Relationship between Stallion Sperm Deoxyribonucleic Acid (DNA) Susceptibility to Denaturation In Situ and Presence of DNA Strand Breaks: Implications for Fertility and Embryo Viability[1]

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ABSTRACT

Thirty-six stallion semen samples demonstrating a relatively wide variation in susceptibility of sperm nuclear DNA to in situ denaturation, as determined by the sperm chromatin structure assay (SCSA), were also measured for the presence of DNA strand breaks in situ. The SCSA is a flow cytometric procedure that measures acridine orange (AO)-stained sperm after exposure of the sperm to low pH to potentially denature the DNA; AO intercalated into native DNA fluoresces green whereas AO associated with denatured DNA fluoresces red. By means of the terminal deoxynucleotidyl transferase assay (TdTA), DNA strand breaks were detected by flow cytometric measurements of fluorescein isothiocyanate (FITC)-tagged avidin used to detect terminal deoxynucleotidyl transferase addition of biotin-tagged dUTP to the ends of DNA strand breaks. The percentage of cells in individual semen samples demonstrating denatured DNA by the SCSA ranged from 5 to 92%, while the percentage of cells demonstrating DNA strand breaks ranged from 4 to 62%. The correlation coefficient observed between these two measurements was $r = 0.65$ ($p < 0.001$). The presence of DNA strand breaks (TdTA) was related to an increased susceptibility of DNA denaturation in situ (SCSA), but this appears to be only one of a number of possible chromatin structural abnormalities that result in an increased susceptibility to DNA denaturation in situ.

INTRODUCTION

Sperm chromatin structure is totally unique in the mammalian body, suggesting that the dramatic alterations of chromatin structure accompanying the exchange of histone-complexed DNA to protamine-complexed DNA during spermiogenesis [Grimes et al., 1977] is required and/or advantageous to fertility potential. During this process, the chromatin is condensed to approximately one sixth the volume of somatic cell metaphase chromosome complement [Ward & Coffey, 1991] with a corresponding reduction of DNA stainability [Evenson et al., 1986; Gledhill et al., 1966]. Protamine-complexed DNA is organized into torroids that in turn are probably organized into a flat sheet array of chromatin [Ward & Coffey, 1991]; this is in sharp contrast to the known nucleosome organization in somatic cells. During passage of the sperm through the epididymis, the -SH groups of the protamine cysteine residues form disulfide bonds [Calvin & Bedford, 1971] to give the nucleus a very rigorous structure that is resistant to sonication. It is likely that this near crystalline-like chromatin structure helps protect the genetic material from damage during storage in the male and migration in the female reproductive tract to the site of fertilization.

During the process of spermiogenesis, as noted, the chromatin structure is dramatically rearranged. In order to accommodate this restructuring, DNA strands are cut by endonucleases and then ligated [McPherson & Longo, 1993]. It is even possible that the sharp bends of the DNA in the near crystalline-like structure of mature sperm require that a DNA nick remain or that the DNA be partially denatured to accommodate the sharp bends. It is reasonable to assume that if nicks were introduced into the DNA for restructuring and if a defect occurred in their repair, then chromosome breaks could result in infertility defined as a nonviable embryo.

In the present study, the structure of stallion sperm chromatin was examined by two methods.

1) One method was the sperm chromatin structure assay (SCSA) as developed in our laboratory [Evenson, 1989; Evenson & Jost, 1994]. The SCSA defines abnormal chromatin struc-

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ture as a heat- or low pH-induced increased susceptibility to DNA denaturation in situ. The metachromatic dye acridine orange (AO) was used to measure by flow cytometry the amounts of double-stranded (green fluorescence) and single-stranded (red fluorescence) DNA. With this method the extent of DNA denaturation per cell is quantitated by the expression alpha $_t$ (α_t = ratio of red/red + green fluorescence). Variables of α_t useful for fertility evaluation include the cells outside the main population (COMPα_t = the percentage of abnormal cells with denatured DNA), the mean of α_t (Xα_t), and the standard deviation of α_t (SDα_t).

