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Protein and DNA Contents in Sperm From an Infertile Human Male Possessing Protamine Defects That Vary Over Time

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ABSTRACT Sperm from 2 semen samples collected 6 months apart from an infertile male and 3 semen samples collected over an 18-month period from a fertile human male volunteer have been analyzed for their protamine and DNA content. Hup1M and Hup2b antibodies were used to detect the presence of protamines and protamine precursors in western blots of nuclear proteins isolated from pools of sperm. Phosphorus and sulfur contents, which can be used to estimate the nuclear DNA and protamine contents of sperm from fertile males, were measured within individual sperm heads from each semen sample by particle induced x-ray emission (PIXE). The single-cell data reveal no significant differences in the phosphorus and sulfur contents of sperm heads in the three semen samples obtained from the fertile male. For the initial semen sample produced by the infertile male, Western blot data show a normal complement of protamine 1, small amounts of mature protamine 2, and reveal large amounts of anti-protamine 2 reactive proteins with electrophoretic mobilities similar to protamine 2 precursors. Data from PIXE show elevated levels of sulfur within sperm heads compared with sperm from the fertile male. Western blot data exhibit no evidence of protamines or protamine 2 precursors in the second semen sample produced by the infertile male. Data from PIXE suggest that these sperm are highly deficient in sulfur and protamines. These results show that the degree of maturation of sperm cells present in the semen of some infertile males can vary with time. *Mol. Reprod. Dev. 50:345–353, 1998.*

Key Words: sperm chromatin; reproduction; male infertility; single-cell elemental analysis

INTRODUCTION

The DNA of human sperm is packed in a highly condensed state inside the head of the sperm by two types or families of small proteins; protamine 1 and protamine 2. Humans possess one form of protamine 1 and two forms of protamine 2: protamine 2a and protamine 2b (Pongsawasdi and Svasti, 1976; Tanphaichitr et al., 1978; Gusse et al., 1986; Balhorn et al., 1987). The amino acid sequence of protamine 2b has been found to be nearly identical to that of protamine 2a (the larger form of the two protamines is designated 2a when both proteins are present in sperm). In human sperm, protamine 2b only lacks the first three amino-terminal amino acids, arg-thr-his, of protamine 2a (Ammer et al., 1986; Gusse et al., 1986; McKay et al., 1986; Balhorn et al., 1987). Biochemical analyses of pooled human sperm obtained from fertile and infertile males suggest that the relative proportion of protamine 1 and 2 bound to DNA is important for fertility. Sperm obtained from infertile individuals that only produce sperm with overly large, round heads (Round-headed sperm syndrome) have been shown to be deficient in protamine 2 (Balhorn et al., 1988; Belokopytova et al., 1993). Deficiencies in protamine content have also been observed in other cases of human male infertility with some individuals appearing to produce sperm lacking in protamine 2 altogether (de Yebra et al., 1993).

While the above studies have correlated protamine defects in sperm with infertility, the conclusions have been drawn from analyses performed on whole semen or pools of millions of cells. In each case the investigator could not rule out the possibility that the observed deficiency might reflect the presence of a sub-population of supporting testicular cells or very abnormal sperm or spermatids arrested at a particular point in their development. Furthermore, the prospect that a significant number of sperm produced by these males may be biochemically normal could not be discounted. Light microscopy studies have indicated that many infertile males produce a number of sperm that at least appear morphologically normal.

In this study, pools of sperm and individual sperm heads from 2 semen samples collected 6 months apart from an infertile male and individual sperm from 3 semen samples collected over an 18-month period from a fertile human male volunteer have been analyzed for their protamine and DNA contents, with the aim of studying chromatin composition in the sperm of infer-

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tile males. Because the cells for PIXE analysis were identified microscopically, we can be certain they are sperm. When combined with biochemical analyses performed on pools of sperm, PIXE data unequivocally reveal that the nuclear protein composition of sperm from this particular infertile individual changed over time.

MATERIALS AND METHODS

Samples

Human semen ejaculates were collected and kept frozen until processed from a fertile volunteer of demonstrated fertility and from an infertile male attending an infertility clinic. The fertility of these men was defined as the ability to produce offspring. The infertile male had not been able to sire offspring for at least six years prior to and during his attendance at the infertility clinic. The fertile volunteer had sired offspring within the preceding two years prior to collection of his first semen ejaculate for this study. For the work reported here, 2 semen samples were processed from the infertile male and will be referred to as infertile 1 and infertile 2. The infertile 1 sample was collected 6 months before the infertile 2 sample. The infertile 1 semen sample had a sperm count of 21.3×10^6 sperm/ml, while the infertile 2 semen sample had a sperm count of 9.0×10^6 sperm/ml. Previous polyacrylamide gel electrophoresis (PAGE) analysis with Coomassie blue detection of the nuclear proteins isolated from a portion of the infertile 1 sample (de Yebra et al., 1993; Fig. 1, lane 2) indicated that sperm in the ejaculate produced by this male appeared to be completely deficient in the mature (fully processed) form of protamine 2. Optical microscopy studies of individual sperm within the infertile 1 and infertile 2 samples revealed that the majority of sperm had an external appearance consistent with that of sperm from fertile males. Three semen samples were processed from the fertile male volunteer for this study and will be referred to as fertile 1, fertile 2 and fertile 3. The fertile 1 sample was collected seven months before the fertile 2 sample and eighteen months before the fertile 3 sample. The fertile 1, fertile 2 and fertile 3 semen samples all had sperm counts between 80 and 100×10^6 sperm/ml.

