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Altered Protamine 2 Expression Is Uncommon In Donors of Known Fertility, but Common Among Men With Poor Fertilizing Capacity, and May Reflect Other Abnormalities of Spermiogenesis

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ABSTRACT: During the spermatid elongation stage of spermiogenesis approximately 85% of sperm nuclear histones are replaced by protamines. Protamines increase the packing ratio of sperm chromatin, presumably facilitating sperm motility and function. In this study we evaluated the incidence of abnormal protamine expression in 75 patients undergoing in vitro fertilization (IVF) and 50 donors of known fertility by isolation of sperm nuclear proteins, quantitative gel electrophoresis, and Western blot analysis. In addition, we evaluated the relationship between abnormal protamine expression and semen quality, sperm penetration ability, chromatin stability, and IVF outcome. Seventeen percent (13/75) of IVF patients had no measurable protamine 2 (P2) versus 0% (0/50) of donors of known fertility ($P < .005$). Sperm penetration rates were decreased in 12 of 13 patients without P2, and mean penetration rates (4.6 ± 1.2 vs 32.8 ± 2.9, $P < .005$), normal morphology (22.4 ± 3.6 vs 48.7 ± 4.2, $P < .05$), and progressive motility (22.3 ± 2.5 vs 35.4 ± 2.1, $P < .05$) were all significantly decreased compared with patients with measurable P2. The mean sperm concentration was not significantly different. The presence of protamine precursor bands was also associated with a diminished penetration capacity (18.4 ± 2.8 vs 36.7 ± 3.0, $P < .05$). Sperm chromatin decondensation following exposure to heparin sulfate was significantly increased in patients without a measurable P2 band. Twelve patients with no measurable P2 underwent intracytoplasmic sperm injection (ICSI), with 6 patients (6/12, 50%) becoming pregnant. ICSI fertilization and subsequent embryo cleavage were not different in patients without P2 compared with other patients undergoing ICSI. These data indicate that abnormal sperm protamine levels are a common defect in infertility patients, but not in donors of known fertility. It appears that abnormal protamine levels may reflect defects of late spermiogenesis, including sperm penetration capacity.

Key Words: Semen, sperm, chromatin, fertilization, IVF, infertility.
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Approximately 85% of human sperm nuclear histones are replaced by protamines during late spermiogenesis (Barone et al, 1994). Histone replacement is universal in higher organisms, and the human is the only species identified in which histones remain following protamine replacement. Protamines are small, highly positively charged proteins that increase the condensation and packing efficiency of sperm chromatin by forming disulfide bonds between the protamine cysteine residues, and toroidal units of chromatin structure (Hud et al, 1993; Khara et al, 1997). Sperm histone replacement by protamines may facilitate improved sperm motility, protect sperm DNA from damage, and may ''reset'' or ''imprint'' the male genome (Oliva and Dixon, 1991; Balhorn et al, 1999).

Two classes of protamines are present in human sperm. Protamine 1 (P1), which is present in all mammalian sperm, contains 50 amino acids and is rich in arginine and cysteine. Protamine 2 (P2) is a family of 3 proteins, all derived from a single gene, but varying in length due to variable amino terminus cleavage. P2 is reduced from 103 amino acids to 53 amino acids due to amino-terminus cleavage after the precursor protein binds to DNA (Balhorn, 1989). The P2 gene and proteins have been found in all mammalian species studied except the bull and boar. In those species the P2 protein is not present in sperm, presumably due to a mutation of the gene (Balhorn, 1989). The ratio of P1:P2 varies in each species, and in humans, is approximately 1.0 (Barone et al, 1994).

Silvestroni et al (1976) first reported that a sperm maturation defect resulting in incomplete replacement of nuclear histones might be associated with human infertility. Subsequent studies have reported that some infertile men have a diminished level of P2 in their sperm, and that the low level of P2 may be due to incomplete processing of P2 precursors (Chevaillier et al, 1987; Balhorn et al, 1988; Belokopytova et al, 1993; deYerba et al, 1993, 1998). We have previously reported that the sperm of some infertility patients demonstrate increased chromatin

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decondensation when exposed to culture medium containing heparin sulfate, and that the level of decondensation is inversely correlated with sperm fertilizing ability (Carrell et al, 1998). In this study we have evaluated the relationship between heparin-induced chromatin decondensation and levels of sperm protamines (P1:P2 ratio) in infertility patients and in donors of known fertility. In addition, we have evaluated in vitro fertilization (IVF) outcome and sperm protamine levels.