Numerous reproductive toxicology studies have shown SCSA data to be highly reproducible, dose-responsive, and biologically meaningful [Evenson et al., 1985, 1989, 1993]. SCSA data have been correlated (r = 0.58–0.94, $p <$ 0.01) with fertility potential in bulls [Ballachey et al., 1987, 1988]. SCSA data correctly predicted the three boars with the highest fertility potential and the three with the lowest fertility potential in a recent heterospermic experiment [Evenson et al., 1994]. Previous [Evenson, 1989; Evenson et al., 1980] and current studies (unpublished results) have shown that chromatin structure in human patients attending an infertility clinic was of poorer quality than that in samples obtained from men known to be fertile.

2) The second method used to examine the structure of stallion sperm chromatin was the terminal deoxynucleotidyl transferase assay (TdTA) as described by Gorczyca et al. [1993]. The TdTA is a flow cytometric assay that measures the presence of DNA strand breaks in situ. In the present study the percentages of cells positive by the TdTA were correlated with the COMPα_t values of the SCSA.

MATERIALS AND METHODS

Aliquots of ejaculated stallion semen samples were frozen on dry ice and shipped to our laboratory. Thawed aliquots were used for the SCSA and TdTA described below.

SCSA

Frozen semen samples were thawed in a 37°C water bath, and an aliquot was diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris, 1 mM EDTA, pH 7.4) to 1–2 × 10^6 sperm/ml. A 200-μl aliquot of sample was admixed with 400 μl of a solution containing 0.1% Triton X-100, 0.08 N HCl, and 0.15 M NaCl (pH 1.2) to permealize cell membranes and potentially denature the DNA. After 30 sec, the cells were stained by addition of 1.2 ml of solution containing 6.0 μg of chromatographically purified AO (Polysciences, Warrington, PA) per milliliter of AO buffer (370 ml of 0.1 M citric acid and 630 ml of 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0 [Darzynkiewicz et al., 1976; Evenson et al., 1985]). The samples were immediately placed in the flow cytometer and allowed to equilibrate in the sheath flow. Data acquisition was begun at 3 min after staining with a flow rate of ~250 cells/sec. The AO-stained cells were excited with a 488-nm argon ion laser (35 mW), and the emitted fluorescence was optically separated and collected with a 515–530-nm bandpass filter (green) and a 630-nm long pass filter (red). Raw data were expressed as dual parameter scattergrams of 5000 cells with each dot representing the amount of green and red fluorescence per cell. Frequency histograms of red and green fluorescence distributions and the α_t distribution were generated by computer protocol.

TdTA

Frozen semen samples were thawed, diluted in Hanks' Balanced Salt Solution (HBSS), centrifuged for 5 min at 3500 × g, placed into fixative (phosphate-buffered saline, with 1% formaldehyde), and incubated on ice for 15 min. After cells were pelleted, they were resuspended in −20°C 70% EtOH and kept up to 3 days at −20°C. The cells were then pelleted and resuspended in 50 μl of a cacodylate buffer containing 0.2 M potassium cacodylate, 2.5 mM Tris-HCl (pH 6.6), 2.5 mM $CoCl_2$, 0.25 mg/ml BSA, 5 U of terminal deoxynucleotidyl transferase (TdT enzyme; Boehringer-Mannheim, Indianapolis, IN), and 0.5 nM biotinylated dUTP (Boehringer-Mannheim). After a 30-min incubation at 37°C, the cells were postlabeled with 100 μl fluorescein isothiocyanate-avidin (FITC-avidin; Boehringer-Mannheim) staining solution and again incubated for 30 min at room temperature. The cells were then postlabeled with 5 μg/ml propidium iodide. Control cells for each sample were treated identically except that the TdT enzyme was omitted.