Preparation of Amembraneous Sperm Nuclei From Fertile Male Semen Sample

To obtain accurate protamine and DNA contents, free of possible contamination from other sperm structures, sperm nuclei were obtained from the fertile 3 semen sample by treating sperm with dithiothreitol (DTT) (Boehringer Mannheim Biochemicals, Indianapolis, IN) and mixed alkyl trimethylammoniumbromide (MTAB) (Sigma Chemical Co., St. Louis, MO) as previously described (Pogany et al., 1981). An aliquot of semen from the fertile 3 sample was diluted with 0.15 M ammonium acetate (Ameresco, Solon, OH), pH 7.4 (2 ml) to wash the sperm. The aliquot was centrifuged (4124*g* for 5 min) to pellet the sperm cells and resuspended in 0.15 M ammonium acetate, pH 7.4 (2 ml). The suspension was centrifuged at 4124*g* for 3 min to selectively pellet the sperm chromatin (amembraneous nuclei). The sperm nuclei were subsequently washed and re-suspended in doubly distilled deionized water twice to remove any remaining DTT and MTAB. Following each resuspension in deionized water the solution was centrifuged at 4124*g* for 3 min to selectively pellet the amembraneous nuclei. Prior to the final centrifugation, the solution was sonicated for 15 sec using a Teflon coated probe to reduce clumping of amembraneous nuclei.

It has been demonstrated that treatment of mammalian sperm from fertile males by this process results in the complete removal and dissolution of the acrosome, nuclear membrane and tail of the sperm, leaving the amembraneous nucleus intact (Pogany et al., 1981). The characteristic shape of the sperm nucleus is also retained. These amembraneous nuclei may then be used for subsequent biochemical analyses avoiding possible contamination with nuclear membranes and tail fragments. Analyses of the MTAB supernatants by gel electrophoresis have shown that no basic nuclear proteins are extracted from the sperm of fertile males by this treatment. Furthermore, previous studies (Bench et al., 1995a, 1996) have shown that measured phosphorus and sulfur masses within individual amembraneous sperm nuclei from the semen of fertile mammals can be used to provide estimates of nuclear DNA and protamine content that agree well with results obtained from biochemical analyses performed on pools of sperm.

Preparation of Intact Sperm Heads From the Semen of the Fertile and Infertile Males

Although the treatment of sperm from fertile males with DTT and MTAB does not result in extraction of basic nuclear proteins from sperm chromatin (Bench et al., 1996), it is not known if the nuclear proteins are quantitatively retained during similar treatment of sperm with protamine defects obtained from infertile males. Consequently, for each semen sample, intact sperm heads that were not treated with DTT and MTAB were prepared for PIXE analysis. To obtain intact sperm heads aliquots of sperm from all semen samples (both fertile and infertile) were vortexed (centrifuged). Aliquots from each semen sample (both fertile and infertile) were diluted with 0.15 M ammonium acetate, pH 7.4 (2 ml) to wash the sperm. All samples were centrifuged (4124*g* for 5 min) to sediment the sperm cells and the pellet was resuspended in 0.15 M ammonium acetate, pH 7.4 (2 ml). Aliquots of sperm suspended in 0.15 M ammonium acetate, pH 7.4 were twice washed and subsequently re-suspended in doubly distilled deionized water to remove any remaining traces of seminal fluids. Following each resuspension in the doubly distilled deionized water the solution was centrifuged at 4124*g* for 3 min to selectively pellet the intact sperm heads. Vortexing only breaks off the sperm tail, leaving the nuclear membranes and acrosome

attached to the intact sperm head. This treatment will not alter the protein content of the sperm head.

Microbeam PIXE Analysis of Individual Amembraneous Sperm Nuclei and Sperm Heads

The sperm heads of MTAB treated nuclei were resuspended in a small volume of doubly distilled deionized water and approximately 5 µl of the suspension was micro-pipetted onto ultra-clean, 1-µm thick, transparent nylon foils stretched over a 15-mm diameter hole in a plastic support frame. The nuclei or heads were allowed to settle onto the nylon surface for 5 min, and the excess buffer was removed with a Pasteur pipette and blotting paper. The nylon foils containing sperm nuclei or heads were rapidly frozen in liquid nitrogen and subsequently lyophilized at a pressure of <100 mTorr for at least 6 h. The freeze dried samples were stored in a clean, dry environment prior to microprobe analysis. Optical studies revealed that the characteristic morphology of the nuclei or heads was retained after lyophilization.

Amembraneous nuclei or sperm heads from individual sperm were examined with the nuclear microprobe facility located at the Lawrence Livermore National Laboratory (Roberts et al., 1995). Proton-induced X-ray emission (PIXE) data were obtained using incident 3 MeV proton microbeams. PIXE is an accurate, absolute, fully quantitative technique that produces sample elemental concentrations from the x-ray yield induced by proton bombardment. X rays were detected with a Si(Li) detector that subtended a solid angle of $\sim$100 msr. The detector was located at an angle of 135° with respect to the incident beam. Charge was collected in a biased Faraday cup located behind the sample. X rays were recorded in list mode along with coincident beam spatial co-ordinates arising from scanning the beam electrostatically over the sample in a point by point raster mode. The use of PIXE to measure elemental concentrations inside sperm has been described in greater detail elsewhere (Bench et al., 1995a).

