Journal of Andrology, Vol. 21, No. 5, September/October 2000
Copyright © American Society of Andrology

Characteristics of Human Sperm Chromatin Structure Following an Episode of Influenza and High Fever: A Case Study

DONALD P. EVENSON,* LORNA K. JOST,* MICHELE CORZETT,† AND ROD BALHORN†

*From the *Department of Chemistry and Biochemistry, South Dakota State University, Brookings, South Dakota; and the †Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore, California.*

ABSTRACT: Semen samples from a fertile patient presenting with influenza and a 1-day fever of 39.9°C were obtained and analyzed at 18–66 days postfever (dpf) for sperm nuclear proteins, DNA stainability, free thiols (−SH), and susceptibility to DNA denaturation in situ. At 18 dpf, 36% of sperm demonstrated denatured DNA as measured by the sperm chromatin structure assay (SCSA), and decreased to 23% by 39 dpf. Samples at 33 and 39 dpf contained 49% and 30%, respectively, of cells with increased DNA stainability (HIGRN). A unique sperm nuclear protein band migrating between histones and protamines on acid-urea gels appeared at 33 and 39 dpf and nearly disappeared by 52 dpf. Amino acid sequencing of the first 8 N-terminal residues identified this protein as the precursor to protamine 2. The protamine P1 and P2 ratio remained normal, whereas the histone to protamine ratio increased slightly at 33 to 39 dpf. Flow cytometric measurements of nuclear −SH groups revealed the greatest reduction in free nuclear thiols at 33 dpf, and returned to normal by 45 dpf. The time of appearance of the unprocessed protamine 2 precursor and the relative increase in histone suggest a fever-related disruption of the synthesis of mRNA that codes for a P2 processing enzyme or enzymes. Increased DNA staining is likely due to the increased histone/protamine ratio. This case study demonstrates that fever/influenza can have latent effects on sperm chromatin structure and may result in transient release of abnormal sperm.

Key words: Infertility, SCSA, DNA denaturation.

J Androl 2000;21:739–746

Mammalian spermatogenesis is a sensitive process involving a continuum of dramatic biochemical events and morphological alterations that occur as diploid spermatogonia differentiate into mature haploid sperm with a unique chromatin structure (see Ward and Zalensky, 1996 for review). The hypothesis of this study is that influenza-related high fever induces alteration in chromatin differentiation. A critical and logical clinical question is whether hypothetical changes in chromatin structure cause genetic alterations that affect fertility and, more importantly, embryo development and pregnancy outcome. During spermiogenesis, somatic-like histones of round spermatids are replaced first by transition proteins (Grimes et al, 1977; Balhorn 1982) and then by the arginine- and cysteine-rich protamines (Bedford and Calvin, 1974). In humans there are 2 major protamines, P1 and P2; and 2 forms of P2, protamine 2α and 2β. Protamine 2β is identical to 2α, except that 2β lacks the first 3 amino-terminal amino acids (Balhorn et al, 1987). The cysteine residues in these protamines form intramolecular and intermolecular disulfide bonds that function to stabilize the protamine-DNA complex (Evenson et al, 1980; Perreault et al, 1987, 1988; Perreault 1993).

Concomitant with the alteration and condensation of the nuclear chromatin is the cessation of gene transcription. However, relevant to observations in this study, it is noted that mRNA for transitional proteins and protamines is transcribed in round spermatids, but not translated until sperm nuclei condense. Recent analyses of 2 semen samples obtained from an infertile man 6 months apart have shown that the nuclear protein composition of sperm produced by an individual can change with time (Bench et al, 1998). This indicates that environmental factors likely affect the level or fidelity of expression of nuclear protein genes and the organization of sperm chromatin. We hypothesized that high fevers could inhibit normal sperm maturation and lead to abnormal sperm chromatin structure.

Both physiological stress and exposures to environmental agents can perturb 1 or more of the biochemical

Supported in part by grants R827019-01-0 from the Environmental Protection Agency and OSR-9452894 from the National Science Foundation, contract W-7405-ENG-48 from the U.S. Department of Energy and South Dakota Futures Funds. This is South Dakota Agricultural Experiment Station publication 3125 of the journal series.

Correspondence to: Dr Donald P. Evenson, Department of Chemistry and Biochemistry, Box 2170, Animal Science Complex 136, South Dakota State University, Brookings, SD 57007 (e-mail: donald_evenson@sdstate.edu).

Received for publication February 7, 2000; accepted for publication April 12, 2000.

events that occur during spermatid differentiation, and this can ultimately lead to alterations in cell differentiation. While total sperm output may be reduced, a more serious outcome could occur in that ejaculated sperm may have altered characteristics that reduce fertility, sustain damage to the genetic material, or both, leading to birth defects or early embryo death.

This hypothesis is supported by previous studies suggesting male mediated-developmental effects after experimental heat exposure. Setchell et al (1998) have shown a transient retardation in embryo growth in normal females made pregnant by males whose testes had been heated. Also, Jannes et al (1998) have shown that male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. In epidemiological studies, fever is considered an important potential modifier (Wyrobek et al, 1997). Studies in Dr Saacke's laboratory have shown that semen obtained from bulls whose testes were subjected to a mild thermal stress were able to fertilize oocytes, but with a significant increase in degenerate and fair to poor embryos at the expense of excellent to good embryos (Saacke et al, 1994, unpublished data). Aliquots of these same sperm samples showed significant alteration of sperm chromatin by the sperm chromatin structure assay (SCSA). A similar study in our laboratory on the effects of heating mouse scrota for 1 hour likewise showed an induction of altered sperm chromatin structure.

