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Sperm Nuclear Histone to Protamine Ratio in Fertile and Infertile Men: Evidence of Heterogeneous Subpopulations of Spermatozoa in the Ejaculate

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ABSTRACT: Sperm protamine deficiency is observed in a subset of infertile men, suggesting that the relative histone to protamine ratio may be altered in the spermatozoa of these men. We measured the ratio of nuclear histones to protamines in the spermatozoa of fertile (n = 10) and infertile men (n = 20). Sperm nuclear proteins were extracted and subsequently separated by acid-urea (AU) polyacrylamide gel electrophoresis. The relative histone (H2B) to protamine (PRM1 + PRM2) and PRM1 to PRM2 ratios were estimated by densitometric analysis of the AU gels. Immunoblotting experiments (using H2B, PRM1, and PRM2 antibodies) were conducted to confirm the specificity of the bands. The pattern and intensity of H2B staining in human spermatozoa was assessed by immunocytochemistry. Sperm samples from the infertile men in this study had a significantly higher proportion of histone H2B to protamine (PRM1 + PRM2) than did samples from the fertile men in this study (0.38 vs 0.08, $P < .001$). Immunocytochemistry experiments demonstrated a punctuated staining pattern (with strong, intermediate, or weak H2B staining intensity) throughout the sperm head. Infertile men had a higher proportion of spermatozoa exhibiting strong and intermediate staining than did samples from fertile men. These findings suggest that infertile men possess a higher proportion of spermatozoa with an increased histone to protamine ratio than fertile controls.

Key words: Sperm DNA, male infertility.
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Sperm chromatin is very tightly compacted by virtue of the unique associations between the DNA and sperm nuclear proteins (Balhorn 1982; Balhorn et al, 2000). During spermatid differentiation (spermiogenesis), nuclear remodeling and condensation are associated with the sequential displacement of histones by transition proteins and then by protamines (PRM1 and PRM2) (Kistler et al, 1996). Inter- and intramolecular disulfide cross-links between the cysteine-rich protamines are responsible for further compaction and stabilization of the sperm nucleus (Rufas et al, 1991; Kosower et al, 1992).

In humans, approximately 15% of the sperm DNA remains packaged by histones in sequence-specific areas (Gatewood et al, 1987; Wykes and Krawetz 2003). The histone-bound DNA sequences are less tightly compacted and may have an important function in the process of fertilization and early embryo development. The retained histones may be associated with telomeric sequences and these are believed to be the first structures in the sperm nucleus to respond to oocyte signals for pronucleus formation (Gineitis et al, 2000; Zalenskaya et al, 2000). Sperm protamine deficiency (partial or complete) is observed in a subset of infertile men and suggests that the relative histone to protamine ratio may be altered in the spermatozoa of these men (Belokopytova et al, 1993; De Yebra et al, 1993, 1998; Carrell et al, 2001). De Yebra et al also observed that infertile men have a high degree of variability in the relative sperm histone to total nuclear protein ratio, although this was not fully characterized (De Yebra et al, 1993).

The unique and exact sperm chromatin architecture implies a specific gene-expression schedule after fertilization (Gatewood et al, 1987; Wykes and Krawetz 2003). Therefore, determining whether or not the composition of nuclear proteins is preserved in the spermatozoa of infertile men is important, as any disruption will likely have profound effects on the integrity of the sperm DNA and its capacity to participate in reproduction. The objective of this study was to further examine the histone (H2B, more accurately designated as *HIST2H2BE,* Marzluff et al, 2002) to protamine ratio in the spermatozoa of fertile and infertile men and determine whether there may be subpopulations of spermatozoa with varying histone content.

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Materials and Methods

Materials

Unless specified, all the chemicals in this study are from Fisher Chemical (Elvet Scientific, Durham, United Kingdom). Hup 1N (protamine PRM1) and 2B (protamine PRM2) antibodies were a generous gift of Dr Rod Balhorn in Lawrence Livermore National Laboratory (Livermore, Calif).

