Sperm Artificially Exposed to Antisperm Antibodies Show Altered Deoxyribonucleic Acid¹

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Purpose: Our purpose was to assess sperm deoxyribonucleic acid (DNA) integrity after exposure to antisperm antibodies.

Methods: Donor semen were divided and exposed to sera containing IgG, IgA, and IgM antisperm antibodies. Untreated portions served as the control. After incubation (1 hr, 23°C), the sperm were centrifuge-washed, resuspended, and incubated (23°C) for 2, 5, 7, or 9 days. Acridine orange staining and kinematic parameters were measured. The sentinel (17q21 from D17S855) and β -globin genes were amplified and analyzed using denaturing gradient gel electrophoresis.

Results: Sperm preexposed to antisperm antibodies had deleted sentinel gene on days 7 and 9. The β -globin gene was intact. There were no differences in acridine orange staining.

Conclusions: Sperm artificially exposed to antisperm antibodies resulted in a subtle deletion of genetic material. The DNA alteration process was slow and was undetectable at the gross level. More studies are needed to confirm the findings and determine whether DNA repair mechanisms can reverse the damage.

KEY WORDS: apoptosis; immunology; antisperm antibodies; spermatozoa; denaturing gradient gel electrophoresis.

INTRODUCTION

The presence of autoantibodies against human sperm or antisperm antibodies were first reported in 1959 by Rümke and Hellinger (1). Antisperm antibodies have

been linked to abnormal fertilization of the oocyte (2–6). Furthermore, antisperm antibodies were linked to abnormal embryo development by retarding the cleavage process (7). There have been some data in the past suggesting that antisperm antibodies can even be a cause of spontaneous abortions or miscarriages (8–16) but recent data do not seem to support this concept (17–19). Reasons offered for the discrepancy ranged from differences in antibodies concentration in serum or seminal plasma fluid to precision and accuracy of the test procedures. Data showing an effect of antisperm antibodies on the actual DNA of the sperm cell is lacking. Hence, the null hypothesis proposed in this study was that sperm DNA was unaffected by antisperm antibodies. The objective was to analyze the integrity of a designated sentinel gene in sperm exposed to antisperm antibodies over a period of time. To accomplish the objective, a sentinel gene was targeted for polymerase chain reaction (PCR) amplification. The primers for the gene (D17S855 section of 17q21) were chosen based on convenience and availability of primers at this laboratory and based on frequency of mutations at that region. PCR was followed by sensitive denaturing gradient gel electrophoresis (DGGE) to analyze for changes in the sperm DNA including point mutation.

MATERIALS AND METHODS

Preparation of Sperm

Liquefied semen from a single fertile donor testing negative for antisperm antibodies was used throughout the study to control for polymorphic mutations. Semen analysis was performed to ensure that each semen specimen had over 20 million/ml sperm with over 50% motility and at least 30% normal forms by WHO criteria (20). The absence of white blood cells and

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immature germ cells in the semen were also confirmed by myeloperoxidase testing (21). Each sperm specimen was divided equally and using a blinded protocol, one portion (0.7 ml) was added to 0.3 ml of mixed sera previously shown to contain antisperm antibodies by indirect immunobead assay (Fertility Technologies, Natick, MA). The mixed sera were derived from males testing positive for antisperm antibodies and stored frozen until use. The remaining portion of untreated sperm specimen served as the control. The treated and control sperm were incubated at room temperature (23°C) for 1 hr.

After incubation, the sperm were washed free of the sera. This was accomplished by mixing an equal volume of phosphate-buffered saline (PBS) supplemented with 3.5% bovine serum albumin fraction V and processed by centrifuge wash at 300g for 10 min. The resultant pellets were resuspended in 0.5 ml PBS medium and the presence of antisperm antibodies on the sperm (Table I) was confirmed by the direct immunobead assay (20). Separate tubes of sperm cells were incubated at room temperature (23°C) for 2, 5, 7, and 9 days. The incubation temperature was based on preliminary studies showing longest sperm survivability *in vitro*.