Sperm chromatin structural integrity, defined here as the susceptibility of DNA denaturation in situ, and measured by SCSA has been shown to be correlated with fertility potential (Evenson et al, 1980, 1999, 2000c; Evenson and Jost, 1994, 2000a,b; Larson et al, 1999) and toxicant-induced chromatin damage (Evenson et al, 1985, 1986, 1989a, 1993b). The merits and intricacies of SCSA have been extensively studied and published on semen from bulls, boars, stallions, humans, rats, and mice (Evenson and Melamed, 1983; Evenson et al, 1985, 1986, 1989a, 1991, 1993a,b,c, 1994, 1995, 1999; Ballachey et al, 1987, 1988; Evenson and Jost, 1993, 1994, 2000a,b; Evenson, 1999a,b; Larson et al, 1999, 2000).

In this study, human semen samples were measured by flow cytometry employing several fluorescent probes and techniques in addition to SCSA. Samples were tested for DNA stainability, as related to protein restriction of DNA stainability, with both acridine orange (AO) and the DNA specific dye, DAPI (4′-6-diamidino-2-phenylindole dihydrochloride). Free nuclear −SH groups were measured using CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; [D-346, Molecular Probes, Eugene, Ore]), an −SH-specific dye, in order to evaluate the amount of −SH groups present or absent due to either or both the lack of exchange of −SH-deficient histones for −SH-rich protamines and/or lack of oxidation of the free −SH groups.

Materials and Methods

Semen Samples

Semen samples were obtained from a 45-year-old man of recent proven fertility who contracted influenza and had an oral temperature of 103.8°F (39.9°C) for 1 day with a decreasing temperature for 3 days before returning to normal. Samples were collected by ejaculation into plastic specimen jars on the following days postfever (dpf): 18, 25, 33, 39, 46, 53, 66, 73, 81, and 101. The 101-day sample was considered a baseline sample because a true baseline (before fever) sample was unavailable. Each sample was collected after a 3- to 4-day abstinence period and aliquots were diluted 10-fold with TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA; pH 7.4) and immediately frozen and stored at −100°C. Early studies used 10% glycerol as a cryoprotectant for storing frozen semen; later studies demonstrated that SCSA data are equivalent whether sperm are frozen with or without cryoprotectant; this observation is likely due to the unique characteristic of sperm chromatin, which has a near crystalline-like structure that is not affected by freezing without cryoprotectant. One undiluted semen aliquot of each sample was frozen (−100°C) and shipped on dry ice to the Lawrence Livermore National Laboratory for nuclear protein isolation and analysis. Because these samples were collected and stored in a clinic without an andrology unit, the classical assays of semen quality were not performed.

Sperm Chromatin Structure Assay

Cell Preparation and Staining—Individual semen samples were thawed for 30 seconds in a 37°C water bath and immediately processed by SCSA as described in full detail elsewhere (Evenson and Jost, 1994, 2000a,b; Evenson et al, 1999). Sonicated and sucrose-purified sperm nuclei were prepared as described (Evenson et al, 1989b, 2000c; Evenson and Jost, 1994, 2000a) for measurements on nuclear −SH groups.

Flow Cytometric Measurements—Each semen sample was measured independently twice by SCSA using a Cytofluorograf II flow cytometer (Ortho Diagnostics Inc, Westwood, Mass) equipped with ultrasense optics and a Lexel 100 mW argon ion laser (488 nm) operated at 35 mW. Sample handling, flow cytometry measurements, and data analysis were performed exactly as described in detail (Evenson and Jost, 1994, 2000a,b; Evenson et al, 1999).

Determination of Nuclear −SH Groups

Cell Preparation and Staining for Nuclear −SH Groups—Sonication-freed and sucrose gradient-purified sperm nuclei were prepared and stained with CPM fluorochrome and measured in an ICP 22 flow cytometer exactly as described in detail (Evenson et al, 1989b, 2000c; Evenson and Jost, 2000a,b).

Isolation of Sperm Nuclear Proteins

Sperm washed with mild sonication in 0.01 M Tris pH 8.0, 0.9% NaCl, 0.1 mM phenylmethylsulfonyl fluoride (T/S-PMSF) were reduced in 10 mM dithiothreitol (DTT), 0.05 M Tris pH 8.0, 0.1 mM PMSF, and the membrane and tails removed with 1% mixed alkyltrimethylammonium bromide (MTAB). Amembranous nu-

clei were washed twice in 1% MTAB, 0.01 M Tris pH 8.0, 0.1 mM PMSF, and twice in T/S-PMSF. Nuclei were dissolved in 5 M guanidine hydrochloride (GuCl; 0.01 M Tris pH 8.0), sonicated, placed on ice for 30 minutes, and the solution was diluted with urea, NaCl, and mercaptoethanol to final concentrations of 0.5 M GuCl, 3.0 M urea, 2.0 M NaCl, and 0.5 M mercaptoethanol. DNA was precipitated overnight at 4°C following the addition of HCl to 0.5 M. After centrifugation at 4200 × *g*, the supernatant was dialyzed against 10 mM HCl, and protein was precipitated with 20% trichloroacetic acid (TCA) at 4°C for 1 hour, pelleted by centrifugation, and the pellet was washed with acidified acetone to remove TCA. The dried sperm protein was dissolved in 0.9 M acetic acid, 0.5 M MSH (2-mercaptoethanol), 20% sucrose, and analyzed on 10-cm acid-urea polyacrylamide gels. The gels were stained overnight in 0.1% napthol blue black, 0.9 N acetic acid, and 30% ethanol; electrophoretically destained, and scanned at 603 nm using a Shimadzu CS9000 Flying Spot microdensitometer (Shimadzu Scientific, Pleasanton, CA) to quantitate the amount of protein.