Study Subjects and Semen Handling

Semen samples were obtained from consecutive asthenospermic (<50% sperm motility with normal sperm concentration [>20 million sperm/mL]) men presenting for infertility evaluation (n = 20). All couples presenting for infertility evaluation had primary infertility (no prior pregnancy) and had been unable to conceive naturally for a period of at least one year. The infertile men were selected based on the observation that abnormalities in sperm motility (asthenospermia) have been associated with aberrant histone to protamine ratio in transgenic animals (Cho et al, 2001). Couples with significant female-factor infertility (tubal obstruction or ovarian failure) were excluded.

Semen samples (n = 10) were also obtained from consecutive fertile controls (men presenting for vasectomy who had previously fathered at least 2 children with 1 in the past 5 years).

Samples were produced by masturbation after 3 to 5 days of sexual abstinence and allowed to liquefy at room temperature. After liquefaction of semen, standard semen parameters (volume, concentration, motility) were obtained according to World Health Organization (1999) guidelines. All of the semen samples had motile sperm and none had significant numbers of round cells or leukocytospermia as per World Health Organization guidelines (<1 million round cells per mL).

All patients signed an informed consent, and the information for this study remained confidential and within the institution. This study was approved by the ethics review board at McGill University.

Nuclear Protein Extraction—Nuclear proteins were extracted from whole semen samples as described previously (de Yebra et al, 1993) and 10×106 spermatozoa were washed in HEPES buffered saline (HBS) (centrifuged at 12 000 rpm for 5 minutes at 4°C). The supernatant was discarded and the sediment was washed 2 more times in HBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The sediment was then resuspended in 50 µl of 20 mM ethylenediaminetetraacetic acid, 1 mM PMSF, 100 mM Tris (pH 8.0). We then added a 50-µl solution of 6M guanidine hydrochloride (Sigma) with 575 mM dithiotreitol (DTT) and a 100-µl solution of 552 mM sodium iodoacetate. After a 30-minute incubation at room temperature, 1 mL of pure ethanol was added. The mixture was stored at −20°C for 1 minute and centrifuged for 15 minutes at 12 000 rpm and the supernatant discarded. Nuclear protein was extracted with 500 µl of 0.5 N HCl at 37°C for 10 minutes. Extracted proteins were precipitated with trichloroacetic acid (TCA, 20% final concentration) for 5 minutes at 4°C, centrifuged for 10 minutes at 12 000 rpm, and then washed twice with 500 µl of 1% β-mercaptoethanol in acetone. Proteins were dried at room temperature for 20 minutes.

