Comparative Genomic Hybridization Analysis of Sperm DNA Apoptosis After Exposure to Heat Shock

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Purpose: A DNA disc chip assay based on comparative genomic hybridization (CGH) was developed to measure sperm DNA integrity. The objective was to correlate DNA integrity of heat-treated sperm with the sperm capacitation index (CI) determined from the sperm penetration assay.

Methods: Basic semen and kinematic parameters were measured ($N = 6$). Sperm were washed in two-layer colloid suspensions and split portions incubated at either 37°C (control) or 40°C for 4 h. Single-stranded DNA of heated sperm were stained in SYBR Gold and hybridized to bisbenzimidazole (Hoechst 33342) stained control DNA in a membrane disc. Fluorescent intensities of the discs were measured and correlation analyses with sperm parameters performed.

Results: Sperm CI was positively correlated ($R = 0.737$) with sperm DNA integrity. Two populations of sperm could be discerned: low capacitating sperm that initiated apoptosis and high capacitating sperm unaffected by heat shock treatment. The remaining parameters were not related to sperm DNA stability.

Conclusions: Fragile DNA were found in a population of sperm associated with poor capacitation characteristics and apoptosis was observed after heat treatment. The results suggested that sperm dysfunction might be due to apoptotic sperm DNA resulting from an elevated temperature in the surroundings. The data suggested that the second population of high capacitating sperm induced chaperones such as heat shock proteins hsp 70 to protect against apoptosis.

KEY WORDS: Comparative genomic hybridization; fluorescence microscopy; microarray; spermatozoa capacitation; sperm heat shock proteins.

INTRODUCTION

The field of molecular biology recently has been energized with an exciting molecular cytogenetic method called comparative genomic hybridization (CGH). This method detects gain or loss in DNA gene sequences and localization and mapping of relative

DNA sequence copy number between control and target cells (1–3). It involves detecting a change in the ratio of the intensities of two fluorochromes separately located on hybridized control and test DNA. Differences in intensities represent DNA alterations attributed to the pathological condition. In practice, the selected gene regions (cDNA from RNA) are divided into small sections and analyzed in automated matrix-CGH arrays or microarrays (4,5).

In the field of assisted reproductive technologies (ART), there is a need for the development of a simple CGH-based assay that takes advantage of resources found in ART laboratories. Hence, a novel DNA disc chip assay was developed in this laboratory based

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on measuring the intensity of SYBR Gold stained single-stranded test DNA that had been hybridized to bisbenzimidazole (Hoechst 33342) stained single-stranded control DNA located in a membrane disc. Several discs were arranged to form a microarray chip. The DNA disc chip assay was then used to test the hypothesis that sperm with low capacity to fertilize oocytes was associated with fragile DNA that would undergo apoptosis or DNA damage when stressed with a mild heat treatment. The objective was to correlate DNA pixel intensities of heat-treated sperm assessed using the DNA disc chip assay with the sperm capacitation index (CI) determined from the sperm penetration assay. The information from this study will help scientists and clinicians develop simple CGH assays for the evaluation of sperm in ART programs. Furthermore, compounds that repair or reverse the damage in DNA can be studied in vitro using the DNA disc chip assay.

MATERIALS AND METHODS

Semen Samples

Sperm cells of male partners ($N = 6$ cases) were analyzed prior to the in vitro fertilization procedures for the treatment of infertility. Data from patients that involved epididymal sperm aspiration, donor oocyte, or donor sperm were not included in the study. Basic semen analyses were performed, and kinematic or motion parameters were assessed using the computer-aided sperm analysis (CASA) method as described below. The sperm in each specimen were also analyzed for percent strict-criteria normal morphology (6), acrosome intactness (7), viability (8), and fertilization capacity using the sperm penetration assay (9). The study was approved by the Loma Linda University Institutional Review Board and written consents obtained from the patients.

The mild heat treatment to induce apoptosis consisted of incubating washed sperm at 40°C for 4 h (10). The sperm wash procedure involved layering aliquots of each specimen above a two-layer 90:45% colloid suspension (Isolate, Irvine Scientific, Santa Ana, CA) in 15-mL centrifuge tubes (11). This was followed by centrifuging the tubes at 300 g for 10 min, diluting the 90% layer with an equal volume of HEPES-modified human tubal fluid (mHTF medium, Irvine Scientific, Santa Ana, CA), and recentrifugation and resuspension of the pellet in 0.4 mL mHTF medium.

