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Protamine Levels Vary Between Individual Sperm Cells of Infertile Human Males and Correlate With Viability and DNA Integrity

VINCENT W. AOKI[*,†], BENJAMIN R. EMERY[*,†], LIHUA LIU[*] AND DOUGLAS T. CARRELL[*,†,‡]

From the [*] Andrology and IVF Laboratories and Department of Surgery, the [†] Department of Physiology, and the [‡] Department of Obstetrics and Gynecology, University of Utah School of Medicine, Salt Lake City, Utah.

Correspondence to: Douglas T. Carrell, University of Utah School of Medicine, Andrology/IVF, 675 S Arapeen Dr, Suite 205 (Andrology), Salt Lake City, UT 84108 (e-mail: douglas.carrell{at}hsc.utah.edu).

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Sperm protamine deficiency has been associated with human male infertility. However, most studies have adopted a global approach to assessing sperm protamine levels. Thus, it is not known whether sperm cells from individual human males possess variations in protamine protein content. The objectives of this study were to evaluate variations in protamine-1 (P1) and protamine-2 (P2) content between individual sperm cells of fertile and infertile men and to correlate DNA integrity and sperm cell viability with protamine levels in individual sperm cells. The semen samples of fertile and infertile men were evaluated globally for protamine protein content using nuclear protein extraction, gel electrophoresis, and densitometry analysis. Individual sperm cell P1 and P2 levels were assessed using immunofluorescence microscopy in conjunction with automated image analysis. The terminal transferase dUTP nick end labeling (TUNEL) assay was performed simultaneously with protamine immunostaining to assess the relationship between protamine levels and DNA integrity in individual spermatozoa. Additionally, the relationship between sperm cell viability and protamine levels was assessed via viability staining concomitant with protamine staining. The protamine fluorescence data demonstrate significant variations in protamine content within individual sperm cells of human males. Overall population-based measures of DNA integrity and sperm cell viability

correlate significantly with population-based measurements of protamine levels. The data also demonstrate individual sperm cells displaying the lowest protamine levels display diminished viability and increased sperm cell susceptibility to DNA damage.

Key words: Spermatogenesis, male fertility

During spermiogenesis the protamine proteins replace the somatic cell histones, a process that results in a highly condensed transcriptionally silent chromatin (Oliva and Dixon, 1991; Aoki and Carrell, 2003). In humans, there are 2 protamine proteins, protamine-1 (P1) and protamine-2 (P2), which occur in a strictly regulated one-to-one ratio (Corzett et al, 2002).

Aberrations in protamine expression have been associated with male infertility (Chevaillier et al, 1987; Balhorn et al, 1988; Chevaillier et al, 1990; Belokopytova et al, 1993; de Yebra et al, 1993; de Yebra et al, 1998; Carrell and Liu, 2001; Aoki et al, 2005,2005). A number of studies have described infertile male populations with abnormally elevated ratios of P1 to P2 (P1/P2) (Balhorn et al, 1988; Chevaillier et al, 1990; de Yebra et al, 1993; de Yebra et al, 1998; Carrell and Liu, 2001; Aoki et al, 2005,2005). Two of these reports document a small population of infertile men with complete selective absence of P2 (de Yebra et al, 1993; Carrell and Liu, 2001). Recently, another population of infertile males was identified with deregulated P1 expression and abnormally reduced P1/P2 ratios (Aoki et al, 2005,2005). Taken together, these studies indicate that abnormal protamine stoichiometry derives from aberrant expression of either P1 or P2.

Human sperm protamine deficiency correlates significantly with diminished semen quality parameters, sperm functional ability, and sperm DNA integrity (de Yebra et al, 1993; de Yebra et al, 1998; Balhorn et al, 1999; Carrell and Liu, 2001; Aoki et al, 2005,2005). Due to diminished sperm counts, motility, head morphology, and sperm penetration assay scores (SPA), the majority of male infertility patients with sperm protamine deficiency are treated using in vitro fertilization (IVF) in conjunction with intracytoplasmic sperm injection (ICSI).

Mouse knockout models clearly demonstrate that sperm protamine haploinsufficiency directly impairs spermatogenesis and subsequent embryo development (Cho et al, 2001; Cho et al, 2003). However, human IVF/ICSI outcomes have diverged from these data, not yet resolving a relationship between sperm protamine deficiency and embryonic development or pregnancy rates (Carrell and Liu, 2001; Aoki and Carrell, 2003; Nasr-Esfahani et al, 2004; Aoki et al, 2005,2005). Given these data, it appears ICSI is able to overcome the diminished semen quality and sperm functional ability associated with sperm protamine deficiency in humans.