Acid Urea Gel Electrophoresis, Immunoblotting, and P1, P2, and H2B Quantification—Extracted nucleoproteins were dissolved in 20 µl of 5.5 M urea, 20% β-mercaptoethanol, and 0.9% acetic acid (w/v). Acetic acid–urea minigel electrophoresis was performed as described before (Spiker, 1980). The stacking gel was prepared with 7.5% acrylamide, 0.1% bisacrylamide, 2.5 M urea, and 0.375 M potassium acetate at pH 4.0. The running gel was prepared with 15% acrylamide, 0.2% bisacrylamide, 2.5 M urea and 0.9 N acetic acid. After a prerun in running buffer (0.7% acetic acid, 30% ethanol) at 100 V for 3 hours, the protein samples were loaded onto the gel in duplicates and then run at 150 V for 2 to 3 hours. The minigel was divided into 2 parts after electrophoresis. Half was transferred to a polyvinylidene fluoride membrane in 0.7% acetic acid, 30% ethanol at 200 mA for 60 minutes, blocked by 5% Carnation dry skim milk in Tris-buffered saline with 0.1% Tween 20 and incubated with anti-P1, anti-P2 (Hup 1N and Hup 2B, monoclonal) (Stanker et al, 1987), or C-terminal-specific anti-H2B (directed against human somatic H2B residues 118–126, Upstate, Lake Placid, NY, Tovich and Oko, 2003) for 1 hour. The membranes were then incubated with horseradish peroxidase (HRPO) conjugated goat anti-mouse IgG diluted 1:5000 (Roche, Indianapolis, Ind) or HRPO conjugated goat anti-rabbit IgG diluted 1:5000 (Jackson ImmunoResearch Laboratories, West Grove, Penn), respectively. Positive reactive bands were detected using Lumi-Light chemiluminescence kit (Roche). Negative immunoblot controls were performed as above without the primary antibody. The other half of the gel was stained with Coomassie brilliant blue (0.25% Coomassie blue, 40% methanol, 10% acetic acid) and subsequently destained in 40% methanol and 10% acetic acid and scanned. The bands corresponding to PRM1, PRM2, and H2B were measured using UN-SCAN-IT software (the total pixel count of each band was recorded). The PRM1/(PRM1 + PRM2) and PRM1/(PRM1 + PRM2 + H2B) ratio of each sample was calculated and the mean values (all samples were tested in duplicate: 2 separate gels were run for each sample) were reported. Moreover, in many cases we ran a third gel with exactly 25% or 50% of the protein sample loaded to verify the accuracy of the optical density measurements. We observed a low variability (~10%) in the histone to protamine ratios when comparing the gels loaded with 100% and 50% (or 100% and 25%) protein load, indicating that the saturation of the lanes did not influence the accuracy of our measurements (data not shown).

Immunocytochemistry—Semen was washed with HBS and smears were prepared on Fisher Superfrost Plus slides (Elvet Scientific, Bearpark, Durham, United Kingdom). The smears were fixed in 100% methanol for 2 hours, air-dried, and then stored at −70°C. Prior to immunostaining, smears were brought to room temperature, rehydrated with PBS for 30 minutes, and decondensed in 5 mM DTT and 0.3 µg/mL heparin for 30 to 60 minutes (to ensure full decondensation of more than 90% of the spermatozoa). Immunostaining for H2B was performed using rabbit anti-H2B (Upstate, Charlottsville, Va). Smears were blocked with 5% goat serum

in PBS for 30 minutes, washed with PBS containing 0.1% Triton X-100 (PBS-T), and incubated with the H2B antibody (Upstate, Charlottsville, Va, dilution 1:300) for 1 hour at 20°C. Smears were then washed with PBS-T and incubated with fluorochrome-conjugated goat anti-rabbit antibody (Invitrogen, Carlsbad, Calif). After that, smears were mounted with Prolong Antifade and observed under a Carl Zeiss Axiophot microscope (exciter filter BP450–490, emission filter BP520) at 1000× magnification. All immunostaining experiments were carried out on the same run, and the data were recorded by two separate and blinded observers (interobserver variability was 7.7%). At least 200 spermatozoa were assessed per slide. The mean percentages (±SE) of sperm exhibiting strong, intermediate, and weak staining was recorded. Negative controls were performed in the absence of primary antibody. Colocalization studies of H2B and PRM1 were performed by dual immunostaining with H2B and PRM1 antibodies (secondary antibodies were conjugated with different fluorochromes to identify the respective antibody). Smears were blocked with 5% goat serum in PBS for 30 minutes, washed with PBS containing 0.1% Triton X-100 (PBS-T), and incubated with the H2B antibody (Upstate, Charlottsville, Va, dilution 1:300) and with PRM1 antibody (dilution 1:300) for 2 hours at 20°C. Smears were then washed with PBS-T and incubated with 1) fluorochrome-conjugated goat anti-rabbit antibody (directed against the H2B antibody, Invitrogen, Carlsbad, Calif) and 2) fluorochrome-conjugated goat anti-mouse antibody (directed against the PRM1 antibody, Jackson ImmunoResearch, West Grove, Penn) at dilution of 1:300 for 1 hour at 20°C. After that, smears were mounted with Prolong Antifade and observed under a Carl Zeiss Axiophot microscope (H2B: exciter filter BP450–490, emission BP520; PRM1: exciter filter BP510–560, emission BP590) at 1000× magnification. At least 300 spermatozoa (from 2 separate samples) were randomly assessed for the colocalization studies. Specifically, PRM1 immunostaining intensity (strong vs weak) was assessed in populations of spermatozoa with strong and intermediate/weak H2B immunostaining, respectively, to determine the relationship, if any, between PRM1 and H2B immunostaining patterns.