Materials and Methods

Materials

Unless otherwise noted, all chemicals were purchased from Sigma Chemical Company (St Louis, Mo). Hams F-10 was obtained from Gibco BRL Life Technologies (Grand Island, NY), and heparin sulfate was purchased from Wyeth-Ayerst (Philadelphia, Pa). Reagents for gel electrophoresis were from BioRad Laboratories (Hercules, Calif).

Evaluation of Semen

Institutional Review Board approval was obtained for all studies. Semen was evaluated from 50 donors of known fertility currently participating in an anonymous sperm donation program, and 75 patients preparing to undergo IVF. Patients in the IVF group were not all categorized as having ''male factor'' infertility; rather, their infertility had a variety of etiologies. A single semen sample was used for all diagnostic assays. A second sample was used for IVF.

Semen was initially evaluated using standard World Health Organization techniques for semen analysis, including sperm concentration, motility, and morphology (World Health Organization, 1999). A sperm penetration assay (SPA) was performed on an aliquot of the semen sample. Briefly, sperm were prepared by density gradient centrifugation and capacitated in Ham F-10 medium containing 10% heat-deactivated, pooled human sera for 4 hours at 37°C. A fraction of the initial semen sample was treated prior to density gradient centrifugation by refrigeration in TEST-yolk buffer containing 20% egg yolk. Both fractions of the patients' semen were tested independently using zona-free hamster ova as previously described (Carrell and Urry, 1997). Data were reported as the percentage of hamster ova penetrated by one or more sperm.

Purification of Nuclear Proteins

Sperm nuclear proteins were extracted using slight modifications of the previously described procedure (de Yerba and Oliva, 1993). Four hundred microliters of semen was mixed with 1.0 mL of Ham F-10 buffer, then centrifuged at 500 × *g* for 5 minutes at 4°C. The pellet was washed a second time in Ham F-10 medium. Forty million of the washed sperm were washed in 1 mM phenylmethylsulfonyl fluoride (PMSF) in distilled water to lyse the sperm membranes by osmotic shock, then resuspended in 100 μL of 100 mM Tris buffer containing 20 mM ethylenediamine tetraacetic acid and 1 mM PMSF (pH 8.0). One hundred microliters of 6 M guanidine and 575 mM dithiothreitol was added, followed by 200 μL of 552 mM sodium iodoacetate. The suspension was protected from light and kept at 4°C for 30 minutes. The suspension was mixed with 1.0 mL of ethanol at −20°C for 1 minute, then centrifuged for 20 minutes at 4°C. The supernatant was decanted and the ethanol wash was repeated. Following the second wash, the pellet was resuspended in 0.8 mL of 0.5 M HCl and incubated for 10 minutes at 37°C, then centrifuged. The supernatant was kept and 100% trichloroacetic acid (TCA) was added to a final concentration of 20% TCA, then cooled for 5 minutes at 4°C. The solution was centrifuged, and the pellet was washed twice in 500 μL of 1% 2-mercaptoethanol acetone. The final pellet was dried and stored at −20°C.

P1/P2 Quantification and Western Blot Analysis

Acetic urea gel electrophoresis was performed as described by Spiker (1980). The stacking gel was 7.5% acrylamide, 0.2% bisacrylamide, 2.5 M urea, and 0.375 M potassium acetate at pH 4.0. The running gel was 15% acrylamide, 0.1% bisacrylamide, 0.9 N acetic acid, and 2.5 M urea. The microgel was stained with Coomassie blue using standard techniques.

The gels were scanned using Adobe Photoshop 4.0 software. The bands corresponding to P1 and P2 were measured using National Institutes of Health Image 1.61/ppc software, and the data were used to calculate the relative P1:P2 ratios. Unstained gels from 15 patients were analyzed by Western blot using standard techniques. Previously developed and analyzed anti-P1 and anti-P2 monoclonal antibodies (Hup 1M and Hup 2B) were generously provided for these studies (Stanker et al, 1993). The secondary antibody was conjugated with alkaline phosphatase.