For the measurements reported here, beam currents of up to 1 nA focused down to spot sizes of between 2 to 3 µm were repeatedly scanned over areas of $\sim 20 \times 20$ µm^2. Within each pass 0.1 nC was deposited on each beam location. Areas were irradiated with an exposure of up to 3.0 µC. Each irradiated area contained the amembraneous nucleus or head from a single sperm. Only sperm nuclei and heads that had the characteristic normal morphology, size and shape were irradiated. Data were reduced off-line so that X-ray spectra corresponding to the nuclei or heads could be extracted from each irradiated region and analysed with x-ray spectrum fitting codes as previously described (Bench et al., 1995a). For the fertile 1, fertile 2, fertile 3, and infertile 1 samples X-ray–counting statistics within individual sperm heads or amembraneous nuclei were better than 2% for phosphorus and better than 3% for sulfur. For the infertile 2 sample, X-ray–counting statistics within individual sperm heads were better than 2% for phosphorus, while sulfur X-ray–counting statistics were better than 9%.

A series of thin-film calibration standards were used to measure the efficiency of the X-ray detection system. To cross check the detector's X-ray detection efficiency two other thin-film standards, one known to contain sulfur at 12.0 ± 0.6 µg/cm^2 and the other known to contain phosphorus at 15.3 ± 0.6 µg/cm^2 were analyzed using 3 MeV proton microbeams. The microbeam PIXE system yielded a measured sulfur thickness of 11.7 ± 0.5 µg/cm^2 for the sulfur-bearing standard and a measured phosphorus thickness of 15.0 ± 0.5 µg/cm^2 for the phosphorus-bearing standard. Phosphorus and sulfur masses (units of gram) inside the nuclei or heads of individual sperm were calculated using the thin film approximation, the total scan area, and the exposure delivered to the total scan area as previously described (Bench et al., 1995a). The measurement of phosphorus and sulfur masses within sperm nuclei or heads has a quantitative accuracy of $\sim$7%.

Biochemical Analyses of Pools of Sperm From the Infertile Male

The proportion of the different forms of protamine present in pools of sperm from the fertile male have been reported previously (Bench et al., 1996). For the semen samples obtained from the infertile male, detection of the various protamines was determined as described below.

Extraction of nuclear proteins. Approximately 10^7 sperm cells from each semen sample were thawed and washed three times (10 min at 3000*g* each time) with a solution containing 9.85 ml of Ham f-10 1× (Sigma Chemical Co.) and 150 µl of NaCl 5 M. The sediment was resuspended in 50 µl of 20 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), 100 mM Tris-HCl (pH 8.0) and then processed as described by Yebra and Oliva (1993) with the variant that no iodoacetate treatment was performed. Both protamine 1 and protamine 2 can be better identified and quantified if the proteins are not modified with iodoacetate. The sediment obtained after 0.5 M HCl extraction of the proteins was used to quantify the DNA using 0.4 N perchloric acid hydrolysis (at 80°C, 30 min) and the diphenylamine reaction (Burton, 1956).

Separation and analysis of proteins. PAGE was performed on 23 cm long gels containing 0.9 M acetic acid, 2.5 M urea, 15% acrylamide, 0.14% TEMED (N,N,N′,N′-tetramethylethylenediamine), 0.09% bisacrylamide and 0.12% ammonium persulfate. The gels were either stained or transferred (0.9 mM acetic acid, 85 volts, 17 min.) to Immobilon-P (Millipore, Bedford, MA) membranes. Staining of the gels was accomplished with 1.1 g of Coomassie blue R-250 (BioRad, Richmond, CA) dissolved in 250 ml methanol, 250 ml H_2O and 50 ml acetic acid for 45 min. The gels were subsequently destained for 5 min in 50% methanol and 10% acetic acid and then destained overnight in 10% methanol and 10% acetic acid. Gels were then dried between 2 sheets

of cellophane film and the intensity of the bands were quantified by scanning each lane (Millipore Bio Image Gel Scanner, Millipore).

Immunostain. Membranes were briefly soaked in methanol, rinsed with H_2O, and blocked in solution 1 (0.01 M Tris HCl, pH 7.4, 0.1 Tween 20, 0.9% NaCl) containing 5% bovine serum albumin and 20 µg/ml calf thymus DNA, for 1.5 h at 37°C and subsequently rinsed three times in solution 1. The membranes were then incubated with 30 ml of primary antibody solution (1:2000 dilution of antibody, HuplM or Hup2b, in stock solution 1) for 2 h at room temperature in a dark environment and then rinsed three times in solution 1. The membranes were then incubated for 45 min at room temperature in a darkened environment with 40 ml of secondary antibody solution (1 drop of Biotinylated anti-mouse IgG [Vector Laboratories, Burlingame, CA], BA-2000, for every 10 ml of stock solution 1) and rinsed three times in solution 1. The primary antibody HuplM specifically binds to human protamine 1; Hup2b is specific for the protamine 2 family proteins and their precursors (Stanker et al., 1987).

Detection was performed with the Vectastain ABC Kit, PK-4000 (Vector Laboratories) by incubating the membranes in ABC solution for 30 min at room temperature in a dark environment. The membranes were then rinsed three times in solution 2 (0.01 M Tris HCl, pH 7.4, 0.9% NaCl) to remove as much detergent as possible from previous steps, and the substrate added (36 mg of 4-chloro-1-naphthol dissolved in 12 ml of methanol, added to 60 ml of solution 2, and supplemented with 30 µl of 30% H_2O_2). The intensity of the bands that were not overloaded in the western blots was quantified and an approximate measure of the amount of protamines was calculated by extrapolation to human protamine standards quantified by HPLC (Balhorn et al., 1987) and included in all Western blots.