Data Analysis

Results are expressed as mean ± SD. Intergroup (fertile and infertile men) differences in semen parameters (and nuclear protein ratios) were assessed by parametric and nonparametric tests as appropriate. All hypothesis testing was two-sided, with a P value of .05 deemed as significant. Statistical analysis was performed using Sigma Stat software (SPSS Inc, Chicago, Ill).

Results

Sperm Parameters—As expected, the mean (±SD) sperm concentration and percent motility were significantly higher in fertile compared to infertile men (140 ± 129 vs 64 ± 37 million per mL and 78% ± 11% vs 36% ± 23%, respectively, $P < .05$). The mean (±SD) semen volume was not significantly different in the fertile compared to the infertile men (4.2 ± 1.8 vs 3.4 ± 2.0 mL, respectively, $P > .05$).

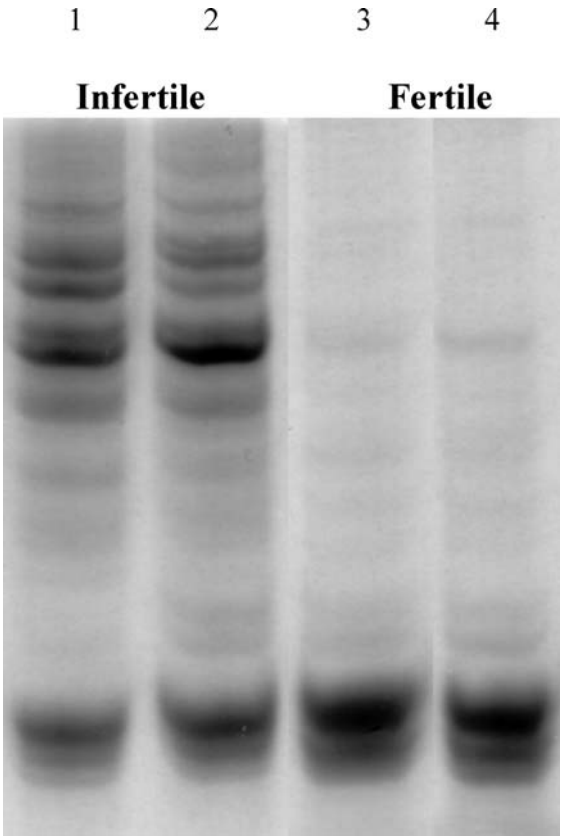

Figure 1. Representative acid-urea PAGE of sperm nuclear proteins from infertile (lanes 1 and 2) and fertile men (lanes 3 and 4).

Acid-Urea Gel Electrophoresis—Evaluation of the Coomassie blue-stained acid urea gels demonstrated the characteristic bands corresponding to nuclear histones and protamines. Acid urea gels of spermatozoa from infertile men demonstrated a higher proportion of histones than that of spermatozoa of fertile men (Figure 1).

Immunoblot Analysis—Immunoblotting experiments using previously characterized antibodies to protamines (PRM1 and PRM2) demonstrated 2 bands corresponding to the 2 protamine bands on the Coomassie blue–stained acid urea gels (Figure 2). Immunoblotting experiments using previously characterized antibodies to H2B (*HIST2H2BE*) demonstrated a single band corresponding to the most intense histone band on the Coomassie blue–stained acid urea gels (Figure 3).