The washed sperm were divided into two portions and incubated at either 37°C (control) or 40°C mild heat treatment for 4 h in room air (10).

After incubation, the sperm cells were stored frozen at -30°C for several weeks and thawed for the DNA assays. Kinematic and sperm function data were analyzed for possible correlation to whole genome DNA integrity status as determined through the novel DNA disc chip assay based on comparative genomic hybridization (described below).

Spermac Stain for Sperm Morphology and Acrosome Intactness

The staining procedure was carried out according to the Spermac kit manufacturer guidelines (Stain Enterprises, Onderstepoort, S. Africa, distributed by SAGE BioPharma, Hartford, CT) and has been previously reported (7). Briefly, a drop of semen from each specimen was smeared on a glass slide and air-dried for 5 min. Each dried smear was fixed in formalin solution (Fixative I) for 5 min at room temperature (23°C) and rinsed in tap water. The fixed smears were stained in Rose Bengal-based solution A for 2 min followed by rinsing with water. Then the smears were stained in solution B for 1 min and rinsed with water. Stain solution C was used to flood the slides for 1 min followed by more rinsing. The stained slides were air-dried before being analyzed in oil immersion and light microscopy (1000 $\times$). Normal morphology analyses was based on the Tygerberg strict criteria developed by Kruger *et al.* (6) and was performed on at least 100 sperm cells.

The percentages of sperm with intact acrosome were also determined from the Spermac-stained morphology slides. Regardless of the shape of the sperm head, sperm were considered as having an intact acrosome when 1) the anterior acrosomal region stained green and 2) the dark green thickened "rubber band" border forming a semicircle line at the tip of the head remained unbroken or continuous (7). The posterior postacrosomal region of each sperm head was red-pink in coloration. Sperm lacking the red-pink counterstain in the posterior head region were indicative of inadequate staining and were not counted.

Sperm with nonintact acrosome (reacted or defective) showed peeled acrosomal membranes, spotting, irregular thickness in the green band, or partial green coloration. Another type of sperm with nonintact acrosome (missing acrosomal enzymes) was stained

either white or red at the acrosomal region without any green color at the head. At least 100 sperm were analyzed for each specimen. The percentage of sperm with intact acrosome was calculated by dividing the number of sperm with intact green acrosome over the total number of sperm analyzed and multiplied by 100.

Computer-Aided Sperm Analyses (CASA)

Sperm dimensions and kinematic parameters were measured using the Hamilton Thorn motility analyzer (HTM-C) CASA system as previously described (7,10). Briefly, the analyses were carried out by pipetting aliquots (10 μ L each) of the semen into 20 μ m deep glass slides with cover slips prewarmed at 37°C on a slide warmer. A minimum of 100 sperm cells were analyzed each time. The percent total motility, percent rapid progression, and curvilinear velocity were measured on the HTM-C based on published settings on this analyzer (7,10). For consistency, the same technician was used throughout the study.

The Enhanced Sperm Penetration Assay: The Capacitation Index (CI)

The sperm were optimized for capacitation by mixing equal volumes of semen with test-yolk buffer (Refrigeration Medium, Irvine Scientific, Santa Ana, CA) and refrigerating at 4°C for 48 h (12). This was followed by thawing the sperm mixture at 37°C for 5 min and centrifuging the mixture at 300 g for 10 mins. The pellets were resuspended in 0.5 mL bicarbonate-based human tubal fluid (HTF) medium supplemented with 3.5% human serum albumin fraction V. The resuspended sperm (10 million/mL) were then used for the insemination of the zona-free hamster oocytes.

The cryopreserved hamster oocytes were obtained from a commercial source (Embryotech Laboratories, Wilmington, MA). The thawed oocytes were treated with 0.1% trypsin to remove the zona pellucida (9). Each group of zona-free oocytes ($N = 7-10$ oocytes per group) was incubated in the presence of washed sperm at 37°C in 5% CO₂ in air mixture for 3 h.

After 3 h, the oocytes were pipetted on to glass slides and gently squashed with cover slips. The number of penetrating sperm with swollen head and visible tail remnants for each oocyte was determined

by visual observation using an inverted phase contrast microscope (500 $\times$). The CI was the mean or the average number of swollen sperm inside each penetrated oocyte. The number of unpenetrated oocytes was also noted for each case.