An aliquot (10 μ l) was removed from each washed sperm suspension and the sperm dimensions and kinematic parameters were analyzed using the Hamilton-Thorn Model C (HTM-C; Hamilton Thorn, Danvers, MA) computer-aided sperm analyzer (CASA). The analyses were carried out in 20- μ m-deep glass slides with coverslips prewarmed at 37°C on a slide warmer. The calibrations for the HTM-C have been reported previously (21,22). The parameters measured on the HTM-C were mean amplitude of lateral head displacement (ALH; distance the spermatozoa head swings from side to side), computer-fitted total motility (percentage of motile sperm in the aliquot which fits the computer criterion for a motile cell), beat cross fre-

quency (BCF; crossing of sperm over the computer-calculated mean path), curvilinear velocity (VCL; velocity calculated from the sum of trackpoint-to-trackpoint velocity), average path velocity (VAP), straight-line velocity (VSL; velocity calculated from the beginning to the end of a digitized sampling interval), and sperm head dimensions.

Acridine Orange Test for Gross-Level DNA Denaturation

After each incubation time period, a drop from each well-mixed sperm specimen was used to make a smear on a glass slide and air-dried (23). Each dried sperm smear was stained with a solution containing 6 μ g/ml acridine orange (AO) dye dissolved in PBS for 3 min. The AO procedure was done with the room lights off and in diffuse room lighting only. The slides were washed with water to remove background staining, and while still wet, they were each covered with coverslips and immediately analyzed. The AO-stained sperm were examined in an ultraviolet (UV) fluorescent microscope at 400 $\times$ magnification. A total of 200 cells was analyzed for each sperm treatment. Special care was taken by narrowing down the UV light diaphragm and permitting only a small beam of UV light to pass through to prevent overexposing the stained sperm to UV light. Overexposure would cause the sperm to bleach to a green coloration and yield inaccurate data.

Sperm undergoing denaturation or apoptosis where the DNA became fragmented to single strands stained orange-red at the head, while healthy sperm with double-stranded DNA stained green at the head (23). The percentage of sperm with denatured DNA was calculated by dividing the number of orange-red sperm by the total number of sperm and multiplying by 100.

Polymerase Chain Reaction (PCR) Protocol

The remaining sperm specimens were transferred to microcentrifuge tubes and centrifuged (500g, 1 min) down to pellets and stored frozen at -20°C until used. On the day of PCR, each sperm specimen was thawed and added to 200 μ l of MilliQ-UF purified water and DNA extracted by boiling at 100°C for 1 hr. An aliquot (10 μ l) from each DNA specimen was added to the PCR reaction cocktail (Perkin Elmer Cetus, Norwalk, CT) in a reaction tube.

Each reaction tube contained 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 4.0 mM MgCl₂, a 200 μ M concentration of each dNTP, 100 μ g/ml gelatin, a 1 μ M

Table I. Immunobead Test Results of Untreated Control Sperm and Sperm Exposed to Antisperm Antibodies: Sperm Were Preexposed to Sera with Antisperm Antibodies for 1 hr (23°C), Followed by Washing to Remove Sera

Specimen	% binding		
	IgG	IgA	IgM
Sperm preexposed to antisperm antibodies	68.1 $\pm$ 16.5*	42.6 $\pm$ 11.2*	1.8 $\pm$ 2.7*
Untreated control sperm	1.0 $\pm$ 1.4	1.5 $\pm$ 1.3	0 $\pm$ 0

* $P < 0.05$.

concentration of each pair of primers targeting the sentinel gene (Bio-Synthesis Inc., Lewisville, TX), 1.3 U of Taq polymerase enzyme (AmpliTaq; Perkin Elmer Cetus), and 50 μ l of mineral oil. The PCR amplified a 434-base pair (bp) product for the sentinel gene. The negative control consisted of the complete PCR reaction cocktail reagents and purified water. The PCR was carried out in a Perkin Elmer Cetus thermal cycler. The hot-start denaturation step was part of the 100°C for 1 hr heat treatment. The cycling parameters were set for 32 step-cycles with 40 sec of denaturation at 94°C, 40 sec of annealing at 60°C, and 40 sec of extension at 72°C, followed by a final extension at 72°C for 7 min. Each amplified product was pipetted out, mixed with 5 $\times$ loading buffer, and loaded into wells and denaturing gradient gel electrophoresis carried out as described below (24).