Conversion of Phosphorus and Sulfur Masses to DNA and Protamine Masses in Individual Sperm Nuclei From Semen Obtained From the Fertile Male

The phosphorus and sulfur mass within an amembraneous sperm nucleus can be used to estimate the nuclear DNA and protamine content in sperm from the semen of a fertile male (Bench et al., 1996). These derivations assume that all the phosphorus is contained in DNA and all the sulfur is contained in the protamine within a sperm nucleus. The phosphorus content of sperm chromatin from fertile males provides a measure of the DNA content that is accurate to within a few percent as sperm do not contain RNA and protamines do not contain significant amounts of phosphorus (Bench et al., 1996). A DNA base pair has a molecular weight of 660 g/Mol and contains 2 phosphorus atoms but no sulfur. Phosphorus has an atomic weight of 30.97 g/Mol. To obtain the DNA mass in sperm chromatin the phosphorus mass is multiplied by

$$[660/(2 \times 30.97)] = 10.655. \quad (1)$$

TABLE 1. Protamine Molecular Weights, Number of Sulfur Atoms in Each Form of Protamine and the Ratio of the Number of Each Form of Protamine Molecule to Total Number of Protamine Molecules in Sperm Chromatin From Fertile Human Males*

Protamine 1 molecular weight (g/Mol)	6692.2
Sulfur atoms/protamine 1 molecule	7
Protamine 2a molecular weight (g/Mol)	7653.4
Protamine 2b molecular weight (g/Mol)	7258.9
Sulfur atoms/protamine 2a molecule	5
Sulfur atoms/protamine 2b molecule	5
Protamine 1/total protamine	0.57 ± 0.03
Protamine 2/total protamine	0.43 ± 0.03
Protamine 2a/total protamine	0.29 ± 0.02
Protamine 2b/total protamine	0.14 ± 0.02

*From Bench G, Friz AM, Corzett MH, Morse DH, Balhorn R (1996): DNA and total protamine content of individual sperm from fertile mammalian subjects. Cytometry 23:263–271.

Cysteine is the only sulfur-containing amino acid in human protamine 2a and 2b. Human protamine 1 also contains sulfur in a single methionine residue. Because the protamines which constitute the majority of the protein inside human sperm nuclei contain a known number of cysteine and methionine residues per molecule, sulfur should be an effective estimate of the total protamine content in fertile males (Bench et al., 1996). Although up to 15% of human sperm DNA may remain packaged by sperm specific histones (Pongsawadi and Svasti, 1976; Tanphaichitr et al., 1978; Gatewood et al., 1987; Balhorn, 1990), none of these histones contain significant amounts of sulfur. The total protamine content can be determined by multiplying the sulfur mass by

$$\sum_{i=1}^{K} \frac{M(P_i) \times R_i}{M(S) \times N(S_i)} \quad (2)$$

where K is the number of forms of protamine, $M(P_i)$ is the molecular weight of protamine i, R_i is the ratio of number of molecules of protamine i to total protamine molecules in the chromatin, M(S) is the atomic weight of sulfur and $N(S_i)$ is the number of sulfur atoms in the i^{th} form of protamine (Bench et al., 1996). Table 1 shows protamine molecular weights and number of sulfur atoms in each form of protamine for sperm from the semen of fertile human males. The ratio of each form of protamine molecule to total number of protamine molecules in human sperm chromatin from fertile males is also listed.

The mass of i^{th} form of protamine $[m(P_i)]$ can be determined from the total protamine mass [m(P)] by:

$$m(P_i) = \frac{m(P) \times M(P_i) \times R_i}{\sum_{j=1}^{K} M(P_j) \times R_j}. \quad (3)$$

TABLE 2. Average Phosphorus Mass and Sulfur Mass Within Amembraneous Nuclei and Intact Sperm Heads From the Fertile 3 Semen Sample

Preparation	Phosphorus mass (10^{-13} g)	Sulfur mass (10^{-13} g)
Amembraneous nuclei[a]	3.07 ± 0.08	0.62 ± 0.03
Intact sperm heads[b]	3.09 ± 0.09*	0.64 ± 0.03**

[a]Data expressed mean ± standard deviation of 9 amembraneous nuclei.
[b]Data expressed mean ± standard deviation of 19 intact sperm heads.
*$P > 0.10$ when compared with phosphorus mass for the amembraneous nuclei by Students' *t*-test.
**$P > 0.10$ when compared with sulfur mass for the amembraneous nuclei by Students' *t*-test.

Data Presentation

Differences in the DNA and protamine contents from different samples of human sperm were assessed by Students' *t*-tests. A significance level of <0.01 was considered meaningful.

RESULTS

Table 2 shows the average phosphorus and sulfur masses present in 9 amembraneous sperm nuclei and 19 intact sperm heads from the fertile 3 sample. Students' *t*-tests performed on the phosphorus and sulfur contents from the sperm heads and the respective values from the amembraneous sperm nuclei revealed no significant differences in the sulfur contents at significance levels of ~0.15 and no significant differences in the phosphorus contents at significance levels of ~0.5.

