reduction of mitochondrial biogenesis. This gene-dosage mechanism may explain the observation that the number of mitochondria remains rather constant but that the number of mtDNA molecules per mitochondrion is increased or decreased under different pathophysiological conditions. In flow cytometric analysis, similar amounts of mitochondrial mass were revealed in the sperm with different motility scores. Extreme depletion of mtDNA was detected in some men with asthenospermia. Electron-microscopic examination revealed helicoidal assembly of mitochondria with essentially normal morphology in the midpiece of sperm from patients with asthenospermia or normal individuals. These results support the notion that mtDNA depletion is not due to the paucity of mitochondria but to the reduction in the number of mtDNA per se.

FIGURE 5

Electron micrographs of human sperm. (**A**) The spermatozoon from a patient with asthenospermia. (**B**) The spermatozoon from a normal subject. The ultrastructures including cell membrane, acrosome (A), nucleus (N), mitochondria (M), and axoneme (An) were essentially normal in the sperm, with only 0.91 relative amount of mtDNA per spermatozoon. Mitochondria in the midpiece did not exhibit any structural abnormalities (original magnification × 20,000). The *bar* on the bottom right represents the scale of 1 μm.



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Furthermore, the man with sperm mtDNA depletion may be infertile. It is highly possible that such a patient would suffer from other symptoms associated with mtDNA depletion. Depletion of mtDNA occurs during the spermatogenesis. Several studies also depicted that high levels of mutant mtDNA strongly correlate with low sperm motility (31, 32) and that heteroplasmy of mtDNA was identified in human sperm. High incidence of mtDNA mutation may be related to the change of the microenvironment surrounding the primordial germ cells (e.g., generation of free radicals, oxygen pressure, contaminants, hormones, and smoking) during spermatogenesis. The mutated mtDNA molecules may be propagated by defective nuclear factors from paternal or

maternal germ cells and are clonally expanded through the germ cells and localized in the primordial germ cytoplasm during gametogenesis (29, 33).

In summary, we developed a new concurrent PCR method to determine the relative content of mtDNA in human sperm. This method has greatly improved the detection limit and has reduced the amount of sperm that is usually required for traditional Southern blot analysis. Taking the findings together, we suggest that mtDNA content may be used for the assessment of the fertility and motility of human sperm and that depletion of mtDNA may play an important role in the pathophysiology of some types of male infertility and subfertility.

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