The presence of DNA in each sperm specimen was also verified by carrying out separate PCR reactions targeting the housekeeping β -globin gene. Aliquots of sperm DNA from each pair of treatment were hot start denatured by heating for 7 min at 95°C and each aliquot (10 μ l) was added to the PCR cocktail (Perkin Elmer Cetus) containing a 1 μ M concentration of each pair of primers (PC04, GH20; Perkin Elmer Cetus) targeting the β -globin gene (268 bp), 2.5 U of Taq polymerase enzyme (AmpliTaq; Perkin Elmer Cetus) and 50 μ l of mineral oil. The PCR was carried out in a Perkin Elmer Cetus thermal cycler. The parameters for the β -globin amplification were 30 step-cycles with 30 sec of denaturation at 94°C, 1 min of annealing at 55°C, and 30 sec of extension at 72°C. A final extension was done at 72°C for 7 min. The amplified product was processed through 0.8% agarose gel electrophoresis, and the separated bands were stained in ethidium bromide and visualized and photographed in UV light. For each agarose run, the *Hae*III digest of plasmid ϕ X174 served as the marker.

Denaturing Gradient Gel Electrophoresis (DGGE)

Detection of deletion, point mutation, frame shift or base substitution was carried out using DGGE as reported previously (24). Briefly, a 40% acrylamide stock solution was prepared from 38.96 g acrylamide, 1.04 g BIS (*N,N'*-methylene-bis-acrylamide) in a 100 ml solution. A 100% acrylamide stock solution was prepared from 16.25 ml of the 40% solution, 2 ml of 50X (50 times concentrate) Tris-acetate-ethylenediaminetetraacetic acid (TAE; 0.04 M Tris-acetate with 0.001 M EDTA), 42 g of urea, 40 ml of

formamide, and water in a 100-ml solution. The third stock solution, the 0% solution, was prepared from 16.25 ml of 40% acrylamide solution, 2 ml of 50X TAE, and water for a final 100-ml volume. The range of denaturant was from 20 to 80% acrylamide solutions for the DGGE. From the first three stock solutions, the final 20% solution was prepared from 4 ml of the 100% stock and 16 ml of the 0% stock solution. The final 80% solution was prepared from 16 ml of the 100% stock and 4 ml of the 0% stock. Each of the solutions was deaerated and added with 10 μ l TEMED (*N,N,N',N'*-tetramethylethylenediamine) and 100 μ l of a 20% ammonium persulfate and poured into a glass plate sandwich setup (80% at the bottom, decreasing to 20% at the top). Each set gel was placed into a 60°C heated aquarium containing 1X TAE solution. The amplified products were mixed with 5X loading buffer (bromophenol blue and glycerol) and pipetted into the wells. The customary markers with known bands were not needed, as the principle of DGGE was based on the melting temperature of DNA domains instead of molecular size. The amplified products were allowed to be drawn into the gel for 20 min at 85 V. Following this, a heated circulating pump was started and electrophoresis continued for 8 hr. The gel was stained in ethidium bromide and visualized under UV light. Photographs were taken, and the results recorded and analyzed. The PCR and DGGE of the sperm specimens were repeated and the results of the second analysis cross-checked and verified with the first analysis.

Statistical Analysis

The results were tabulated as the mean $\pm$ standard deviation. Differences in means were tested using Student's paired *t*-test. *P* < 0.05 was considered significant.

RESULTS

The results showed deletion of the sentinel gene in antisperm antibodies treated sperm on day 7 of incubation (Fig. 1). The deletion of this sentinel gene was corroborated by another set of antisperm antibodies treated sperm on day 9 of incubation. There were no deletions of DNA in the same gene in untreated control sperm. There were no observed changes in the DNA of sperm on days 2 and 5 of incubation. Heteroduplex formation was not observed. In the antisperm antibody-exposed sperm specimens where deletion of the sentinel gene was observed, the presence

Fig. 1. Denaturing gradient gel electrophoresis (20:80 DGGE) analysis for deletion in the sentinel gene in 17q21 from the D17S855 region after polymerase chain reaction amplification of DNA of sperm exposed to antisperm antibodies (ASAB) and incubated for different time periods. Bands are observed at 51.4%, at a calculated melting temperature of 77.2°C, after staining in ethidium bromide and visualization in UV light. The absence of amplified bands in lanes 6 and 8 suggests a deletion mutation. The lack of mobility shifts suggests the absence of point mutations in the targeted gene region. The bands are 2-day incubation in lanes 1 and 2, 5-day incubation in lanes 3 and 4, 7-day incubation in lanes 5 and 6, and 9-day incubation in lanes 7 and 8. Antisperm antibody-positive sperm DNA is in lanes 1, 3, 6, and 8. The negative controls are in lanes 2, 4, 5, and 7. Lane 9 is a repeat of lane 1 for alignment control.

of DNA was verified by amplification of the β -globin gene, thus ruling out artifacts.