Flow Cytometry

SCSA. Flow cytometer measurements on 5000 cells per sample were made in a Cytofluorograf II interfaced to a 2150 data handler (Or-

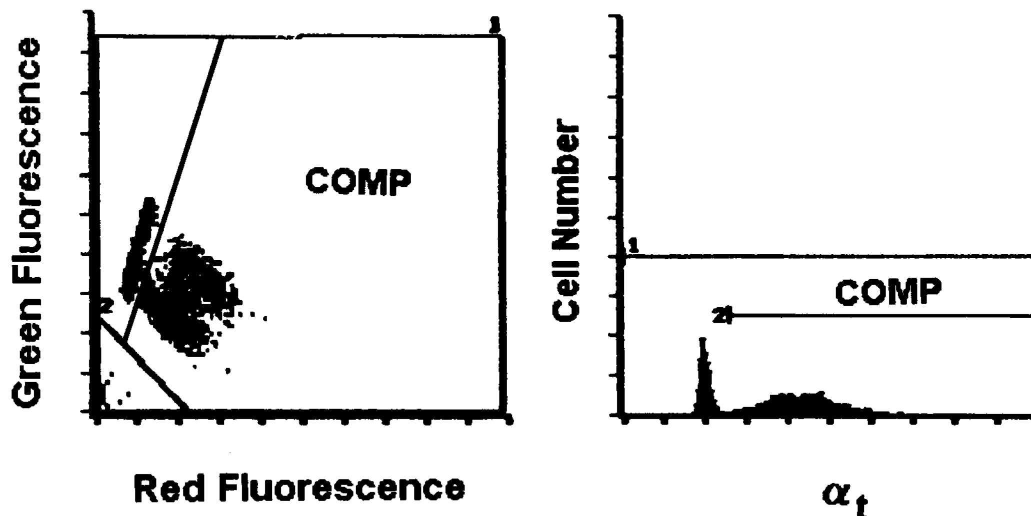

FIG. 1. Typical data from the SCSA measurement of stallion sperm; a green vs. red fluorescence cytogram is shown on the left, and an α_t frequency histogram is shown on the right.

tho Diagnostic Systems, Westwood, MA) exactly as detailed in previous publications [Evenson, 1989; Evenson & Jost, 1994]. To maintain instrument constancy, a reference semen sample was identified that had about 10–20% cells with a heterogeneous range of DNA denaturation. Several hundred 0.25-ml reference sample aliquots were placed into small snap-cap vials and frozen at − 100°C. These reference aliquots were used to set up the flow cytometer parameters initially and then were measured after every ~10 samples to monitor instrument reliability and laser focus and stability. Mean red and green fluorescence values of the reference sample were maintained within ± 5 channels and used as calibration markers.

List mode data were transferred to a PC, and the alpha $_t$ variables $X\alpha_t$ (mean), $SD\alpha_t$ (standard deviation), and $COMP\alpha_t$ (cells outside the main population showing DNA denaturation) were calculated using Multi2D software (Phoenix Flow Systems, San Diego, CA).

TdTA. Cells prepared for the TdTA were measured by flow cytometry, with red fluorescence (> 630 nm) representing DNA stainability (PI) as a positive marker for all cells. The red-fluorescing cells were used as a computer gate for measuring TdT-labeled cells. Green fluorescence (515–530 nm) represented DNA strand breaks having incorporated biotinylated dUTP/FITC-avidin. Data were based on 10,000 cells per sample. List mode data files were converted to histogram files by the Ortho 2150 Data Handler, transferred to a PC computer by means of a custom interface, and analyzed through use of Multi2D software (Phoenix Flow Systems). TdTA analysis consisted of subtracting the control (no TdT enzyme; negative) green fluorescence histogram from the TdT-positive green fluorescence histogram to obtain the percentage of cells incorporating biotin-16-dUTP onto DNA strand breaks.

Statistical Analysis

Linear regression and correlation coefficients were calculated through use of the student edition of Minitab (MINITAB, Inc., State College, PA).

RESULTS

Figure 1 shows a typical distribution of normal stallion sperm cells with native DNA and a subpopulation of cells containing denatured DNA. The wide coefficient of variation (CV) of the green fluorescence is due to an optical artifact that has been previously discussed [Gledhill et al., 1979]. The panel on the right shows the narrow CV of the normal population and the wide CV of the COMP population.

Figure 2 shows the TdT results from an aliquot of the same stallion semen sample described in Figure 1. The TdT-negative (no TdT) histogram was subtracted from the TdT-positive sample by computer software to derive the percentage of TdT-positive cells.