Estimation of Sperm P1 to P2 and H2B to P1 Ratios—We observed no significant difference in the relative proportion of sperm PRM1 to PRM2 [using the formula PRM1/(PRM1 + PRM2)] between fertile and infertile men (Table 1). In contrast, we observed a significantly higher relative proportion of sperm H2B to protamine [using the formula H2B/(H2B + PRM1 +PRM2)] in the infertile compared to the fertile men in this study (Table 1). The

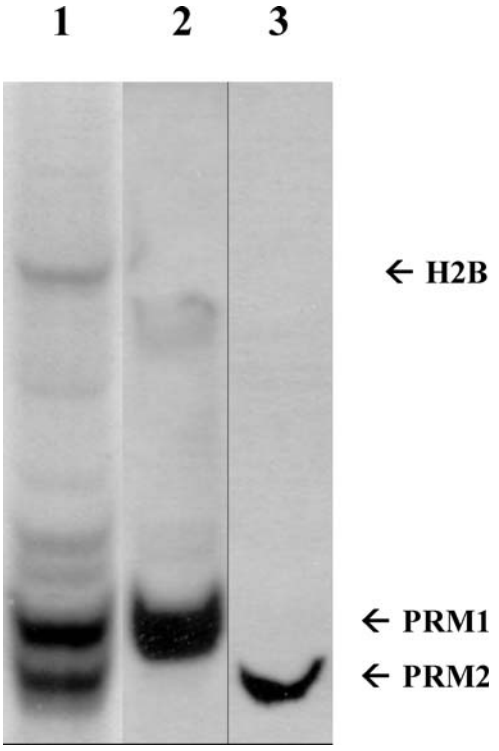

Figure 2. Acid-urea PAGE of sperm nuclear proteins and confirmation of protein bands (PRM1 and PRM2) by immunoblotting. Lane 1: Sperm nuclear proteins separated by acid-urea PAGE and stained with Coomassie blue. Lanes 2 and 3 show the results of the immunoblotting of the same gel using PRM1 (Hup 1N) and PRM2 (Hup 2B) antibodies, respectively. PRM1 and PRM2 antibodies are a gift from Dr Rod Balhorn.

intra- and interassay variability for the relative H2B to protamine levels are 17% (95% CI: 5%–34%) and 13% (95% CI: 1%–26%), respectively.

Immunocytochemistry Analysis—Immunocytochemistry experiments using H2B antibody demonstrated a punctuated staining pattern throughout the sperm head, with 3 populations of spermatozoa based on the H2B immunostaining intensity: strong, intermediate, and weak (Figure 4). Samples from infertile men demonstrated a higher proportion of spermatozoa exhibiting strong and intermediate staining than did samples from fertile men (Table 2). Colocalization studies using H2B and PRM1 antibodies (Figure 5A and B) demonstrated that spermatozoa with strong H2B immunostaining consistently exhibited weak PRM1 immunostaining. In contrast, spermatozoa with intermediate or weak H2B immunostaining consistently exhibited strong PRM1 immunostaining.

Discussion

Several histone isoforms (H2A, H2B, H3, H4) and isoform variants are present in human spermatozoa, with the predominant isoform being histone H2B (Gatewood et al, 1990). Recently, two distinct human testis/sperm-specific H2B variants (hTSH2B, H2BFWT) have been cloned and characterized (Zalensky et al, 2002; Churikov et al, 2004). It is interesting to note that these isoform variants may not be uniformly distributed throughout the sperm population. It has been shown that only 20% of spermatozoa (in semen from fertile men) possess TSH2B, suggesting the presence of different sperm populations in the human ejaculate (Van Rojen et al, 1998; Zalensky et al, 2002). The exact role of these histone H2B variants is largely unknown. However, the temporal accumulation of histone variants during spermatogenesis indicates their potential involvement in meiosis, spermiogenesis, and fertilization (Govin et al, 2004). Specifically, H2BFWT may be associated with telomeres, a finding that suggests a putative role in early chromatin remodeling at fertilization (Churikov et al, 2004).