Sperm Chromatin Decondensation Assay

Sperm chromatin stability was evaluated in 42 patients and 15 donors by measuring the decondensation of sperm chromatin after exposure to heparin sulfate (Carrell et al, 1998). Semen was washed in Ham F-10 medium, then resuspended in 1.0 mL of medium containing 50 USP/mL of heparin sulfate. The suspension was incubated at 37°C for 60 minutes, then washed in heparin-free medium. A smear of the sperm suspension was made on a clean microscope slide and dried at 37°C. The slide was then stained using standard hematoxylin/eosin staining procedures. Two hundred sperm were observed from each sample using bright-field microscopy at a magnification of 1000×. Sperm were classified as either condensed or decondensed and the data were reported as the percentage of sperm undergoing decondensation. Sperm decondensation was considered increased if >8% of sperm decondensed.

In Vitro Fertilization

Ovarian stimulation was performed using standard gonadotropin-releasing hormone agonist down-regulation and gonadotropin stimulation of the ovaries. Oocytes were obtained using ultrasound-guided, transvaginal aspiration. The oocytes were incubated in human tubal fluid culture medium supplemented with 15% heat-deactivated maternal sera for 4 hours prior to sperm insemination. Insemination and culture were performed using standard ''microdrop'' techniques. Transfer of 2–3 embryos was performed 72 hours after oocyte retrieval.

Intracytoplasmic sperm injection (ICSI) was performed in cas-

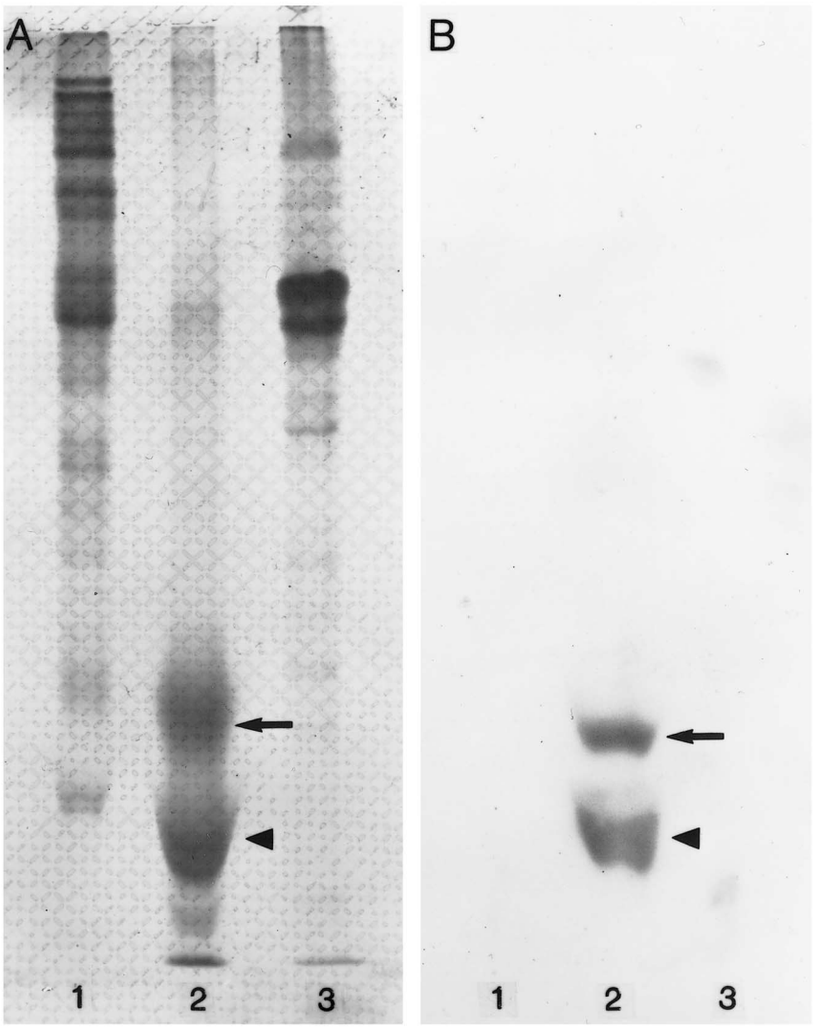

Figure 1. **(A)** Coomassie-stained gel electrophoresis results of purified nuclear proteins from white blood cell (WBC) control (lane 1), donor sperm (lane 2), and abnormal patient sperm (lane 3). Low-mobility histones are visible in lane 1 in increased relative quantities, whereas P1 (arrow) and P2 (arrowhead) are shown in lane 2. **(B)** Western blot of unstained duplicate lanes 1–3 demonstrates the specificity of P1 and P2 bands for binding to monoclonal antibodies to those protamines.

es in which the sperm penetration assay was diminished (<15% penetration) in both the standard and refrigerated aliquots of evaluated samples. ICSI was performed using standard techniques at 4 hours after oocyte retrieval.