Table 3 shows the average phosphorus mass and sulfur mass present in intact sperm heads isolated from the semen of the fertile and infertile males. Nineteen intact sperm heads were analyzed from each semen sample. Students' *t*-tests reveal no significant differences in the average phosphorus content, sulfur content and the sulfur to phosphorus mass ratio of sperm heads from the fertile 1, fertile 2, and fertile 3 semen samples. Consequently, data from all individual sperm heads analyzed from these three semen samples have been combined to improve statistics, and the results are shown on the bottom row of Table 3. Figure 1 shows a plot of the number of sperm versus the ratio of sulfur mass to phosphorus mass for the infertile 1 and infertile 2 samples and the combined fertile human male data. For this figure sulfur/phosphorus ratios for individual sperm heads have been rounded down to the nearest hundredth, and the number of sperm have been plotted against sulfur/phosphorus ratio in bin sizes of 0.01.

Electrophoresis of the basic nuclear proteins isolated from the sperm cells of fertile males combined with Coomassie blue detection exhibit a pattern of proteins characteristic of sperm containing the normal proportion of protamine 1 and 2 (Fig. 2, lanes 2 and 3). Lower mobility proteins corresponding to histones, the protamine 2 precursor, and its partially processed forms are also observed but these other proteins are less abundant than the protamines. In contrast, the proteins isolated from the infertile 1 sample were observed to be deficient in protamine 2. Protamine 1 and the lower mobility proteins were present, but no proteins with a mobility characteristic of the protamine 2 family could be detected in the Coomassie blue stained gels (Fig. 2, lane 4). Electrophoretic analysis with Coomassie blue detection of the proteins isolated from the sperm of the same infertile individual 6 months later did not reveal any detectable protamines or protamine precursors (data not shown).

Hup2b antibodies used to detect and identify protamine 2 on western blots indicate that sperm present in the first semen sample obtained from the infertile male (infertile 1) do actually contain a small amount of protamine 2 (Fig. 3, panel A, lane 3). The amount of protamine 2 present was most likely too small to be detected by the less sensitive Coomassie staining technique. The Hup2b antibody stain reveals that a number of the lower mobility proteins that were detected in the Coomassie stained protein gels are intact or partially processed protamine 2 precursors. Subsequent reprobing of the filter from panel A with the Hup1M antibody reveals the presence of protamine 1 (Fig. 3, panel C, lanes 3 and 4). In keeping with the results obtained with Coomassie blue staining, neither antibody detected protamine 1, protamine 2 or the intact or intermediate processed forms of the protamine 2 precursor in the infertile 2 sample (Fig. 3, panel B, lane 2, and panel D, lane 2).

The biochemical analyses of pooled sperm from the infertile 1 sample in this study also revealed the ratio of protamine 1 molecules to total protamine molecules in the sperm chromatin to be 0.93 ± 0.04, while the protamine 1 to DNA mass ratio was 0.29 and the protamine 2 to DNA mass ratio was 0.02. Our analyses of pooled sperm from the infertile 2 sample showed the sperm did not contain detectable amounts of protamine 1, protamine 2 or protamine 2 precursors. While the antibody approach used to quantify the protamine contents of the samples is prone to errors in quantitation, the purpose here is only to suggest whether the content of a particular protamine is consistent with that expected from biochemically normal semen.

DISCUSSION

The similarity in the average phosphorus and sulfur contents of amembraneous sperm nuclei and intact sperm heads shown in Table 2 suggest that phosphorus and sulfur are unlikely to have been removed from or added to the amembraneous nuclei during the DTT and MTAB treatment. Conversely, the data suggest that the phosphorus and sulfur signals in intact sperm heads arise almost completely from nuclear DNA and protamine. The presence of the acrosome and nuclear membranes in intact heads do not add significantly to the phosphorus and sulfur contents obtained from the nucleus. The data also indicate that for fertile males the

TABLE 3. Average Phosphorus Mass, DNA Mass, Sulfur Mass, and the Ratio of the Sulfur Mass to Phosphorus Mass in Sperm Heads From the Semen Samples†

Sample	Phosphorus content (10^{-13} g)	DNA content (pg)	Sulfur content (10^{-13} g)	Sulfur/ phosphorus ratio
Infertile 1	3.13 ± 0.08	3.33 ± 0.09	0.74 ± 0.04*	0.24 ± 0.01**
Infertile 2	3.06 ± 0.08	3.26 ± 0.09	0.10 ± 0.02*	0.034 ± 0.006**
Fertile 1	3.07 ± 0.09	3.27 ± 0.10	0.63 ± 0.03	0.21 ± 0.01
Fertile 2	3.05 ± 0.09	3.25 ± 0.09	0.62 ± 0.05	0.20 ± 0.01
Fertile 3	3.09 ± 0.09	3.29 ± 0.10	0.64 ± 0.03	0.21 ± 0.01
Fertile average[a]	3.07 ± 0.09	3.27 ± 0.10	0.63 ± 0.04	0.21 ± 0.01

†Data expressed as sample mean ± standard deviation of 19 sperm heads.
[a]Average values of the 57 sperm heads analyzed from the fertile 1, fertile 2, and fertile 3 semen samples.
*$P < 0.01$ when compared with the sulfur mass from each of the fertile average and fertile 1, fertile 2, and fertile 3 samples by Students' *t*-test.
**$P < 0.01$ when compared with the sulfur/phosphorus mass from each of the fertile average and fertile 1, fertile 2, and fertile 3 samples by Students' *t*-test.