Statistical Analysis

A repeated measures analysis was used for the data set. Analysis of variance (Proc GLM; SAS, 1985) was used to test for differences in time after onset of fever. For SCSA, time and treatment (whole cells or sonicated) were the main classes with all SCSA variables tested for significant difference from control values. Least squares means (± SEM) were calculated for time for each dye. For DAPI and CPM measurements, time was the main classification and all time points were tested for significant difference from the control value.

Results

SCSA, DNA Stainability, and Nuclear Thiol Stainability

Green versus red fluorescence dot plots of AO-stained sperm samples are shown in Figure 1 with dpf noted in each box. The control sample was obtained from the same individual 4 months following the fever; this sample has a normal cytogram consistent with those observed from fertile donors (Evenson et al, 1999). The dots in the lower left-hand corner correspond to fluorescent debris and were excluded from the analysis. Sperm to the right of the main population, shown by the line in the control box, are sperm with fluorescence values that fall outside the main α_t population (COMPα_t; ie, the percentage of sperm that exhibit an increased susceptibility to DNA denaturation). Sperm with high green stainability (%HIGRN, immature sperm) are those above the horizontal line as shown in the control box.

Alterations in α_t parameters occurred over time (Figure 2). Maximum Xα_t and COMPα_t may have already occurred by the time the first sample was obtained (18 dpf). Because the literature is not consistent with regard to epididymal transport times, with estimates ranging from 2 to 22 days, it is not possible to extrapolate backward in the reproductive tract with precision to the sensitive cells affected by the influenza/fever. However, if epididymal transport were estimated to be approximately 12 days, this first sample would represent testicular sperm exposed to the fever about 6 days prior to release into the epididymis. Maximum SDα_t and red fluorescence (denatured DNA) occurred at 33 dpf. Using 12 days as the estimated epididymal transport time, these sperm would represent testicular spermatids that were exposed to the fever about 21 days prior to testicular release.

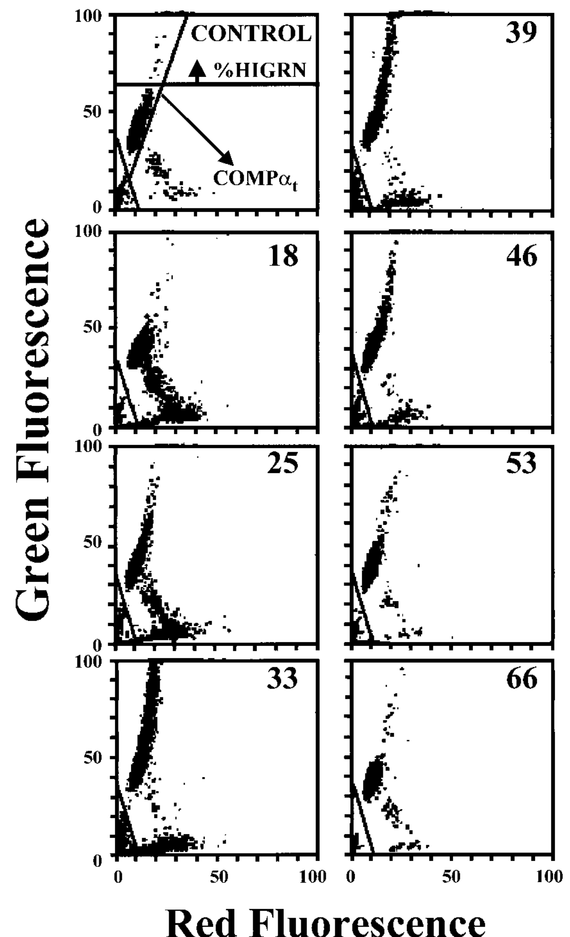

Figure 1. Green versus red fluorescence cytograms of acridine orange-stained sperm nuclei measured by SCSA. Numbers in each cytogram indicate dpf. Each dot represents a sperm characterized by the amount of native DNA (green fluorescence) and denatured DNA (red fluorescence).

Note that the 18 and 25 dpf samples contain a significant number of sperm with an increased susceptibility to DNA denaturation (36.3% and 33.3% COMPα_t, respectively; Figures 1 and 2). In addition, samples 33 and 39 dpf (31.1% and 23.1% COMPα_t, respectively; mid-spermatogenic cycle at time of fever) contain sperm with increased high green fluorescence (49% and 30.2%, respectively; Figures 1 and 2). By 53 and 66 dpf (near the completion of the human 74-day testicular sperm maturation