In the present study, we have found that semen samples from infertile men with asthenospermia possess a significantly higher ratio of sperm nuclear histone H2B (*HIST2H2BE*) to protamine (PRM1 + PRM2) than do those from fertile men. There is little doubt regarding the specificity of protamine PRM1 and PRM2 measures (the protamine bands are distinct and separate from other proteins, and the antibodies used in the experiments are well characterized). However, we acknowledge that there may be some question regarding the specificity of the H2B measures. Although we have verified our results by extended separation of nuclear histones on the gels and by immunoblotting experiments (showing increasing immunoreactivity with H2B antibody as a function of an increase in the corresponding band intensity on Coomassie-stained gels), we cannot exclude the possibility that nonsomatic H2B variants (TSH2B and H2BFWT) have comigrated with the somatic H2B on the acid urea gels. We know that the antibody that we have used is directed against the C-terminal of somatic H2B and that both TSH2B and H2BFWT share sequence homology at the C-terminal. Our data must therefore be interpreted with the understanding that the H2B measures (used in the estimation of H2B to protamine ratio) may include one or more H2B variant(s).

The sperm nuclear H2B immunohistochemistry data indicate that all spermatozoa contain H2B but that the human ejaculate possesses subpopulations of spermatozoa with variable histone H2B content. This is further supported by the H2B and PRM1 colocalization studies, which demonstrate an inverse relationship between the H2B and PRM1 staining intensities. Taken together, our studies suggest that semen samples from infertile men possess a higher proportion of spermatozoa with a high