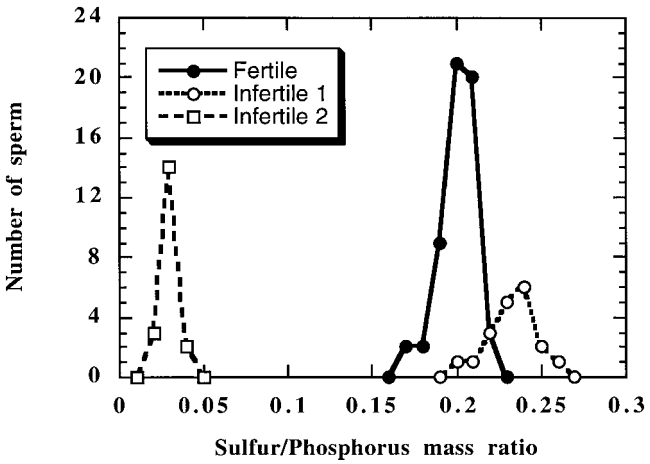

Fig. 1. Plot of number of intact sperm heads versus the ratio of sulfur mass to phosphorus mass from infertile 1 and infertile 2 male semen samples and the combination of the three fertile male semen samples.

phosphorus and sulfur contents within the heads of intact sperm can reliably be used to estimate the nuclear DNA and protamine content.

Using the phosphorus and sulfur contents of the amembraneous nuclei shown in Table 2, the data in Table 1 and equations 1 and 2 to provide estimates of the DNA and protamine content yield a DNA mass of 3.27 ± 0.08 pg and a protamine mass of 2.30 ± 0.11 pg per nucleus. These values are consistent with previous microbeam PIXE measurements of DNA and protamine contents in amembraneous nuclei from the semen of fertile human males (Bench et al., 1996). The average DNA content for the amembraneous human sperm nuclei is similar to the 3.14 pg obtained by White et al. (1953) but substantially higher than the 2.46 pg obtained by Leuchtenberger et al. (1956) and the 2.66 pg determined by Ackerman and Sod-Moriah (1968). To the best of our knowledge no other measurements of protamine content within sperm nuclei from fertile human males have been reported in the literature. Using the phosphorus data in Table 3 and equation 1 to estimate DNA content, sperm heads within the five semen samples appear to contain similar amounts of DNA (3.25 to 3.33 pg/cell). The differences in DNA content are small and may not be real.

Our PAGE analyses with Coomassie blue detection of the nuclear proteins isolated from the infertile 1 sample (Fig. 2, lane 4) are similar to previous PAGE analyses of proteins from the infertile 1 sample that included an iodoacetate modification of the proteins and Coomassie blue detection (deYebra et al., 1993; Fig. 1, lane 2). Both these analyses failed to detect the mature (fully processed) form of protamine 2. However, Hup2b and Hup1M antibodies used to detect and identify the two protamines on Western blots, indicate that sperm present in the first semen sample obtained from the infertile male (infertile 1) do actually contain a small amount of protamine 2 in addition to protamine 2 precursors and protamine 1. The presence of large amounts of protamine 2 precursors in the proteins extracted from the infertile 1 sample indicates either a defect in protamine 2 processing or the presence of large amounts of immature sperm cells. Similar analyses of pooled sperm obtained from the same individual 6 months later (infertile 2) indicated the composition of the sperm chromatin had changed. Sperm present in the infertile 2 sample were deficient in both protamine 1 and protamine 2. The only other clinical symptom that had changed in this 6-month period was the sperm count from the individual: The infertile 2 semen sample was a factor of 2 lower than that of the infertile 1 semen sample.

The biochemical analyses of pooled sperm from the infertile 1 sample in this study revealed the ratio of protamine 1 to total protamine to be 0.93 ± 0.04, which is substantially different from the value of 0.57 found in the sperm chromatin of fertile males and displayed in Table 1. The protamine 1 to DNA mass ratio in the infertile 1 sample was ~0.3. This value is similar to the protamine 1 to DNA mass ratio of ~0.4 found in sperm