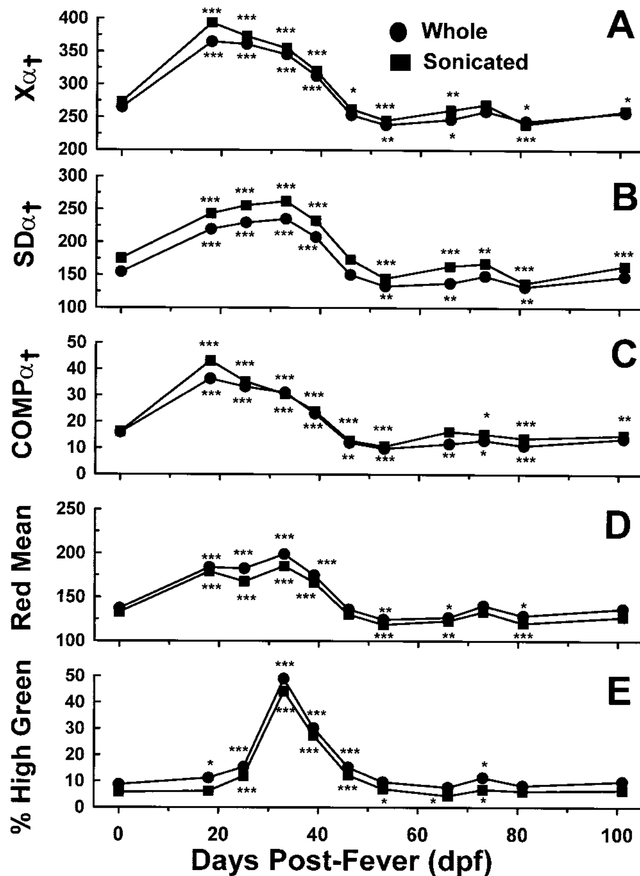

Figure 2. Relationship between the alpha-t (α_t) parameters **(A)** $X\alpha_t$, **(B)** $SD\alpha_t$, and **(C)** $COMP\alpha_t$ (denatured cells), **(D)** red fluorescence, and **(E)** percentage of cells with high green fluorescence (%HIGRN; immature cells) of acridine orange-stained whole and sonicated cells over dpf. Note that the control value shown here was taken at 4 months after fever. *P* values indicate significant differences from control values; **P* < .05; ***P* < .01; ****P* < .001.

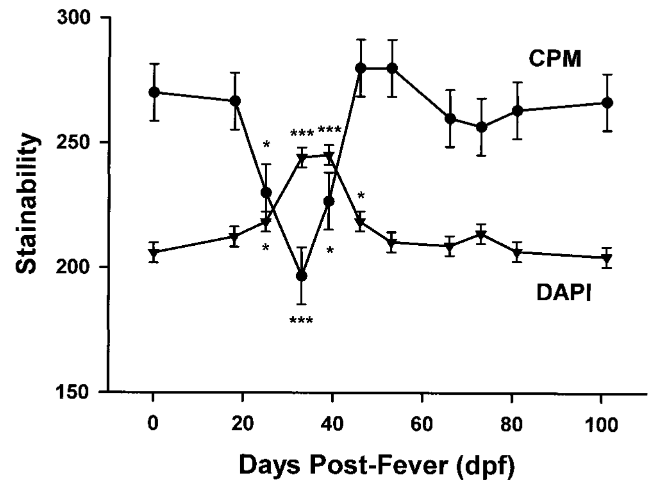

Figure 3. Comparison of measuring sperm obtained over dpf stained with DAPI or CPM. The mean (± SEM) channel of 5000 DAPI-stained cells per sample measured on the ICP22A and Cytofluorograf versus 3 measurements of 5000 CPM-stained free −SH groups in sperm nuclei are plotted. *P* values indicate significant differences from control values (from sample obtained 4 months after fever) for each stain: **P* < .05; ****P* < .001.

cycle), the DNA staining and denaturation patterns returned to normal. The %HIGRN data were verified by an increase in DNA-specific DAPI staining (Figure 3).

The DNA in ejaculated sperm 33 dpf exhibited maximum stainability with both AO and DAPI. Similar data were obtained whether whole cells or isolated nuclei were used (sonicated cells for AO; Figure 2). Therefore, cytoplasmic droplets potentially containing AO-stained RNA were not responsible for the increase in red fluorescence, and histone-complexed DNA is not eliminated by sonication.

CPM-stainable nuclear −SH groups changed as a function of time with the lowest relative amount of free −SH groups observed at 33 dpf (Figure 3).

Protein Analysis: Gel Electrophoretic and Amino-Terminal Sequencing

The sperm in samples obtained at 33 and 39 dpf contained a unique protein that had not been previously observed in hundreds of gels of sperm nuclear proteins from normal individuals analyzed in R.B.'s laboratory. The protein, identified as protein X (Figure 4), migrates between histones and protamines in acid-urea polyacrylamide gels. Microdensitometer scans of the sperm basic proteins separated in acid-urea gels with panels (top to bottom) corresponding to 33, 39, and 73 dpf, respectively (Figure 5). At 33 and 39 dpf, protein X represents 11%, 13%, and 7% of P1, $P2_\alpha$, and $P2_\beta$ nuclear protein. Although ratios of protamine 1 and 2 remained normal in all samples collected, the histone/protamine ratio nearly doubled for samples obtained 33 and 39 dpf. These same samples also exhibited a high ratio of protein X to protamine (Table).

Proteins separated by gel electrophoresis were subsequently blotted onto Immobilon, the stained bands corresponding to protein X were cut out, and the amino terminal sequence was determined by automated protein sequence analysis. The sequence obtained, VRYRVR?L (residue 7 could not be identified), is identical to the amino terminal sequence determined previously for the human protamine 2 precursor (Domenjoud et al, 1988).