The majority of studies describing human sperm protamine content have utilized global assessments of protamine levels in whole ejaculates. Thus, it is not known whether sperm cells from individual human males possess variations in protamine levels. The objectives of this study were to evaluate variations in P1 and P2 content between individual sperm cells of fertile and infertile men and to correlate those protamine levels with the DNA integrity status and viability in individual sperm cells.

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Institutional Review Board approval was obtained prior to initiation of this study. Semen was collected from 10 fertile donors and 69 male infertility patients. A single semen sample was used for nuclear protein extraction and immunofluorescence microscopy. Unless otherwise noted, all chemicals were obtained from Sigma Chemical Company (St Louis, Mo). Reagents for gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, Calif).

Nuclear Protein Extraction

Sperm nuclear proteins were extracted from the cryopreserved semen aliquots as previously described (Aoki et al, 2005,2005). The P1/P2 ratio and P1 and P2 concentrations were subsequently quantified in each of the study subjects. All samples were run in duplicate, and the average P1 and P2 concentrations and P1/P2 ratio from the 2 runs were reported.

Prior to extraction, sperm cell concentrations were evaluated in order to quantify the protamine concentrations (ng/10^6 cells). Briefly, semen aliquots with a known number of sperm were centrifuged (500 x *g*, 5 minutes, 4 °C) and the pellet was washed in 1 mmol phenylmethylsulfonyl fluoride (PMSF). After centrifugation (500 x *g*, 5 minutes, 4 °C), the pellet was resuspended in 100 µL of Tris buffer (100 mmol) containing EDTA (20 mmol) and PMSF (1 mmol, pH 8.0). One hundred microliters of guanidine (6 M) and dithiothreitol (575 mmol) was added to the suspension, followed by the addition of sodium iodoacetate (200 µL, 522 mmol).

The suspension was protected from light, kept at room temperature (30 minutes), and mixed with 100% ethanol (1.0 mL, 4 °C). After centrifugation (12 000 x *g*, 10 minutes, 4 °C) the ethanol wash was repeated, and the pellet was resuspended in 0.8 mL of 0.5 mol HCl, incubated (15 minutes, 37 °C), and centrifuged (10 000 x *g*, 10 minutes). The supernatant was retained, and the nuclear proteins were precipitated by the addition of 100% trichloroacetic acid (TCA) to a final concentration of 20% TCA. The solution was incubated (4 °C, 5 minutes) and centrifuged (12 000 x *g*, 10 minutes). The pellet was washed twice in 500 µL 1% 2-mercaptoethanol in acetone, and the final pellet was dried and stored at −20 °C until gel electrophoresis analysis.

Protamine Protein Quantification

A highly purified human protamine standard was used to quantify the P1 and P2 concentrations as previously described (Aoki et al, 2005,2005). The P1 and P2 standard concentrations were calculated from the percent composition of each of the protamines multiplied by the total protamine concentration (determined using the RC DC protein assay kit, Bio-Rad).

Acetic-acid urea gel electrophoresis was used to evaluate the intensity of the P1 and P2 bands. A serial dilution of the standard (1.52, 0.76, 0.38, and 0.19 µg) was loaded in each gel, and a standard regression curve was generated, which afforded quantification of sperm protamine concentrations in the unknowns. The r^2 value of the regression curve was .96 or better for each gel run. Identity of P1 and P2 bands were verified using Western blot analysis as reported in a previous study (Carrell and Liu, 2001).

Immunofluorescence Microscopy and TUNEL Detection

Variations in P1 and P2 levels were assessed in individual spermatozoa using quantitative immunofluorescence microscopy. The terminal transferase dUTP nick end labeling (TUNEL) assay was used simultaneously to assess the relationship between sperm DNA damage and protamine levels (both globally and within individual cells, Figure 1). Semen samples were washed in PBS (0.1 M, pH 7.4), and the sperm pellet was smeared onto several precleaned glass slides and air-dried for a minimum of

1 hour (VWR Scientific, West Chester, Pa).