The data (Table II) from the acridine orange analyses of DNA denaturation in antisperm antibody-treated sperm were not significantly different, suggesting negligible gross level changes in sperm DNA. Furthermore, the percentages of the denaturation of sperm DNA in the control sperm were not different from the treated sperm and remained similar for the 9 days of incubation in vitro. The percentages of normal DNA were consistent with those of washed fertile donor sperm.

In the present study, direct immunobead testing of the antisperm antibody-treated sperm indicated the presence of $68.1 \pm 16.5\%$ IgG, $42.6 \pm 11.2\%$ IgA, and $1.8 \pm 2.7\%$ IgM, while the untreated control sperm

was negative for antisperm antibodies (Table I). There were no differences in semen characteristics and kinematic parameters between the sperm specimens with antibodies and the negative control (Table III).

DISCUSSION

The present study demonstrated an association between sperm that had been artificially exposed to sera containing antisperm antibodies and the deletion of genetic material such as the designated sentinel gene in 17q21 from the D17S855 region. The deletion transformation process appeared to require time, at least 7 days of incubation, for detection using the present method of analysis. The in vitro design of this study does not necessarily correspond to the in vivo situation. The mechanisms involved in acquiring antibodies naturally and the exposure period may be different. For example, sperm exposed to seminal fluid containing antibodies at the time of ejaculation would have a short duration of exposure or the fertilizing capacity of the sperm would not extend into 1 week. Hence, studies evaluating males with naturally acquired antibodies are needed to confirm the in vitro findings.

The sentinel gene was chosen to signal subtle changes in DNA based on the high frequency of DNA mutations in this region (25–29). The deletion of the sentinel gene does not appear to be an artifact of failed amplification of DNA because PCR amplification of the β -globin gene in those sperm specimens yielded confirming bands. Repeat PCR confirmed the deleted sentinel gene in the treated sperm while amplifying the gene in control sperm.

In this study, the exposure time used was up to 9 days in the presence of sera containing a mixture of IgG, IgA, and IgM antibodies. Immunobead testing (20) showed that the sera contained 68.1, 42.6, and 1.8% IgG, IgA, and IgM, respectively. Gross-level denaturation of sperm DNA was not detectable in either the treated or the control sperm even after 9 days of incubation as shown by the AO assay (23) data. The results suggested that the effects of antisperm antibodies on sperm DNA are subtle and remain undetectable at the gross level. Past reports have suggested an association between antisperm antibodies and spontaneous abortion (8–11), but more recent data seem to refute this association (17–19). The mechanism involved in terminating the viable fetuses by antisperm antibodies has yet to be elucidated, although an immunological response was suspected in the spontaneous

Table II. Acridine Orange Test of Control Sperm and Sperm Exposed to Antisperm Antibodies

Specimen	Day 2	Day 5	Day 7	Day 9
Sperm preexposed to antisperm antibodies	99.0 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	98.0 $\pm$ 2.0
Untreated control sperm	98.0 $\pm$ 1.0	100 $\pm$ 0	99.5 $\pm$ 0.5	100 $\pm$ 0

Table III. Basic Semen Characteristics and Kinematic Parameters of Sperm with and Without Antisperm Antibodies: Posttreatment Sperm Were Measured Immediately After Sperm Washing^a