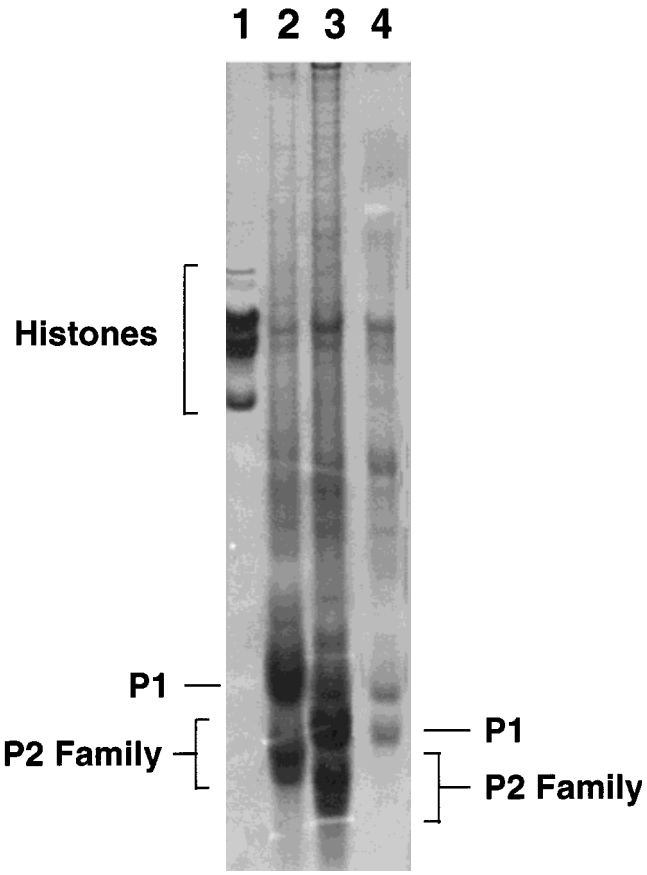

Fig. 2. Sperm nuclear proteins analyzed by 15% PAGE 23-cm gels and visualized by Coomassie blue staining. In this figure P1 stands for protamine 1 and P2 stands for protamine 2. Lane 1: Chicken erythrocyte histones as a control. Lane 2: Sperm proteins from pooled fertile male semen with a normal protamine 1/protamine 2 ratio extracted following the methods described in Yebra and Oliva (1993) including an iodoacetate modification of the proteins. The migration of the protamine 1 and protamine 2 family proteins in this lane is indicated at the bottom left of the figure. Lane 3: The same sample described in lane 2 except that no iodoacetate modification was performed. The migration of the protamine 1 and protamine 2 family proteins in this lane is indicated at the bottom right of the figure. Lane 4: Sperm proteins extracted without iodoacetate modification from cells present in the infertile 1 semen sample.

from the fertile 3 sample by PIXE (using equations 1, 2, 3, data in Table 1, and the average phosphorus and sulfur masses from the amembraneous nuclei in Table 2).

In conjunction with the results obtained from biochemical analyses, several observations can be drawn from the microbeam PIXE measurements. The very low ratio of sulfur to phosphorus in the sperm from the infertile 2 sample compared to sperm from the fertile samples indicates that the total protamine (protamine 1 + protamine 2) content in individual sperm nuclei from the infertile 2 sample is extremely low. Students' *t*-tests performed on the sulfur to phosphorus mass ratio distributions for the infertile 2 and all three fertile samples yields a significance level of <0.01 in each instance, which indicates that these sulfur-to-phosphorus mass ratio distributions are significantly different. Likewise, Students' *t*-tests performed on the sulfur mass distributions for the infertile 2 and all three fertile samples yield a significance level of <0.01 in each instance, which indicates that the sulfur ratio distributions are significantly different. The measured sulfur masses indicate the sperm from this individual cannot contain more than one sixth the normal complement of protamines. Consequently, these sperm must be deficient in both protamines. Furthermore, the data shown in Figure 1 and Table 3 reveal that all the sperm measured from this infertile sample have significantly lower protamine contents than those from the fertile male. The likelihood of there existing a sub-population of sperm possessing an external appearance consistent with that of sperm from fertile males and with a normal biochemical composition is vanishingly small based on the data shown in Figure 1.

On the other hand, the average sulfur to phosphorus mass ratio in individual sperm heads from the infertile 1 sample is ~15% higher than the corresponding value from the fertile samples. A Students' *t*-test performed on the sulfur to phosphorus ratio distributions for the infertile 1 and all three fertile samples yields a significance level of <0.01 in each instance, which indicates that these sulfur-to-phosphorus mass ratio distributions are significantly different. Likewise, Students' *t*-tests performed on the sulfur mass distributions for the infertile 1 and all three fertile samples yield a significance level of <0.01 in each instance, which indicates that these sulfur mass distributions are significantly different.

One hypothesis we considered to explain the increase in sulfur content is that the total mass of protamine bound to DNA in the cells from the infertile 1 sample is similar to that found in normal sperm, but in the absence of sufficient protamine 2 synthesis, additional protamine 1 is deposited onto the DNA to compensate for the deficiency in protamine 2. Since each human protamine 1 molecule contains seven sulfur atoms and human protamine 2 molecules contain only five, the measured sulfur content for such sperm would yield a total protamine content of ~2.25 pg per sperm head (using the protamine 1/total protamine ratio of 0.93, equation 2, and assuming the sulfur signal derives entirely from protamine 1 and protamine 2). This value is similar to the ~2.3 pg of protamine found in the sperm heads of the fertile male. However, such a hypothesis neglects any contribution made to the sulfur content by the protamine 2 precursors and is not substantiated by the total protamine (protamine 1 plus protamine 2) to DNA mass ratio ~0.3, which is markedly lower than the value of ~0.7 often found in sperm chromatin from fertile males (Bench et al., 1996).

The increase in sulfur content may also indicate that the sperm measured have a nuclear protein content that reflects the presence of other proteins that are rich in sulfur. Such an hypothesis is not unreasonable as mouse spermatids containing protamine 2 precursors have been shown to have higher sulfur contents than mature mouse sperm (Bench et al., 1995b). The electro-