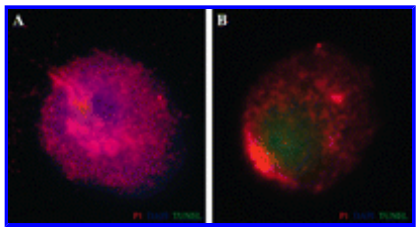

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Figure 1. Immunofluorescence micrographs showing simultaneous evaluation of individual sperm cell P1 levels and DNA damage via TUNEL assay. **(A)** TUNEL-negative mature spermatozoa with P1 immunostaining. **(B)** TUNEL-positive mature spermatozoa with P1 immunostaining.

To ensure antibody accessibility to the nuclear proteins, sperm nuclei were mildly decondensed by incubating the slides in 10 mmol DTT (0.1 mol Tris buffer) followed by a 2-hour incubation in a solution containing 10 mmol lithium diiodosalicylate (LIS) and 1 mmol DTT in 0.1 mol Tris buffer. Sperm cells were subsequently fixed (4% paraformaldehyde in 0.1 mol PBS, pH 7.4 for 1 hour) and permeabilized (2% Triton X-100 and 0.1% bovine serum albumin in 0.1 mol PBS for 15 minutes).

One set of slides was incubated overnight (20 °C) with a cocktail containing P1 (1:500) and transition protein 1 (1:1000) primary antibodies (HuP1N monoclonal mouse anti-human, kindly donated by Dr Rodney Balhorn, Livermore, Calif, and TP1 polyclonal rabbit anti-human, kindly donated by Dr Stephen Kistler, Columbia, SC). A second set of slides was incubated overnight (20 °C) with a cocktail containing P2 (1:500) and transition protein 2 (1:1000) primary antibodies (HuP2B monoclonal mouse anti-human, kindly donated by Dr Rodney Balhorn, and TP2 polyclonal rabbit anti-human, kindly donated by Dr Stephen Kistler). TP1 and TP2 were included as internal controls, since mature sperm would not be expected to exhibit transition protein immunofluorescence. Slides were washed in 0.1 mol PBS with 2% Triton X-100 (2 x 15 minutes) and treated with a secondary antibody cocktail containing Alexa Fluor 594 (goat anti-mouse, 1:1000), and Marina Blue (goat anti-rabbit, 1:1000; Invitrogen, Carlsbad, Calif). Slides were washed in 0.1 mol PBS with 2% Triton-X (2 x 15 minutes) and prepared for TUNEL detection using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany). Slides were cover-slipped using an anti-fade solution (25 µL) containing 25% glycerol and 125 mg/mL p-phenylenediamine in PN buffer.

White blood cells were used as negative controls for protamine and transition protein antibody staining. Human testicular tissue homogenates were used as a positive control for antibody staining. The homogenates were prepared as outlined above and evaluated for the presence of the protamine and transition proteins in mature spermatozoa as well as round and elongating spermatids (Figure 2). Negative controls were performed in the evaluation of DNA damage via the application of fluorescent marker without the TUNEL-reaction mixture (per manufacturer recommendations). Positive TUNEL controls were performed on DNAase-treated sperm. The TUNEL assay was also performed on samples before decondensation and antibody treatment to control for differential effects of sample preparation between cases and controls.

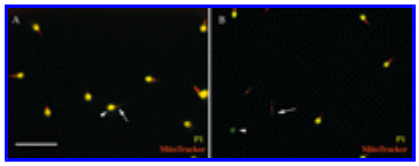

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Figure 2. MitoTracker and P2 immunofluorescence in **(A)** fertile donor sperm with normal protamine content and **(B)** patient sperm with globally-assessed P2 deficiency. **(A)** P2 localizes to the sperm head (arrowhead), while MitoTracker staining is present in the mid-piece of viable sperm cells (arrow). **(B)** The patient sample shows significant variability in P2 fluorescence intensity between individual sperm cells, with viable MitoTracker-positive cells (arrow) and nonviable MitoTracker-negative cells (arrowhead). Bar = 50 µm.

Immunofluorescence Quantification

Fluorescence intensity was quantitatively assessed using the Metafer 4.0 automated cell signal analyzer (MetaSystems, Altlussheim, Germany). An algorithm was developed that identified decondensed sperm nuclei based on cell size, circularity, and aspect ratio using the fluorescence emission at a wavelength of 594 nm. The fidelity of these criteria was verified by manual review of randomly selected data sets to ensure a majority of the mature sperm cells were included in analysis, while immature spermatozoa and amorphous cells were excluded.