Discussion

Biochemical and flow cytometry measurements were made on serially obtained semen samples from 18 to 66 dpf. Attempts to obtain additional volunteers to provide semen samples following a high fever were not successful. Thus, this manuscript is presented as a case study of a healthy, fertile donor. Because human SCSA variables often differ between individuals, the power of this study

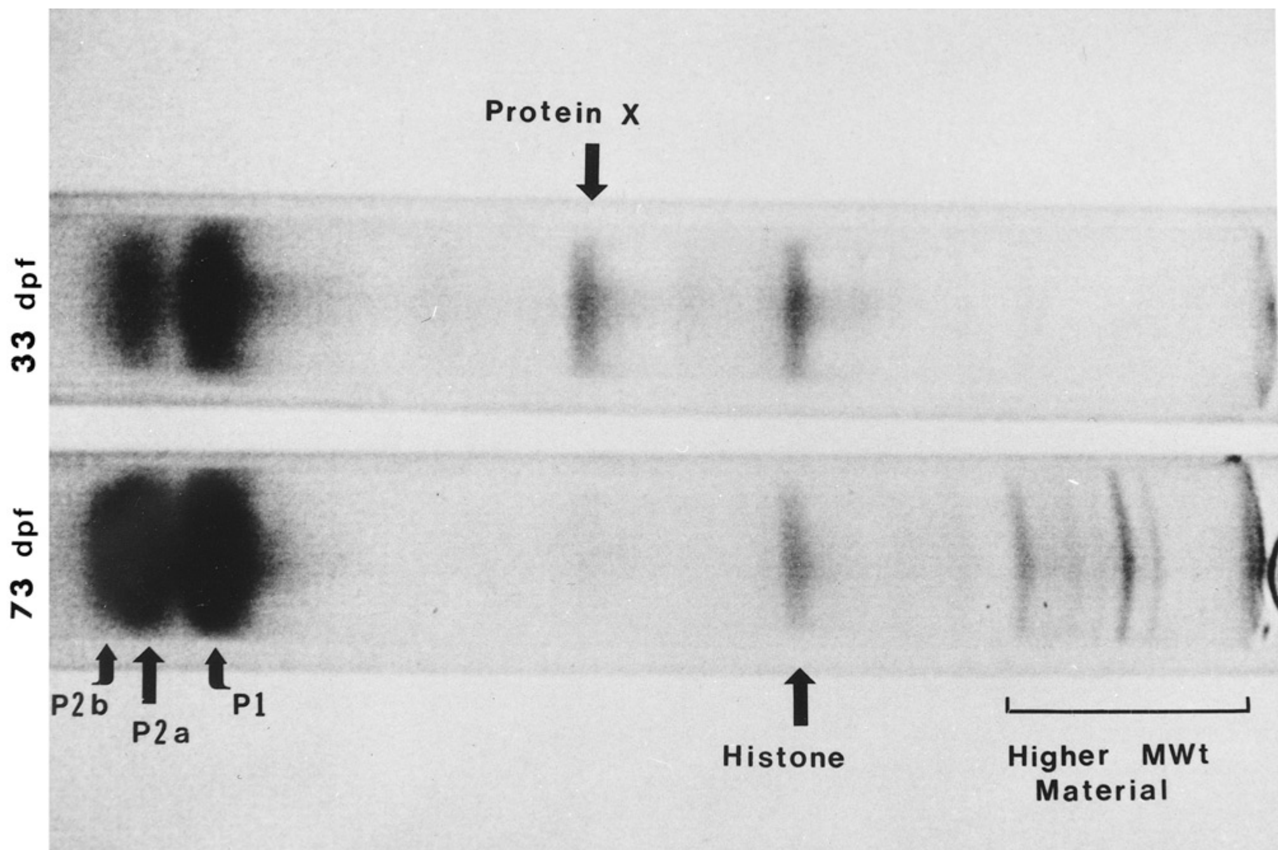

Figure 4. Photograph of acid-urea gels showing migration of sperm basic nuclear proteins from samples obtained at 33 and 73 dpf. A band corresponding to protein X is seen at 33 dpf.

is that of a longitudinal sampling from the same individual showing the effect of the influenza/fever over time. Due to the previously observed negative effects of heat and fever on reducing semen quality, we favor the view that the primary effect of influenza/fever is increased body heat to the testes. However, we cannot rule out the possible effects of viral infection or some other unknown factor. The data show that the effects of this influenza/fever episode was manifested as an increased susceptibility to DNA denaturation in situ, an increased DNA stainability, a decreased number of free −SH groups, and an alteration in the nuclear protein composition of ejaculated sperm.

Each of these changes in chromatin structure and composition appear to reflect an effect of the fever that occurred early in spermiogenesis. The observed effect of this is manifest several weeks later at a time when a variety of biochemical and structural changes are induced (histone replacement, protamine 2 precursor processing, disulfide bond formation) to bring about chromatin reorganization in the maturing sperm cell. Electrophoretic analyses of the nuclear proteins have shown 2 differences in nuclear protein content that appear to be correlated with the observed changes in dye binding: 1) an increased histone content and 2) an apparent block in the processing of the precursor to protamine 2.

This abnormal increase in DNA staining is very similar to that observed in studies of sperm obtained from subfertile/infertile males (Engh et al, 1992; Evenson et al, 1999). Human sperm with denatured DNA similar to that observed in the 33 and 39 dpf samples also exhibit extensive DNA fragmentation as shown by single-cell gel electrophoresis (COMET; Aravindan et al, 1997) and terminal uridine nick end-labeling, (TUNEL) also known as the terminal deoxynucleotidyl transferase assay (TdTA) of DNA strand breaks (Gorzcyca et al, 1993; Sailer et al, 1995; Aravindan et al, 1997). The extensive DNA strand breaks are similar to those observed in apoptotic somatic cells (Gorzcyca et al, 1993), and our laboratory has suggested that these fever-altered sperm could be the result of a sperm-specific apoptosis (Evenson, 1999b). Because SCSA data do not correlate well with classical sperm quality criteria (Evenson et al, 1991, 1999), sperm with fragmented DNA might be genetically compromised, and because they are motile and representative of normal sperm, they could be selected in an intracytoplasmic sperm injection procedure as suitable for injection. These data warrant an advisory to in vitro fertilization labora-