Parameter	Before-treatment group (<i>N</i> = 420)	Posttreatment	
		Antisperm antibody positive (<i>N</i> = 448)	Antisperm antibody negative (<i>N</i> = 424)
Count (millions/ml)	52.7 ± 21.0	40.9 ± 13.8	46.2 ± 17.6
Motility (%)	64.0 ± 8.5	64.3 ± 8.5	54.5 ± 6.4
Progressive (%)	17.5 ± 8.7	13.5 ± 5.6	13.3 ± 3.7
Average path velocity (μm/sec)	32.3 ± 5.6	37.3 ± 3.3	32.8 ± 2.5
Curvilinear velocity (μm/sec)	43.8 ± 6.1	51.3 ± 6.3	43.8 ± 5.1
Straight line velocity (μm/sec)	22.5 ± 4.6	23.3 ± 2.6	22.0 ± 1.6
Lateral head displacement (μm)	2.1 ± 0.2	2.3 ± 0.6	2.2 ± 0.5
Beat cross-frequency (Hz)	10.1 ± 2.1	10.1 ± 0.7	8.8 ± 1.4
Mean width of sperm head (μm)	3.2 ± 0.3	3.4 ± 0.5	3.0 ± 0.2
Mean length of sperm head (μm)	6.2 ± 0.7	6.4 ± 1.6	5.4 ± 0.4

^a Sperm were preexposed to control or sera with antisperm antibodies for 1 h (23°C), followed by washing and analyses.

abortion cases. Indeed, anti-DNA antibodies are frequently found in patients with antisperm antibodies (30). The possibility that anti-DNA antibodies were in the test sera was ruled out by (a) the observed long delay, after 7 and 9 days of incubation, before changes in DNA occurred and (b) the *in vitro* conditions of the experiment. Regardless, the results suggested the deletion of genes in sperm artificially exposed to antisperm antibodies (31). It is possible that embryos resulting from the union between afflicted sperm cells and oocytes may have intrinsic DNA repair mechanisms that provide sufficient restructuring so as to permit embryo survival. More studies are needed, particularly to examine matched controls with males with antisperm antibodies and their partners undergoing IVF with and without ICSI, to gain insight into whether the presence of sperm antibodies leads to an increased incidence of poor or failed fertilization, implantation failure, or retarded embryonic development or an increased risk of spontaneous abortion.

Interestingly, the present finding challenges the dogma that the tightly packed condensed chromatin in the sperm head is invincible to mutational events. Deletions, a form of mutation, are possible and are similar to DNA fragmentation during apoptosis, although on a molecular scale. Immature sperm are also susceptible to changes in DNA due to the incomplete exchange of protamines for the histones. Indeed, it is hypothesized that the mechanism for the observed mutation in antibody-treated sperm involves antibody disruption of the sperm membranes, leading to lipid peroxidative damage to exposed parts of the sperm DNA that are missing protamine proteins. The antisperm antibodies may also affect sperm motility parameters, depending on the presence of components

such as complement proteins. The similarity in motility parameters between sperm with and sperm without antisperm antibodies at the start of the experiment (Table III) suggested that the deletion of the sentinel gene in treated sperm was not due to motility modifiers *in vitro*. It is evident that there is much to be done to elucidate fully the mechanisms involved in antibody-mediated DNA events.

While the focus is on sperm exposed to antisperm antibodies in the male reproductive tract (32), the effect of maternal antisperm antibodies on the sperm cells *in vivo* also deserves attention, and we have no information other than the contentious observation of an increased incidence of miscarriages at this time (8–11). Assisted reproductive technologies such as intracytoplasmic sperm injection (ICSI) of oocytes have been used to bypass problems associated with sperm having antisperm antibodies. When ICSI cases involve teratozoospermic patients (33), spontaneous abortion has been observed in some cases. The actual proportion of ICSI cases that involve antisperm antibodies cannot be determined with accuracy because the data are not available due to the trend toward reducing testing of sperm and performing ICSI for all cases without regard to the antisperm antibodies status. More studies are clearly needed to reevaluate the effect of ICSI involving sperm with antisperm antibodies and the DNA effects in the offsprings. If the data with sperm antibodies and ICSI failed to show any significant decrease in implantation rate or increase in miscarriage (thus suggesting that subtle DNA defects are not a cause of these problems), future efforts may involve a focus on follow-up of the health of the offsprings.

In summary, the data showed that sperm artificially preexposed to antisperm antibodies resulted in deletion of genetic material. In this case, detection of the deleted sentinel gene was observed only at 7 and 9 days of incubation, suggesting that DNA damage by antibodies was a slow process. Point mutations of control sperm were not observed. Changes in sperm DNA at the gross level were not detectable as shown by the AO assay. The results suggested that antisperm antibodies may be one of many factors that affect the integrity of DNA.

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