Images were captured using the Metafer 4.0 software package, which subtracted background from total fluorescence intensity. Appropriate filter sets were used to measure the fluorescence intensities of the protamine, fluorescein (TUNEL), and transition protein staining (emission wavelengths of 594, 488, and 365 nm, respectively). The total area and mean and total fluorescence intensity of each cell were automatically recorded in approximately 1000 cells per patient. TUNEL results were recorded alongside the protamine and transition protein fluorescence intensities in individual cells. Cells were classified as TUNEL positive or negative based on a threshold fluorescence intensity determined by negative TUNEL control in each study sample.

Immunofluorescence Microscopy and Sperm Viability Analysis

In addition to DNA damage, sperm cell viability was evaluated (both globally and within individual sperm cells) simultaneously with protamine immunofluorescence quantification using MitoTracker staining (Figure 2; Invitrogen). Mito-Tracker fluorescence staining is dependent on the presence of a proton gradient in actively metabolizing mitochondria. Thus, it provides a convenient approach to assessing viability in sperm cells.

MitoTracker (500 nM) was incubated (30 minutes) with a washed suspension of sperm cells. Subsequently, the sperm cell suspensions were washed and a single drop (20 µL) was smeared on a plain glass slide. Two slides were prepared for each patient, one used for a negative control (no primary antibody) and the other for the treatment slide. After the slides were allowed to dry, the sperm were fixed for 1 hour using a 3.7% paraformaldehyde in 0.1 mol PBS (pH 7.4). The sperm were blocked and permeabilized overnight using 0.5% Triton-X in 0.1 mol PBS with 10% goat sera.

The slides were then incubated (6 hours) with primary affinity-purified mouse anti-human protamine antibodies (1:500; Dr Rodney Balhorn) and Alexa Fluor 488 goat antimouse IgG conjugate (1:1000, 1 hour; Invitrogen). Slides were washed (0.1% Triton-X in 0.1 mol PBS) and cover-slipped using an anti-fade solution (25 µL) containing 25% glycerol and 125 mg/mL p-phenylenediamine in PN buffer. Negative controls were performed using the same methods except for the exclusion of the primary antibody. Additionally, a negative control was prepared using white blood cells (which have no protamine proteins) following the same methods.

Individual sperm cells were evaluated for protamine fluorescence and graded as viable or nonviable.

Appropriate filter sets were used to evaluate protamine and MitoTracker fluorescence intensities (emission wavelengths of 488 nm for protamine and 594 nm for MitoTracker). Protamine immunofluorescence was quantified as outlined above. MitoTracker signals were classified as positive or negative based on threshold fluorescence intensity.

Statistical Analysis

Based on the P1/P2 ratio obtained from global assessments of the total sperm population, study subjects were stratified into 4 groups: fertile men, normal P1/P2 patients, low P1/P2 patients, and high P1/P2 patients. The values defining abnormally low (<0.8) and high (>1.2) P1/P2 ratios have been established in a previous report, which calculated these critical values from the 2-tailed normal distribution for the P1/P2 ratios of fertile donors with 90% confidence limits (Aoki et al, 2005,2005).

Mean P1 and P2 fluorescence intensities in the total sperm populations were compared between P1/P2 ratio groups using Kruskal-Wallis evaluation. Intraindividual variation in protamine content between individual sperm cells was assessed using coefficient of variation (CV) analysis for P1 and P2 fluorescence intensities. Mean CV values were compared between the P1/P2 ratio groups using a one-way ANOVA.

Mean levels of DNA damage and viability in the whole sperm cell populations were compared between P1/P2 ratio groups using Kruskal-Wallis evaluation. Intrastudy subject DNA damage and viability were compared between individual sperm cells in the lower 5% and upper 95% of P1 and P2 fluorescence intensities using a paired *t* test. The same comparison was conducted between individual sperm cells in the upper 5% and lower 95% of P1 and P2 fluorescence intensities.

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Protamine-1 and Protamine-2 Quantification

Nuclear protein extraction and gel electrophoresis resolved 10 fertile donors with normal P1/P2 ratios (0.92 ± 0.05), 14 patients with abnormally low P1/P2 ratios (0.43 ± 0.06), 29 with normal P1/P2 ratios (1.02 ± 0.02), and 26 with abnormally high P1/P2 ratios (1.62 ± 0.06). Mean P1 and P2 fluorescence intensities in the total sperm cell populations correlated well with population-based nuclear protein extraction quantification. The P1/P2 ratio resolved by these 2 techniques was statistically similar (1.11 ± 0.07 vs 1.03 ± 0.12, NS).