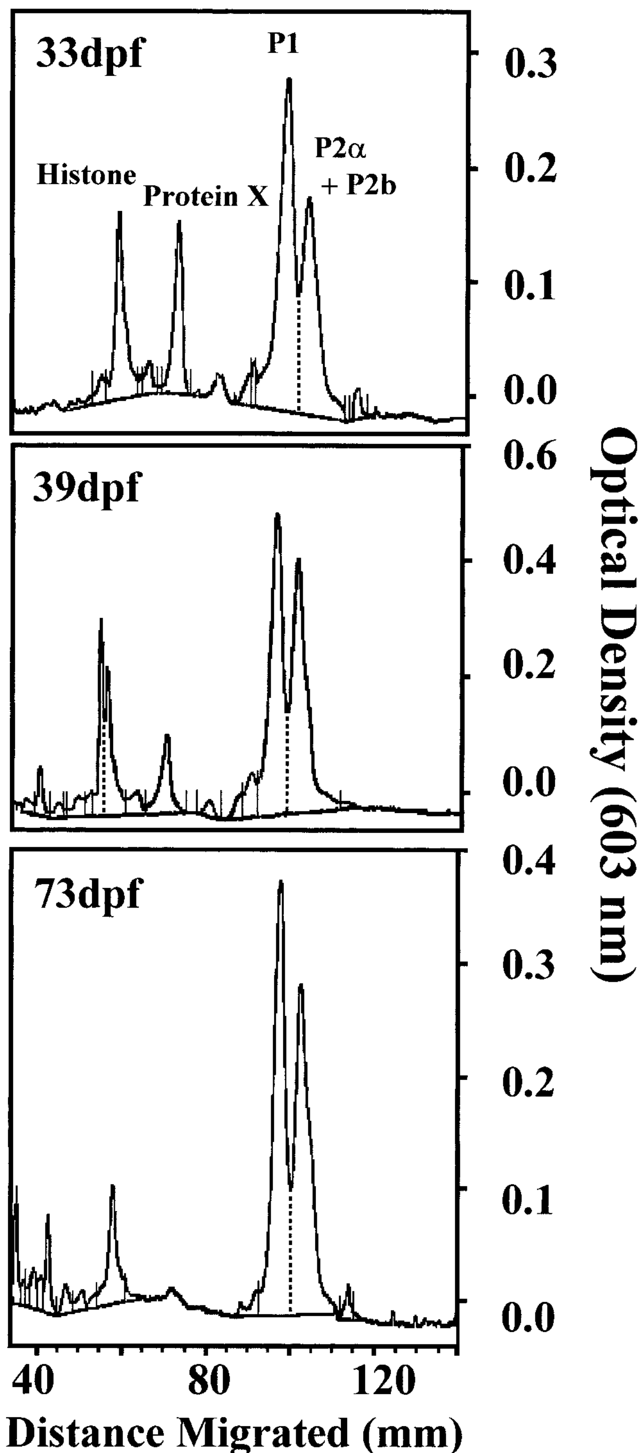

Figure 5. Scan tracings of acid-urea gels showing migration of basic nuclear proteins from samples obtained at 33, 39, and 73 dpf.

tories that potential fathers who experienced a fever in the 104°F range will likely have sperm with compromised chromatin integrity. The data suggest that such individuals should wait for about 2 months postfever before providing sperm for fertilization. These results also provide

Proportion of histone and protein X

Day Postfever	Histone Area/Protamine Area	X Area/Protamine Area
25	0.0980	0.0417
	0.1182	
33	0.2096	0.1659
	0.2312	0.1832
39	0.2636	0.1064
46	0.1182	0.0404
	0.1000	0.0322
53	0.0589	0.0359
	0.0513	0.0239
66	0.0687	
73	0.1035	
	0.0927	0.0260
81	0.1214	
	0.1172	
101	0.0690	
	0.0977	

increased rationale for controlling for recent fever in epidemiology studies that include SCSA.

Previous studies have shown that histone-complexed DNA is more accessible to DNA stains than protamine-complexed DNA, and as a consequence, the DNA stainability of round spermatids with AO as described has a 3.2-fold greater stainability than mature, ejaculated sperm (Evenson and Melamed, 1983). Histones have fewer −SH groups relative to the cysteine-rich protamines, and the retention of histones likely accounts for the observed reduction in CPM staining at 33 dpf. An alternate explanation is that the −SH groups have oxidized to form disulfide bonds, but this explanation is not consistent with the protein gel data showing an abnormally high histone/protamine. Coincident with increased DNA stainability, electrophoretic analyses of sperm nuclear proteins revealed the appearance of a new protein migrating in acid-urea gels between protamines and histones. Amino acid sequence obtained from the amino-terminus of the protein identified it as the precursor to protamine 2. This protein is not normally observed as a component of the basic nuclear proteins isolated from mature sperm cells. Experiments performed in vitro using mouse testes (Green et al, 1988) and radiolabeling studies in mice (Balhorn, unpublished data) have both shown that the protamine 2 precursor is bound to DNA before processing is initiated. During processing, 40% of the amino-terminal of the protein sequence is removed through a series of proteolytic steps, leaving the arginine- and cysteine-rich carboxy-terminal domain of the protein, commonly referred to as protamine 2. Processing is completed before sperm enter the epididymis, and only a trace amount of the intact precursor can be detected in mature sperm.