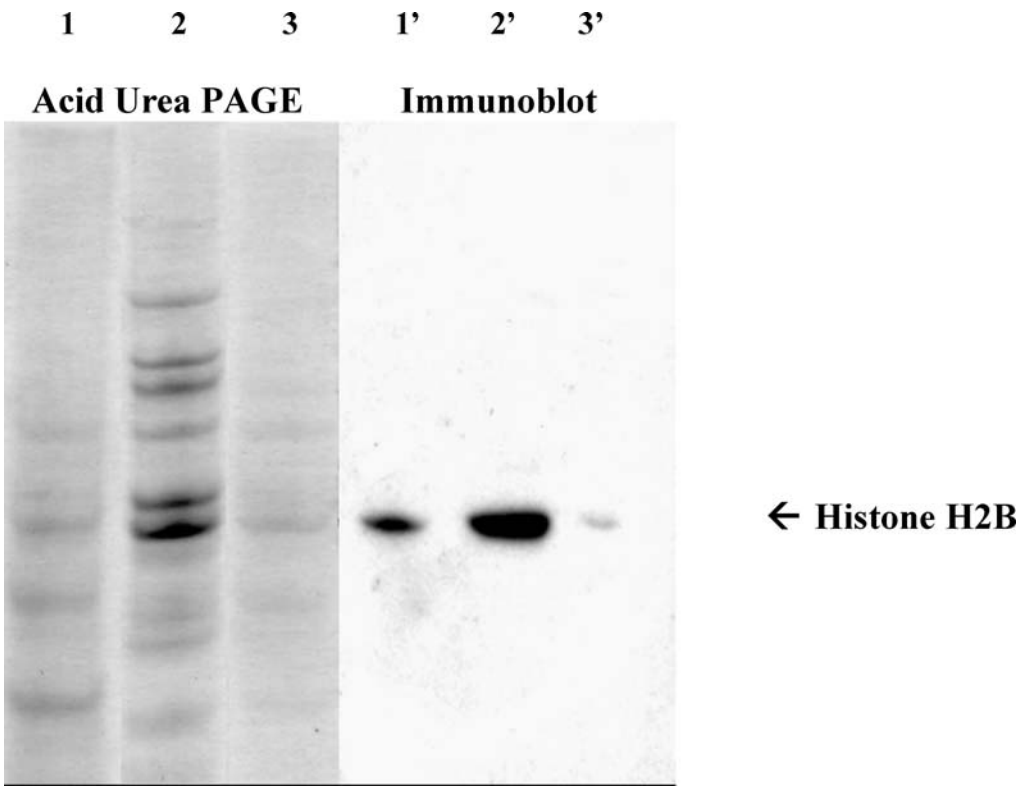

Figure 3. Acid-urea PAGE of sperm nuclear proteins and confirmation of histone H2B band by immunoblotting. Lanes 1, 2, and 3: Representative acid-urea PAGE stained with Coomassie blue (Lanes 1 and 3: fertile men, Lane 2: infertile man). Lanes 1′, 2′, and 3′: Immunoblot (of the corresponding acid urea gel) using histone H2B antibody. Note the increased H2B levels in the infertile compared to fertile men.

(and presumably abnormal) histone H2B to protamine ratio than that of fertile men. The immunocytochemistry data also suggest that in samples from infertile men a subpopulation of spermatozoa possess grossly normal H2B to protamine ratio.

Our data are in keeping with reports showing that sperm protamine deficiency (and by extension histone excess) is common in infertile men. Indeed, an important subset of infertile men (~5%–15%), but not of fertile men, produce spermatozoa that are protamine deficient (de Yebra et al, 1998; Carrell and Liu 2001). Furthermore, de Yebra et al (1993) have demonstrated that infertile men have a high degree of variability in the relative sperm histone to total nuclear protein ratio (ranging from 5% to 55%). These data suggest that those infertile men with high sperm nuclear histone H2B to protamine ratio have a defect in spermiogenesis (the later stage of spermatogenesis), as this is the specific step in spermatogenesis where the final assembly of sperm proteins (displacement of histones by transition proteins and then by protamines) occurs. The immunocytochemistry data (demonstrating heterogeneous populations of spermatozoa) suggest that the putative defect in spermiogenesis (or perhaps earlier in the process of spermatogenesis) is likely focal (affecting only some areas of the testicle) rather than global.

Table 1. *Mean (±SD) sperm PRM1 to PRM2 ratio [using the formula PRM1/(PRM1 + PRM2)] and sperm H2B (HIST2H2BE) to PRM1 ratio [using the formula H2B/(H2B + PRM1 + PRM2)] in infertile and fertile men*

	Fertile	Infertile	*P**
n	10	20	
PRM1/(PRM1 + PRM2)	0.65 ± 0.08	0.67 ± 0.09	.65
H2B/(H2B + PRM1 + PRM2)	0.08 ± 0.02	0.38 ± 0.24	.0004

* Comparison between fertile and infertile men by Mann-Whitney U test.

In contrast to the observations on the nuclear histone H2B to protamine ratio in fertile and infertile men, we have found no significant difference in the ratio of PRM1 to PRM2 in fertile men compared to our population of infertile men. Our data on the PRM1 to PRM2 ratio are in keeping with those of other investigators (Mengual et al, 2003). Mengual et al (2003) have reported that the sperm nuclear PRM1 to PRM2 ratio in the subgroup of men with asthenosper-