Mean P1 fluorescence intensities measured in the total sperm cell populations were slightly reduced in patients with low P1/P2 ratios (866 ± 84) vs fertile controls and patients with normal and high P1/P2 ratios (1116 ± 94, 1080 ± 93, 1000 ± 191, respectively). Mean P2 fluorescence intensity was slightly reduced in patients with elevated P1/P2 ratios (840 ± 85) vs fertile controls and patients with normal and elevated ratios (1140 ± 95, 1038 ± 76, 1086 ± 75, respectively).

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*Protamine fluorescence characteristics among P1/P2 ratio groups**

Significant variations in protamine fluorescence intensities (CV range 30% to 80%) were observed between individual sperm cells of study subjects (Table). The variation in individual sperm cell P1 and P2 fluorescence intensities was statistically similar between fertile men (P1-CV: 68.9 ± 3.9 and P2-CV: 64.5 ± 6.5), patients with normal P1/P2 ratios (P1-CV: 64.9 ± 4.8, P2-CV: 53.8 ± 4.3), patients with low P1/P2 ratios (P1-CV: 58.7 ± 6.0, P2-CV: 56.9 ± 3.9), and patients with high P1/P2 ratios (P1-CV: 64.9 ± 4.8, P2-CV: 53.8 ± 4.3, Table). Protamine fluorescence intensities among individual sperm cells were normally distributed in patients and fertile controls. Skew and kurtosis analysis verified that the shape of these distributions was similar between the fertile controls and patients in all 3 P1/P2 ratio groups (Table). Positive signals for TP1 and TP2 were not detected in any of the mature spermatozoa showing P1 or P2 fluorescence (Figure 3).

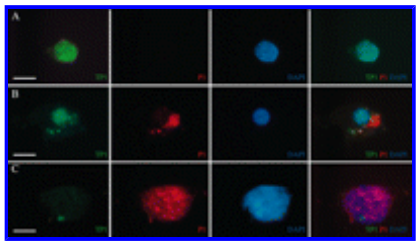

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Figure 3. Immunofluorescence micrographs showing P1 and TP1 expression in round and elongating spermatids. **(A)** Early round spermatid displaying TP1 nuclear localization without detectable P1 immunofluorescence. **(B)** Early elongating spermatid showing TP1 nuclear localization in concert with P1 cytoplasmic production. **(C)** Later elongating spermatid showing P1 nuclear localization with minimal residual TP1. Bar = 10 µm for **A**, **B**; 25 µm for **C**.

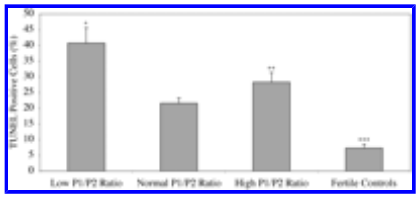

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Figure 4. Population-based DNA damage assessments within P1/P2 ratio groups. Mean TUNEL positive scores were significantly increased in patients with low P1/P2 ratios vs all other groups (*). Patients with high P1/P2 ratios had significantly elevated DNA damage vs fertile controls and patients with normal P1/P2 ratios (**). Fertile controls had significantly reduced TUNEL positive cells vs all patient groups (***). Values are expressed as mean ± SE.

The Relationship Between Sperm Protamine Levels, DNA Damage, and Viability

DNA damage (assessed in the total sperm cell populations) was significantly elevated in patients with low and high P1/P2 ratios (40.8 ± 4.9% and 28.3% ± 3.1% respectively) vs those with normal P1/P2 ratios (21.6 ± 1.7%, $P < .001$, Figure 4). This population-based DNA damage assessment showed an inverse correlation with the P1/P2 ratio (R_S −0.29, $P < .05$), P1 concentration (R_S −0.38; $P < .01$), P2 concentration (R_S −0.39, $P < .01$), and total protamine concentration (R_S −0.40; $P < .005$).