Remarkably little is known about the function of protamine 2 processing. Even less is known about potential

differences that may exist in the interactions of the intact precursor and fully processed protamine 2 with DNA. As a result, it is difficult to hypothesize how the presence of a significant amount of unprocessed protamine 2 may affect the stability of sperm chromatin. The time interval between fever and observed effect (ie, the appearance of the precursor) occurs 33 to 39 days after the fever subsided, suggesting that the processing event must be indirectly affected. The appearance of the unprocessed protamine 2 precursor in sperm at 33 dpf suggests that this individual's fever may have inhibited or blocked some event that normally occurs right after meiosis. This is a time of active mRNA synthesis and the observed block in P2 processing could be explained if RNA synthesis were temporarily inhibited by the increase in body temperature, preventing synthesis of the message that codes for the P2 processing enzyme(s). Fever effects on transcription of protamine mRNA would not be evident until the message was translated several weeks later, and would not appear in sperm until they transited the epididymis; this may explain the latency of this effect. Induction of heat shock proteins would be expected by such a fever and could play a role in the effects seen. The observed increase in sperm histone may also be explained by a similar mechanism in which synthesis of mRNAs coding for proteins that facilitate the replacement of histones are also blocked. In both cases, fever could totally block mRNA synthesis in cells at a particular stage of development. Because sperm can reside in the epididymis for a full week or more, biochemical analyses of pools of sperm provide an average composition for the nuclear proteins; in this case up to 3 to 4 days of abstinence. Methods capable of measuring protein content of single cells would be required to determine if all the sperm contain the same ratio of histone/protamine or unprocessed precursor/protamine 2. While it is not yet possible to determine the histone or P2 precursor contents of single sperm cells, analyses of single sperm by Bench et al (1998), using proton induced X-ray emission spectroscopy, have shown that it is possible to detect differences in protamine content of individual sperm. This technique should prove useful in future studies to confirm the existence of a population of sperm that contain more histone than normal by detecting the associated deficiency in protamine.

This study has provided some new insights into relationships between fever-altered components of sperm chromatin:

1) The presence of abnormal, unprocessed protamines does not cause an increased susceptibility to DNA denaturation (Figure 2, 33 dpf), but does cause an increase in DNA stainability, which is likely the result of less compacted chromatin.
2) Decreased free −SH groups do not cause an increased susceptibility to DNA denaturation (Figures 2 and 3, 33 dpf).
3) Abnormal fever-induced chromatin consisting of retained histones and precursor forms of protamines is sonication-resistant and the nuclei have the same DNA stainability as whole sperm (Figure 2).
4) Although COMPα_t and Xα_t decreased (recovery phase) steadily from that seen in the first sample (18 dpf), the variation (SDα_t) increased for more than 2 weeks, suggesting heterogeneity of recovery, or heterogeneity of response by different cell types.

Unfortunately the first semen sample was not obtained until 18 dpf, so it is not known from this study whether epididymal sperm were affected, which limits our interpretation of the etiology of susceptibility to DNA denaturation in situ. The study shows that fever caused altered protein synthesis and a consequential altered chromatin structure. In addition, however, the data are still consistent with the hypothesis that DNA denaturation is a result of extensive DNA strand breaks caused by a fever-induced sperm-specific or aborted apoptosis.

References

Aravindan GR, Bjordahl J, Jost LK, Evenson DP. Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp Cell Res.* 1997;236:231–237.

Balhorn R. A model for the structure of chromatin in mammalian sperm. *J Cell Biol.* 1982;93:298–305.

Balhorn R, Corzett M, Mazrimas JA. HPLC separation and partial characterization of human protamines 1, 2 and 3. *Biotechnol Appl Biochem.* 1987;9:82–88.

Ballachey BE, Evenson DP, Saacke R. The sperm chromatin structure assay: relationship with alternate tests of semen quality and heterospermic performance of bulls. *J Androl.* 1988;9:109–115.

Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to fertility of bulls. *Biol Reprod.* 1987;36:915–925.

Bedford JM, Calvin HI. The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to Eutherian mammals. *J Exp Zool.* 1974;188:137–156.

Bench G, Corzett MH, Oliva R, Balhorn R. Protein and DNA contents in sperm from an infertile human male possessing protamine defects that vary over time. *Mol Reprod Dev.* 1998;50:345–353.

Domenjoud L, Fronia C, Uhde F, Engel W. Sequence of human protamine 2 cDNA. *Nucleic Acids Res.* 1988;16:7733.

Engh E, Clausen OP, Scholberg A, Tollefsrud A, Purvis K. Relationship between sperm quality and chromatin condensation measured by sperm DNA fluorescence using flow cytometry. *Intl J Androl.* 1992; 15:407–415.

Evenson DP. Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. *Reprod Fertil Dev.* 1999a;11:1–15.

Evenson DP. Alterations and damage of sperm chromatin structure and early embryonic failure. In: Jannsen R, Mortimer D, eds. *Towards Reproductive Certainty: Fertility and Genetics Beyond 1999.* London: Parthenon Publishing Group Ltd; 1999b:313–329.

Evenson DP, Baer RK, Jost LK. Long-term effects of triethylenemelamine

exposure on mouse testis cells and sperm chromatin structure assayed by flow cytometry. *Env Mol Mutag.* 1989a;14:79–89.

Evenson DP, Baer RK, Jost LK. Flow cytometric analysis of rodent epididymal spermatozoal chromatin condensation and loss of free sulfhydryl groups. *Mol Reprod Dev.* 1989b;1:283–288.

Evenson DP, Baer RK, Jost LK, Gesch RW. Toxicity of thiotepa on mouse spermatogenesis as determined by dual parameter flow cytometry. *Toxicol Applied Pharmacol.* 1986;82:151–163.