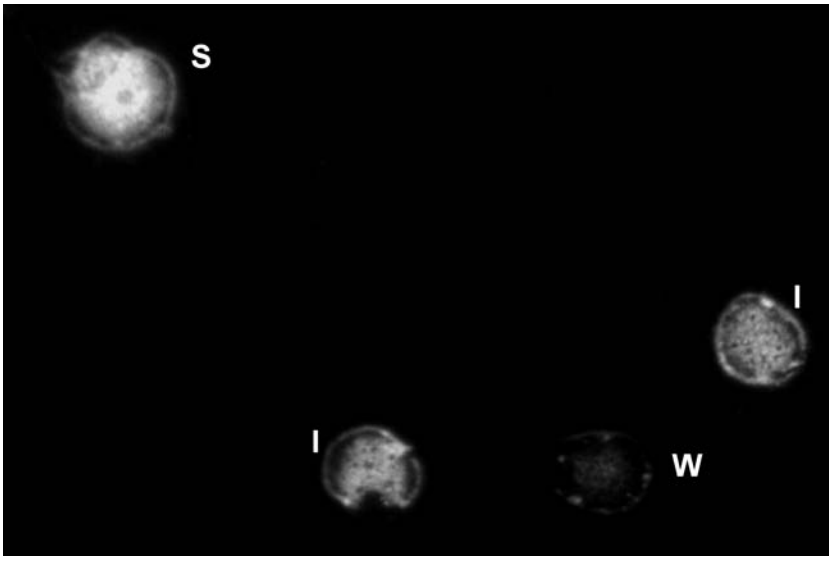

Figure 4. Photomicrograph of human spermatozoa immunoreacted with H2B antibody showing strong ("S"), intermediate ("I"), and weak ("W") punctuated staining.

Table 2. *Percentage (±SE) of spermatozoa in samples from fertile and infertile men exhibiting strong, intermediate, and weak H2B (HIST2H2BE) immunostaining*

	Fertile	Infertile	*P**
n	9	19	
Strong	1.6 ± 0.4	8.0 ± 1.1	.0003
Intermediate	26.6 ± 2.6	36.5 ± 1.6	.002
Weak	72.4 ± 2.6	55.2 ± 1.7	<.0001

* Comparison between fertile and infertile men by Mann-Whitney U test.

mia (characteristic of the men in our infertile population) was not significantly different from that of the fertile men. However, Mengual et al (2003) noted that the sperm nuclear PRM1 to PRM2 ratio was significantly higher in the subgroup of men with oligospermia compared to the fertile controls.

Normal sperm nuclear composition is essential to maintain sperm DNA integrity (Cho et al, 2001, 2003). Our results suggest that those infertile men with relatively lower histone to protamine ratio may also have poor sperm DNA integrity and enhanced susceptibility to DNA damage (Cho et al, 2001, 2003). Sperm DNA damage is clinically relevant, as it has been associated with reduced fertility potential in vivo and in vitro (Evenson et al, 1980, 1999; Lopes et al, 1998; Ahmadi and Ng 1999a, b; Spano et al, 2000; Zini et al, 2001; Morris et al, 2002; Benchaib et al, 2003; Bungum et al, 2004).

In summary, we have found that infertile men possess a higher proportion of spermatozoa with a high histone H2B to protamine ratio than that of fertile men. The unique and exact sperm chromatin architecture is likely

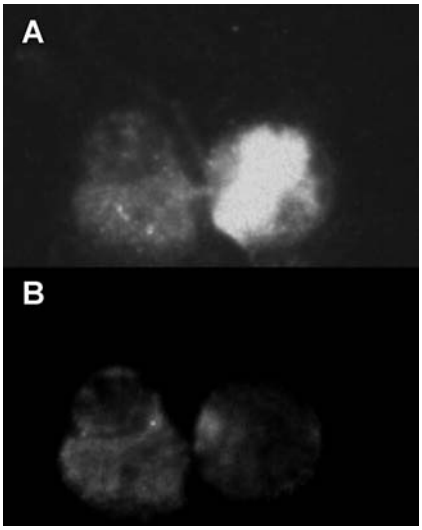

Figure 5. Colocalization experiments using H2B and PRM1 antibodies. **(A)** Photomicrograph of human spermatozoa immunoreacted with H2B antibody showing weak (left) and strong (right) punctuated staining. **(B)** Photomicrograph of the same spermatozoa (as in 3A) immunoreacted with PRM1 antibody showing strong (left) and weaker (right) punctuated staining.

critical for spermatogenesis and gene-expression after fertilization. The altered composition of sperm nuclear proteins in infertile men is therefore suggestive of abnormal spermatogenesis and reduced sperm fertilizing potential.

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