Statistical Evaluation

Comparisons between the patient and donor groups were performed using analysis of variance. Chi-square analysis was used to evaluate the differences in the incidence of abnormal protamine expression. All statistical descriptions and tests were performed using Statview software for the Macintosh.

Results

Thirteen infertility patients had no measurable P2. Five of those 13 had fewer mobile bands observed on the acid-urea gels (Figure 1). The mean P1:P2 ratio for the other 62 patients was 1.12 ± 0.04 (range = 0.63–2.4), compared with a mean P1:P2 ratio of 0.83 ± 0.05 (range = 0.53–0.99) for the donor group. These values were significantly different ($P < .01$). The distribution of P1:P2 ratios for donors was within a much tighter range than the distribution of patient ratios (Figure 2). Fourteen patients with measurable P2 had more low-mobility bands on the gel. The low-mobility bands (possible precursor bands) were not observed in any of the 50 donor samples analyzed. Some patients had lower levels of P1 observed relative to the precursor bands.

Western blot analysis confirmed the identity of P1 and P2 bands. Western blot analysis also showed that the less-mobile bands were not immunoreactive with the monoclonal antibodies for P1 and P2.

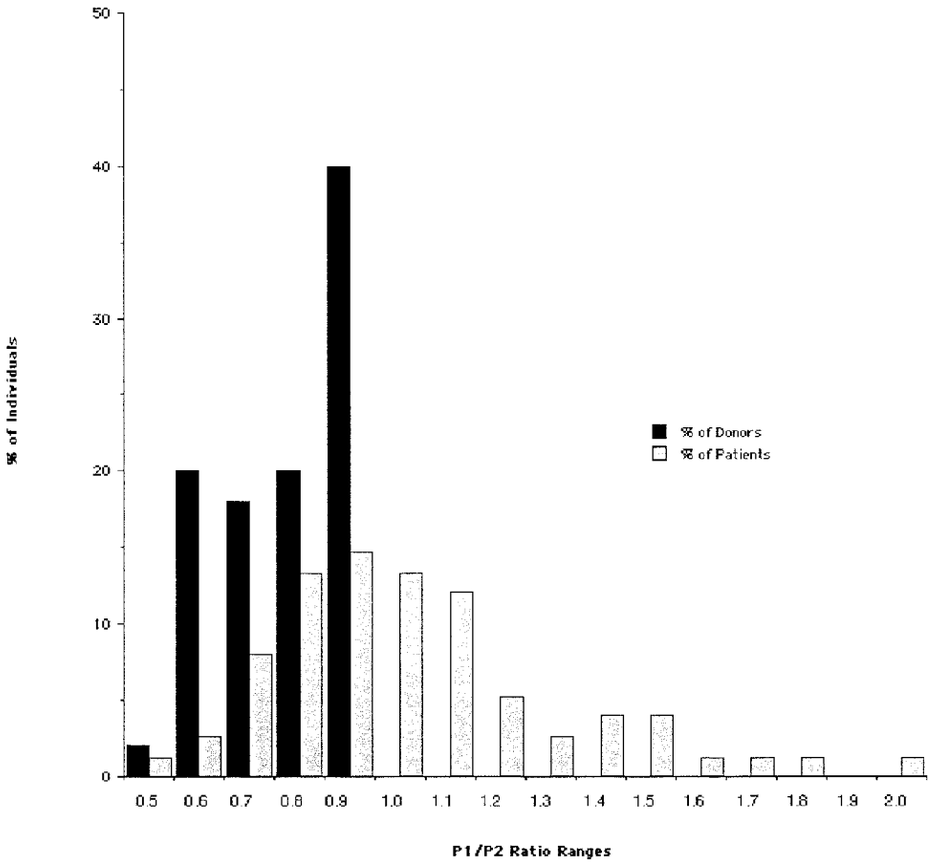

Figure 2. The percentage of individuals from the patient and control groups within given P1:P2 ranges.