The DNA Disc Chip Assay

The DNA disc chip assay procedure was as follows: The frozen heat-treated and control sperm cells were thawed at room temperature for 10 min and pipetted into polymerase chain reaction (PCR) tubes. Each batch of control sperm (previously incubated at 37°C for 4 h) was divided into two portions to serve as a matching internal quality control set. The tubes of sperm were centrifuged at 3000g for 1 min to remove the culture media. Each pellet was resuspended in 0.1 mL TE buffer (10 mM Tris-Chloride, pH 7.4, and 1 mM EDTA, pH 8.0).

The DNA staining procedure was carried out in diffuse lighting or darkened room. One portion of the control sperm was added with 9 μ M bisbenzimidazole (Hoechst 33342, Sigma, St. Louis, MO) while the second portion of control sperm was added with 1:10,000 diluted SYBR Gold stock stain (Molecular Probes, Eugene, OR) as recommended by the manufacturer. This set of bisbenzimidazole and SYBR Gold stained control sperm served as the internal calibration control. Each tube of heat-treated sperm was added with the 1:10,000 diluted stock SYBR Gold stain. It was unnecessary to adjust sperm concentration in the tubes because of the matched control-test design and saturation of the positive-charged miniature disc.

The sperm cells were stained for 5 min and centrifuged (3000g for 1 min) to remove the excess stain. The pellets were resuspended in TE and recentrifuged and the resultant pellet resuspended in 4°C alkaline lysis buffer (1% *N*-Lauroylsarcosine, 1.0 M Tris-HCl pH 7.5, 0.5 M EDTA, 0.3 M mercaptoethanol, pH adjusted to >10 with sodium hydroxide pellets) for 20 min with periodic vortexing to release sperm DNA. The tubes of sperm DNA were placed in a PCR thermal cycler and heated at 94°C for 5 min to denature the DNA into single strands similar to the "hot start" PCR protocol. The tubes of single-stranded DNA were then rapidly placed into shaved ice to retard re-naturation of the DNA strands similar to the loading process in a gel sequencing protocol.

The tiny round discs used in the DNA disc chip assay were made by pressing a 2-mm Acupunch biopsy punch pen (Acuderm, Ft. Lauderdale, FL) into the

Nytran (Schleicher & Schuell, Keene, NH) membrane sheet and using a plastic stylet to expel the round discs. The size of the disc was chosen to fit the 40X magnification of the fluorescent microscope used in this laboratory. The discs could be attached into the wells of a microtiter plate to process greater volume of samples, similar to a microarray design. For this study, each disc was held using a pair of microforceps and first dipped into the tube of control DNA for a few seconds and blotting off the excess. Then, the disc was dropped into the tube of test DNA still in the ice. This process was repeated several times to obtain replicated data points. Then, each tube with submerged discs was placed at room temperature for 10–20 s for annealing. Several discs were removed using a pair of microforceps and lined up in a glass slide to form a DNA chip.

Each glass slide with the DNA discs was examined using an ultraviolet (UV) epi-fluorescent microscope at 40X magnification. The fluorescent images were captured by placing an inexpensive QuickCam Pro camera (Logitech, Fremont, CA) over the microscope eyepiece and saving the 640 × 480 pixels images to hard disk (Fig. 1). For comparison purposes, the color images were computer-cut and pasted on to a photo of a 50-well microtiter plate in a microarray design using the Microsoft Paint program. The images were converted to gray scale and the pixel intensity of each disc analyzed using Paint Shop Pro 6 software (Jasc Software, Eden Prairie, MN). Greater fragmentation of sperm DNA was reflected in lower pixel intensity of the disc.

Fig. 1. The CGH-based DNA disc chip assay showing (A) reduced DNA fluorescence in the hybridized membrane disc (2-mm diameter) of a sperm specimen with low capacitation index (CI = 1.0), an index of fertilization capacity, and (B) a sperm specimen with high capacitation index (CI = 16.2) exhibiting bright fluorescence in the hybridized membrane disc. To prepare for the assay, the sperm cells were washed in two-layer 90:45 colloid suspension and the washed sperm heat shocked at 40°C for 4 h before DNA extraction, staining, and hybridization.

Statistical Analysis

The pixel intensity of each fluorescent disc containing hybridized DNA was obtained from the computer imaging software and expressed as mean $\pm$ SEM (standard error of the mean). The pixel intensity of each disc from heat-treated (40°C) sperm DNA were matched or paired with the pixel intensity of corresponding internal calibration control (37°C) sperm. The ratio of heat-treated to control (40:37°C pixel intensity ratios) were calculated and used in the analyses. All pixel data as well as sperm parameters were stored in a Microsoft Excel spreadsheet. Linear correlation coefficient statistical analysis was used and the significance tested using Students' *t*-test statistic. A value of $P < 0.05$ was considered significant.