DNA damage was significantly elevated in individual sperm cells showing the lowest 5% of P1 and P2 fluorescence intensity (59.7 ± 2.2%) vs sperm cells in the upper 95% (20.9 ± 1.64%, $P < .05$, Figure 5A). This relationship was present within fertile controls (43.6 ± 5.2% vs 5.2 ± 1.3%, respectively), patients with normal P1/P2 ratios (59.5 ± 3.8% vs 17.8 ± 1.8%, respectively), patients with low P1/P2 ratios (62.2 ± 4.8% vs 37.6 ± 5.3%, respectively), and patients with high

P1/P2 ratios (62.3 ± 2.8% vs 23.7 ± 3.2%, respectively; Figure 5A).

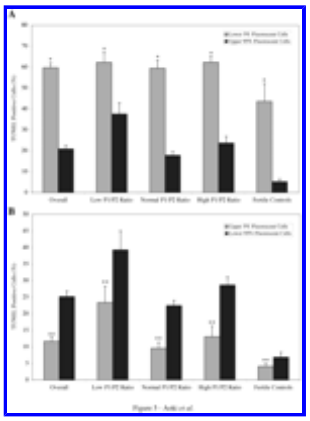

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Figure 5. Relationship between DNA damage and protamine levels in individual sperm cells. **(A)** Within all groups included in the study, TUNEL scores were significantly increased (*) in sperm cells with the lowest 5% of protamine fluorescence intensities vs those with the upper 95% of protamine signals. **(B)** TUNEL scores were significantly reduced (**) in sperm cells with the highest 5% of protamine fluorescence intensities vs those in the lower 95%.

Conversely, DNA damage was significantly reduced in individual sperm cells showing the highest 5% of protamine fluorescence intensity (11.7 ± 1.3%) vs the lower 95% of protamine fluorescent cells (25.2 ± 1.6%, $P < .005$, Figure 5B). This relationship was present within fertile controls (4.1 ± 0.9% vs 6.9 ± 1.5%, respectively), patients with normal P1/P2 ratios (9.6 ± 1.8% vs 22.5 ± 1.5%, respectively), patients with low P1/P2 ratios (23.4 ± 5.7% vs 39.3 ± 5.7%, respectively), and patients with high P1/P2 ratios (13.1 ± 0.9% vs 28.7 ± 2.4%, respectively; Figure 5B). Untreated samples (no decondensation or antibody application) showed slightly lower population-based DNA damage than treated samples. However, these differences were nonsignificant and comparable between fertile donors and patients with normal P1/P2 ratios, patients with low P1/P2 ratios, and patients with elevated P1/P2 ratios.

Population-based sperm cell viability assessments revealed a significantly reduced mean viability in the protamine-deficient infertility patients (41%) and normal-protamine patients (59%) vs fertile donors (75%; $P < .05$). Individual sperm cells displaying in the lowest 5% of total protamine fluorescence displayed reduced viability (41%) vs sperm cells in the upper 95% (59%, $P < .05$). However, no significant differences in sperm cell viability were observed between individual sperm cells within the upper 5% of protamine fluorescence vs those in the lower 95%.

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This is the first study to quantitatively assess protamine variations in individual sperm cells from fertile men and infertility patients with and without protamine deficiency. The data suggest that significant variations in P1 and P2 levels exist within sperm populations from individual men. These protamine levels significantly relate to the susceptibility of sperm cells to DNA damage induction and overall sperm cell viability.

During spermiogenesis, the nuclear chromatin undergoes significant remodeling (Hecht, 1988; Hecht, 1998; Fuentes-Mascorro et al, 2000; Aoki and Carrell, 2003; Dadoune, 2003). The protamine proteins facilitate these nuclear changes by replacing the somatic cell histones in a 2-step process

(Fuentes-Mascorro et al, 2000). In this study, the nuclear protein replacement sequence was elegantly demonstrated via protamine and transition protein immunofluorescence microscopy of human testicular cells. The first step occurs during the round-spermatid stage and involves replacement of the histones with the transition proteins. Later, during the elongating stage of spermatogenesis, the protamine proteins replace these transition proteins. An interesting aspect of these data is that the transition proteins do not appear to be retained in mature spermatozoa, even in patients diagnosed with sperm protamine deficiency.