Evenson DP, Darzynkiewicz Z, Jost L, Janca F, Ballachey B. Changes in accessibility of DNA to various fluorochromes during spermatogenesis. *Cytometry.* 1986;7:45–53.

Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science.* 1980;240:1131–1133.

Evenson DP, Emerick RJ, Jost LK, Kayongo-Male H, Stewart SR. Zinc-Silicon interactions influencing sperm chromatin integrity and testicular cell development in the rat as measured by flow cytometry. *J Anim Sci.* 1993a;71:955–962.

Evenson DP, Higgins PH, Grueneberg D, Ballachey B. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. *Cytometry.* 1985;6:238–253.

Evenson DP, Jost LK. Hydroxyurea exposure alters mouse testicular kinetics and sperm chromatin structure. *Cell Prolif.* 1993;26:147–159.

Evenson DP, Jost LK. Sperm chromatin structure assay: DNA denaturability. In: Darzynkiewicz Z, Robinson JP, Crissman HA, eds. *Methods in Cell Biology, Vol 42, Flow Cytometry.* Orlando, Fl: Academic Press Inc; 1994:159–176.

Evenson DP, Jost LK. Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci.* 2000a: (submitted).

Evenson DP, Jost LK. Sperm Chromatin Structure Assay for fertility assessment. In: Robinson P, ed. *Current Protocols in Cytometry.* Vol 1. New York: John Wiley & Sons Inc, 2000b:in press.

Evenson DP, Jost LK, Baer RK. Effects of methyl methanesulfonate on mouse sperm chromatin structure and testicular cell kinetics. *Env Mol Mutag.* 1993b;21:144–153.

Evenson DP, Jost LK, Baer RK, Turner T, Schrader S. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod Toxicol.* 1991;5:115–125.

Evenson DP, Jost LK, Gandy J. Glutathione depletion potentiates ethyl methanesulfonate-induced damage to sperm chromatin structure. *Reprod Toxicol.* 1993c;7:297–304.

Evenson D, Jost L, Sailer B. Flow cytometry of sperm chromatin structure as related to toxicology and fertility. In: *Proceedings of the 7th International Spermatology Symposium.* Cairns, Australia; 1995.

Evenson DP, Jost LK, Varner DD. Stallion sperm nuclear protamine −SH status and susceptibility to DNA denaturation are not strongly correlated. *J Reprod Fertil Suppl.* 2000c; in press.

Evenson DP, Jost LK, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Clausen OP. Utility of the sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod.* 1999;14:1039–1049.

Evenson DP, Melamed MR. Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. *J Histochem Cytochem.* 1983;31:248–253.

Evenson DP, Thompson L, Jost L. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology.* 1994;41:637–651.

Gorzcyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* 1993;53:945–951.

Green GR, Balhorn R, Yelick PC, Hecht NB. Synthesis, processing and phosphorylation of protamines and TPs in rat and mouse late-step spermatids. Spermatogenesis II: molecular aspects. *J Cell Biol.* 1988; 105:167a.

Grimes SR, Meistrich ML, Platz RD, Hnilica LS. Nuclear protein transitions in rat testis spermatids. *Exp Cell Res.* 1977;110:31–39.

Jannes P, Spiessens C, Van der Auwera I, D'Hooghe T, Verhoeven G, Vanderschueren D. Male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. *Hum Reprod.* 1998; 13:372–375.

Larson KL, Brannian JD, Timm BK, Jost LK, Evenson DP. Density gradient centrifugation and glass wool filtration of semen remove sperm with damaged chromatin. *Hum Reprod.* 1999;14:2015–2019.

Larson K, DeJonge C, Barnes A, Jost L, Evenson D. Relationship between assisted reproductive techniques (ART) outcome and status of chromatin integrity as measured by the Sperm Chromatin Structure Assay (SCSA). *Hum Reprod.* 2000:in press.

Perreault SD. Importance of sperm chromatin packaging and unpacking for reproductive success. In: Zirkin BR, Whitcomb RW, eds. *Understanding Male Infertility: Basics and Clinical Approaches.* Serono, Italy: Raven Press; 1993.

Perreault SD, Barbee RR, Elstein KH, Zucker RM, Keefer CL. Interspecies differences in the stability of mammalian sperm nuclei assessed in vivo by sperm microinjection and in vitro by flow cytometry. *Biol Reprod.* 1988;39:157–167.

Perreault SD, Naish SJ, Zirkin BR. The timing of hamster sperm nuclear decondensation and male pronucleus formation is related to sperm nuclear disulfide bond content. *Biol Reprod.* 1987;36:239–244.

Saacke RG, Nadir S, Nebel RL. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. *Theriogenology.* 1994;41:45.

Sailer BL, Jost LK, Evenson DP. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J Androl.* 1995;16:80–87.

Setchell BP, Ekpe G, Zupp JL, Surani MA. Transient retardation in embryo growth in normal female mice made pregnant by males whose testes had been heated. *Hum Reprod.* 1998;13:342–347.

Statistical Analysis Systems. *SAS User's Guide: Statistics.* Version 5. Cary, NC: Statistical Analysis Systems Institute Inc; 1985.

Ward WS, Zalensky AO. The unique, complex organization of the transcriptionally silent sperm chromatin. *Crit Rev Eukaryot Gene Expr.* 1996;6:139–147.

Wyrobek AJ, Schrader SM, Perreault SD, Fenster L, Huszar G, Katz DF, Osorio AM, Sublet V, Evenson D. Assessment of reproductive disorders and birth defects in communities near hazardous chemical sites. III. Guidelines for field studies of male reproductive disorders. *Reprod Toxicol.* 1997;11:243–259.