RESULTS

The CI, a marker of sperm fertilization capacity, was positively correlated ($R = 0.737$) with the DNA integrity status of sperm cells exposed to the heat shock treatment of 40°C for 4 h (Table I). In other words, the DNA of sperm with poor fertilization capacity became damaged upon exposure to mild heat as suggested by the split matching portions of sperm exposed to different temperatures. In contrast, the DNA of sperm with high fertilization capacity showed bright stable fluorescent hybridized DNA in the assay (mean $\pm$ SEM: 40:37°C pixel intensity ratios of 3.1 ± 1.7 versus 0.8 ± 0.3 for specimens with low capacitation indices (<7.0)).

The remaining sperm parameters were not correlated with the status of the sperm DNA after heat shock treatment. Interestingly, in addition to the CI parameter, the percentages of total motile sperm and rapid progressive motility showed correlation coefficients of $R = 0.564$ and 0.518 with the DNA integrity status of the sperm cells but these were not statistically significant.

DISCUSSION

This study demonstrated a weakness in DNA structural integrity in sperm associated with poor capacitation characteristics. Sperm capacitation is a necessary preparatory step in the series of prefertilization events characterized by efflux of membrane cholesterol and sterols (13), generation of free radicals, plasma membrane conformation changes, redistribution of surface glycoproteins, and an increase in protein tyrosine

Table I. Sperm Parameters of the Semen Specimens ($N = 6$ cases) Used in the Study and the Correlation with DNA Apoptosis Measured as Pixel Intensities in the DNA Disc Chip Assay

Parameter	Mean $\pm$ SEM	Linear correlation coefficient (R value)	P value
Volume (mL)	4.3 $\pm$ 0.4	0.139	<0.25
Concentration (million/mL)	38.0 $\pm$ 7.0	0.246	<0.25
Total motility (%)	50.2 $\pm$ 8.9	0.564	<0.12
Rapid progression (%)	27.0 $\pm$ 9.5	0.518	<0.15
Curvilinear velocity ($\mu\text{m/s}$)	53.7 $\pm$ 6.1	0.229	<0.25
Viability (%)	66.6 $\pm$ 12.9	0.259	<0.25
Strict normal morphology (%)	7.3 $\pm$ 1.8	0.184	<0.25
Acrosome intactness (%)	25.3 $\pm$ 6.4	0.088	<0.25
Zona-free oocytes penetrated (%)	91.7 $\pm$ 9.1	0.207	<0.25
CI ^a	11.0 $\pm$ 3.4	0.737	<0.05*

^aCapacitation index (CI) is the mean number of penetrated sperm with swollen heads in each penetrated zona-free hamster oocyte.

* $P < 0.05$; Significant correlation.

phosphorylation (13,14). The fragility of DNA was observed in sperm specimens with low CIs (<7.0) after exposure to mild heat treatment. Sperm specimens with low CIs have been associated with failed in vitro fertilization (15). Chromosomal instability has been reported in human sperm even immediately after the intracytoplasmic sperm injection procedure to bypass conventional fertilization (16,17).

Single-stranded DNA derived from low capacitating sperm became damaged or apoptotic after heat exposure and were unable to pair efficiently with homologous matching single-stranded control DNA during hybridization. Consequently, the fluorescence intensity of the stained DNA under UV illumination was diminished. The data corroborates previous reports that failed fertilization after assisted reproductive procedures might be due to apoptotic sperm DNA (16). It was proposed that heat-initiated DNA apoptosis in sperm might be one plausible reason for infertility in certain patients. Interestingly, it has been observed that the human female body could achieve an internal temperature of 40°C during certain activities and would affect sperm with suboptimal mechanisms. Furthermore, it has been reported that at 40°C, poor quality sperm do not exhibit enhanced motility (18) or hyperactivation (10), markers of capacitated sperm. On the basis of results presented here, the heat shock treatment carried out after test-yolk buffer refrigeration as part of the sperm processing procedure would not be useful in cases of sperm derived from low capacitating specimens (12).

A possible explanation for the two different populations of sperm and their DNA that respond differently to heat exposure is the ability of the high

capacitating sperm population to induce heat shock proteins such as hsp 27, hsp 70, and hsp 72 to inhibit apoptosis and protect DNA integrity (19,20). Inhibition of apoptosis by these proteins involve blocking mitochondrial cytochrome c release and inhibiting the series of caspases. Clearly, more studies are needed to develop diagnostic tests to detect sperm with suboptimal DNA chaperones and to examine heat-induced sperm apoptosis.