The intrasample protamine heterogeneity observed in this study is consistent with other reports using chromomycin A_3 (CMA_3) and aniline blue staining to indirectly measure protaminization of human sperm cells (Manicardi et al, 1995; Hammadeh et al, 2001). Aniline blue selectively stains lysine-rich histone proteins, leaving the arginine-rich protamine proteins unstained (Hammadeh et al, 2001). CMA_3 directly competes with protamine for access to the DNA (Manicardi et al, 1995). Thus, high levels of protamine relate to low levels of aniline blue or CMA_3 staining. Results of these indirect staining experiments indicate that, within individuals, protamine levels may vary between individual sperm cells, exemplified by variations in aniline blue and CMA_3 staining within sperm cell populations (Bianchi et al, 1993; Manicardi et al, 1995; Manicardi et al, 1998; Hammadeh et al, 2001).

Intrasample protamine variability is of particular clinical significance for assisted reproductive techniques. Patients with protamine deficiency typically present with significantly diminished semen quality parameters, sperm functional ability, and sperm DNA integrity (de Yebra et al, 1993; de Yebra et al, 1998; Balhorn et al, 1999; Carrell and Liu, 2001; Aoki et al, 2005,2005). Due to diminished counts, motility, head morphology, and SPA scores, the majority of these infertility patients are treated using IVF in conjunction with ICSI (Aoki and Carrell, 2003; Aoki et al, 2005,2005).

Protamine-deficient patients undergoing human IVF/ICSI treatment have shown normal embryo quality, implantation, and pregnancy rates (Carrell and Liu, 2001; Nasr-Esfahani et al, 2004; Aoki et al, 2005,2005). These data are inconsistent with animal studies showing that mouse protamine haploinsufficiency directly impairs spermatogenesis and subsequent embryo development (Cho et al, 2001; Cho et al, 2003). However, in these mouse models the protamine deficiency was reported to be homogenous throughout the sperm cell population.

The heterogeneity in protamine levels reported in this study may serve to clarify how ICSI is able to overcome the diminished semen quality and sperm functional ability associated with sperm protamine deficiency in humans. The data indicate that, in patients diagnosed with protamine deficiency via global assessments, a small population of sperm cells is present with normal protamine content, which may be selected for injection during IVF/ICSI. Zhang et al (2006) have recently confirmed that semen samples with a low protamine level contain increased levels of histone 2B, and have confirmed their results using immunohistochemistry analysis of individual sperm. In their study, intrasample variation was observed, similar to our data, indicating focal regions of abnormal spermatogenesis. The sperm cell preparation techniques used in conjunction with IVF/ICSI, such as density gradient centrifugation, may be able to increase the relative concentrations of sperm cells with normal protamine content (Colleu et al, 1996; Sakkas et al, 2000). Additionally, the data indicate compromised viability in protamine-deficient sperm, which may further aid in positive sperm selection during ICSI. Further studies should evaluate protamine levels in fresh vs prepared semen samples. In addition, it will be important to evaluate variations in the P1/P2 ratio between individual sperm cells.

The data also suggest DNA damage is compromised in protamine-deficient human sperm. These results are consistent with other reports which document a relationship between sperm protamine levels and DNA integrity (Bianchi et al, 1993; Manicardi et al, 1995; Manicardi et al, 1998; Aoki et al, 2005,2005). Elegant studies in mice demonstrate protamine haploinsufficiency leads to increases in sperm cell DNA damage (Cho et al, 2003). In the present study, sperm cells with the lowest protamine levels were shown to have significantly increased DNA damage within individuals. Meanwhile, sperm cells with the highest protamine levels demonstrated significantly reduced DNA damage as measured by the TUNEL assay.

These results suggest that normally expressed sperm protamines may serve a protective function against DNA damage. Protamine-deficient sperm appear to be more susceptible to DNA strand breaks, evidenced by the significantly increased DNA damage in sperm cells with the lowest levels of protamine proteins. Sperm with low protamine levels retain higher levels of histone 2B, which may be less effective in protecting sperm DNA from damage (Zhang et al, 2006). Because a mild sperm chromatin decondensation protocol was used in this study, it is likely there was an artificial induction of DNA strand breaks. Indeed, DNA damage was slightly elevated postdecondensation. However, this treatment-induced increase in DNA damage was nonsignificant and comparable between all comparison groups, indicating that decondensation did not bias the DNA damage comparison between cases and controls.

To conclude, this is the first study to demonstrate that significant variations exist in the protamine levels of individual human sperm cells. These protamine variations relate significantly to sperm cell viability and DNA damage and may be of clinical significance, since infertile human males diagnosed with protamine deficiency via global assessments may possess a small population of cells with normal protamine content.

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