For the 36 stallion semen samples analyzed, the range of cells demonstrating DNA denaturation (COMP cells) was 5–92% and the range of TdT positive cells was 4–62%. The cor-

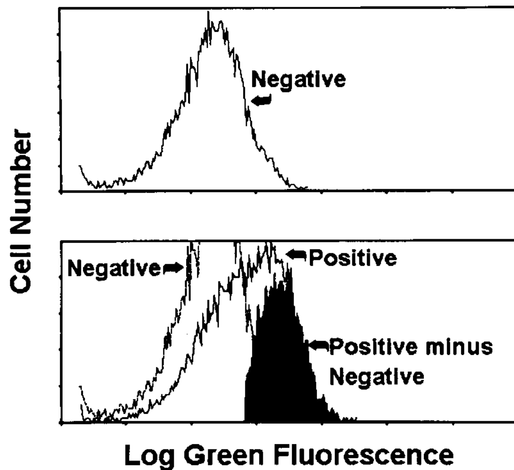

FIG. 2. The upper panel shows the green fluorescence frequency distribution of the control (no TdT enzyme; negative). The lower panel shows the same negative control as in the upper panel plus an overlaid frequency histogram of the TdT-positive sample. The darkened area represents the difference when the negative is subtracted from the positive. The subtraction procedure results in a value of 35% cells that are positive for DNA strand breaks.

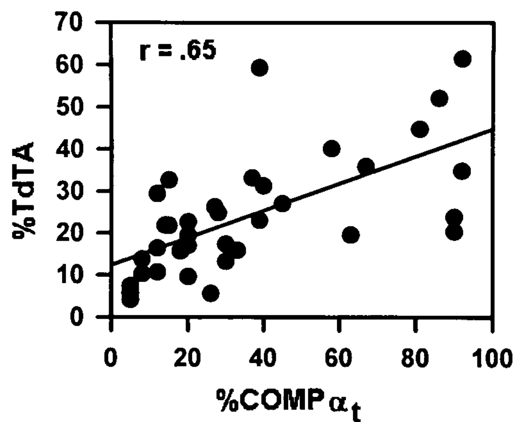

FIG. 3. Regression analysis of the relationship between percentage of cells positive by the SCSA (COMPα_t) and the percentage positive cells by the TdTA (%TdTA) for the 36 stallion semen samples measured. A correlation coefficient of $r = 0.65$ ($p < 0.001$) was found.

relation between percentage of cells with increased level of DNA denaturation (as determined by the SCSA) and the percentage cells of with increased DNA strand breaks was $r = 0.65$ ($p < 0.001$; Fig. 3).

DISCUSSION

This study has shown a significant correlation between altered chromatin structure, defined as an increased susceptibility to DNA denaturation in situ, and the presence of DNA strand breaks accessible to dUTP incorporation. We suggest that the DNA strand breaks that are introduced into sperm nuclei as the chromatin is changed from a somatic cell histone complex to a sperm protamine complex [McPherson et al., 1993] are not properly ligated, resulting in altered chromatin and residual DNA strand breaks. The upper range of cells positive by the SCSA was 50% higher than the percentage positive for DNA strand breaks (92 vs. 62%). Perhaps chromatin structure needs to be at a certain level of abnormality before DNA strand breaks are detected. Alternatively, the SCSA may be a more sensitive assay due to the greater accessibility of the compacted nuclear chromatin to the smaller AO probe relative to the much larger TdT enzyme molecule.

NA strand breaks do not account for all cases of altered chromatin structure identified by the SCSA. Preliminary data on sperm derived from animals exposed to several reproductive toxins were negative for DNA strand breaks as determined by the TdTA (in preparation).

The origin of the DNA strand breaks is not clear. Gorczyca et al. [1993] have suggested that the DNA strand breaks are related to an aborted apoptosis phenomenon. We favor the viewpoint that, during spermiogenesis, the numerous endonuclease-derived strand breaks needed for chromatin reorganization are not normally ligated, resulting in residual DNA strand breaks. This may lead to an altered chromatin structure and infertility defined by either a lack of fertilization of the oocyte or an early embryo death.

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