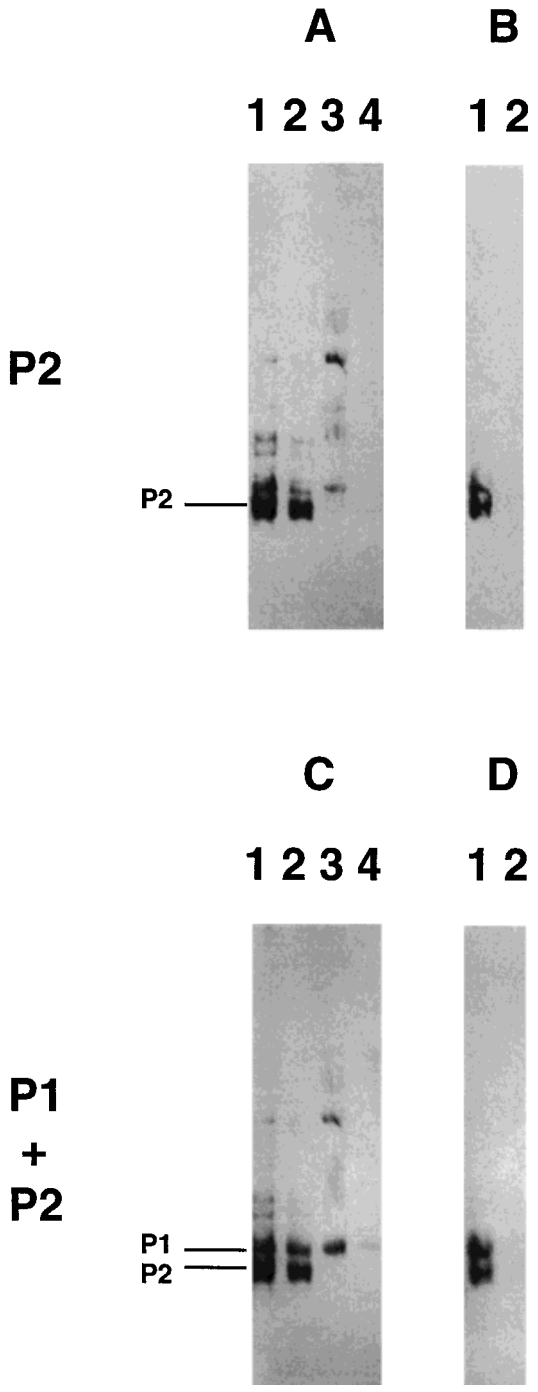

Fig. 3. Western blot analysis of sperm proteins separated by PAGE and detected using the anti-protamine 2 antibody (Hup 2b; panels A and B) or the anti-protamine 1 antibody (Hup 1M; panels C and D). In this figure P1 stands for protamine 1 and P2 stands for protamine 2. Panels **A** and **C**, lane 1: 0.4 µg of protamines extracted from pooled fertile male semen with a normal protamine 1/protamine 2 ratio extracted following the methods described in Yebra and Oliva (1993), except that no iodoacetate modification was performed; lane 2: 1/2 fraction of proteins loaded in lane 1; lane 3: 0.14 µg of the infertile 1 sample; and lane 4: 1/4 fraction of the sample loaded in lane 3. Panels **B** and **D**, lane 1: 0.2 µg protamines extracted from pooled male semen with a normal protamine 1/protamine 2 extracted following the methods described in Yebra and Oliva (1993), except that no iodoacetate modification was performed; lane 2: Protein extracted from 0.68 µg of DNA from the infertile 2 semen sample.

phoretic pattern of the isolated proteins offers partial support to such an hypothesis. As shown in Figure 3 (panels A and C), the lower mobility proteins as a group are more abundant in the infertile 1 sample (lanes 3 and 4) relative to protamine 1 than in the 0.4 µg of protamines extracted from pooled fertile male semen (lanes 1 and 2). One band migrating with a mobility characteristic of the intact protamine 2 precursor in the infertile 1 sample appears substantially more abundant than the rest. However, since the sulfur content of all the lower mobility proteins extracted from the infertile 1 sample have not been measured, it is not known whether some of these proteins are richer in sulfur than the fully processed protamine 2 or if the increased sulfur content derives from an increased proportion of protamine 2 precursors. Clearly, our understanding of the processes that result in the increased sulfur content within sperm heads from the infertile 1 sample is far from complete. It is also possible that there is a subtle interplay of many factors that result in the observed increase. However, further discussion of such factors is beyond the scope of the available data.

The ability of PIXE to provide accurate measurements of the phosphorus and sulfur contents in individual sperm heads is crucial in studies such as these. Biochemical analyses alone are difficult to interpret, because they reflect analyses of pooled samples of cells that may contain variable percentages of normal or arrested spermatids, or supporting testicular cells in addition to mature sperm. It is also extremely difficult to obtain accurate measurements of the relative proportion of protein and DNA in sperm using biochemical methods due to significant losses in both components during the fractionation procedure. Even when these data are obtained, one cannot examine the variation that exists among cells within the population.

In this study, the PIXE data obtained for sperm produced by the same infertile individual 6 months apart has provided unequivocal evidence that the nuclear protein composition of this individual's sperm changed over time. Because the cells selected for analysis were identified microscopically, we can be certain that they were sperm. At the population level, all measured sperm appeared to be affected. This suggests that infertility in this particular individual is not caused by a stable genetic defect, but that it may instead be controlled by factors (potentially environmental) that alter the efficiency of translation of protamine mRNAs late in spermiogenesis. The PIXE data obtained for sperm in the second sample (infertile 2), which contained at most one sixth the normal content of protamine, also provides evidence that chromatin packaging and the formation of mature appearing sperm cells can take place in the absence of protamines. Such an observation is consistent with other studies that show that shaping of the sperm head starts before protamines are deposited within the sperm nucleus. Shaping of the sperm head starts in stage-9 spermatids (Kierszenbaum and Tres, 1975), whereas protamines

start to be deposited onto nuclear DNA in stage-12 spermatids (Balhorn et al., 1984).

In conclusion, the results reported in this work demonstrate the presence of immature protamine 2 in the sperm cells of some infertile patients. The data also show that the packaging of the genome in sperm produced by certain infertile patients can vary with time. Variation of the deficiency in protamine content in such individuals may be caused by alterations in the efficiency of protamine mRNA translation late in spermiogenesis. However, the significance of the differences in protamine contents and its relationship to infertility remain unknown.

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