In this study, a novel DNA disc chip assay was used to perform inexpensive and rapid analyses of comparative changes in DNA. The assay did not require the degenerate oligonucleotide primed-PCR step (DOP-PCR), lengthy hybridization periods to metaphase chromosomes, or randomly targeted hybridization arrays and confocal microscopes (1–5). However, the disadvantages of the disc chip assay include manual processing steps, requirement of a fluorescent microscope, a computer with a digital camera, and a 94°C heating block. Nevertheless, the assay procedure was not complicated and it would allow personnel in small clinics and laboratories to join in the CGH microarray excitement. It should be emphasized that the assay only measured large changes in DNA and not single mutations; hence, the requirement for more detailed studies before the question could be fully answered. In this laboratory, we have begun work using the PCR to generate DNA fragments of specific gene regions or using reverse transcriptase to produce DNA from RNA and then carrying out the DNA disc chip assay to detect alterations in specific genes. More work is needed in the exciting field of CGH and microarrays and it is hoped that they will become part of routine ART programs.

REFERENCES

1. Kallioniemi A, Kallioniemi O-P, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992;258:818–821
2. Voullaire L, Slater H, Williamson R, Wilton L: Chromosomal analysis of blastomeres from human embryos by using comparative genomic hybridization. *Hum Genet* 2000;106:210–217
3. Lichter P, Joos S, Bentz M, Lampel S: Comparative genomic hybridization: Uses and limitations. *Semin Hematol* 2000;37:348–357
4. Schena M, Shalon D, Davis RW, Brown PO: Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995;270:467–470
5. Freeman WM, Robertson DJ, Vrana KE: Fundamentals of DNA hybridization arrays for gene expression analysis. *Biotechniques* 2000;29:1042–1055
6. Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S: Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril* 1988;49:112–117
7. Chan PJ, Corselli JU, Jacobson JD, Patton WC, King A: Spermac stain analysis of human sperm acrosomes. *Fertil Steril* 1999;72:124–128
8. Eliasson R, Treschl L: Supravital staining of human spermatozoa. *Fertil Steril* 1971;22:134–137
9. Yanagimachi R, Yanagimachi H, Rogers BJ: The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod* 1976;15:471–476
10. Chan PJ, Corselli JU, Patton WC, Jacobson JD, King A: Heat-induced hyperactivation. *J Assist Reprod Genet* 1998;15:32–38
11. McClure RD, Nunes L, Tom R: Semen manipulation: Improved sperm recovery and function with a two-layer Percoll gradient. *Fertil Steril* 1989;51:874–877
12. Falk RM, Silverberg KM, Fetterolf PM, Kirchner FK, Rogers BJ: Establishment of TEST-yolk buffer enhanced sperm penetration assay limits for fertile males. *Fertil Steril* 1990;54:121–126
13. Suzuki F, Yanagimachi R: Changes in the distribution of intramembranous particles and filipin-reactive membrane sterols during in vitro capacitation of golden hamster spermatozoa. *Gamete Res* 1989;23:335–347
14. Naz RK, Ahmad K, Kumar R: Role of membrane phosphotyrosine proteins in human spermatozoal function. *J Cell Sci* 1991;99:157–165
15. Honig SC, Thompson S, Lipshultz LI: Reassessment of male-factor infertility, including the varicocele, sperm penetration assay, semen analysis, and in vitro fertilization. *Curr Opin Obstet Gynecol* 1993;5:245–251
16. Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF: Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril* 1998;69:528–532
17. Peschka B, Leygraaf J, Van der Ven K, Montag M, Scharmann B, Schubert R, van der Ven H, Schwanitz G: Type and frequency of chromosome aberrations in 781 couples undergoing intracytoplasmic sperm injection. *Hum Reprod* 1999;14:2257–2263
18. Alvarez JG, Minaretzis D, Barrett CB, Mortola JF, Thompson IE: The sperm stress test: A novel test that predicts pregnancy in assisted reproductive technologies. *Fertil Steril* 1996;65:400–405
19. Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, Kroemer G: Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* 2001;3:839–843
20. Samali A, Robertson JD, Peterson E, Manero F, van Zeijl L, Paul C, Cotgreave IA, Arrigo AP, Orrenius S: Hsp27 protects mitochondria of thermotolerant cells against apoptotic stimuli. *Cell Stress Chaperones* 2001;6:49–58