The mean SPA penetration score for patients with no measurable P2 was 4.6 ± 1.2 (range = 0–25) compared with 32.8 ± 3.9 for patients with measurable P2 ($P < .005$). Significant differences were also observed between patients with or without P2 bands in the percentage of morphologically normal heads ($P < .01$) and the percentage of progressively motile sperm ($P < .05$), but not in the sperm concentration (Table 1). Twelve of the 13 patients with no measurable P2 underwent IVF with ICSI. The fertilization rate was 74.8%, and embryo morphology was not different than other ICSI patients (Table 2). The clinical pregnancy rate was 50% (6/12). The single patient without measurable P2, who did not undergo ICSI, had a normal SPA rate of 25% penetration. In that patient, fertilization was diminished (20%, 1/5), so rescue ICSI was performed (4/4 fertilized). The patient did not become pregnant.

Patients with measurable P2 and increased low-mobility bands compared with the donor group had a significantly ($P < .05$) decreased SPA rate (18.4 ± 2.8) than those without the low-mobility bands (36.7 ± 3.0; Figure 3). There was no difference in sperm concentration, morphology, or progressive motility (Table 1).

No significant differences were observed in any IVF outcome for the patients included in this study compared with the general IVF outcome at our laboratory. Using SPA to indicate the need for ICSI, 93% (12/13) of patients without P2 underwent ICSI compared with 9.6% (6/62) of patients with measurable P2 ($P < .05$). Six of 14 (42.8%) patients with measurable P2 but increased precursor bands had an abnormal SPA rate compared with 0% (0/48) of patients in which P2 and precursors were both normal ($P < .01$).

The mean percentage of sperm undergoing heparin-induced decondensation was 4.5 ± 0.9 for donor sperm vs 18.6 ± 3.7 for the patients ($P < .01$). Sperm decondensation was increased (>8% reacted) in 75% (9/12) of patients without a P2 band and in 6.6% (2/30) of patients with normal P2 bands ($P < .01$). Decondensation was increased in 1 of 3 patients (33%) with a normal P2 band but increased protamine precursors.

Discussion

Protamines are ubiquitous among higher organisms, but the pattern of expression in sperm varies. Protamine 2

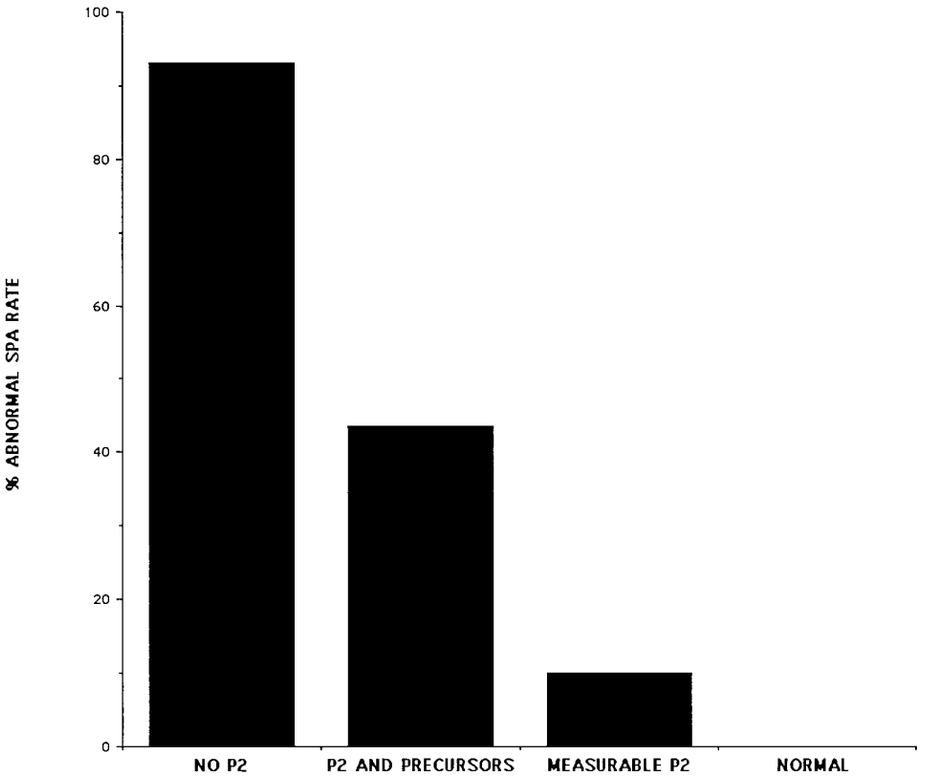

Figure 3. Relationship of P2 expression and penetration ability as measured by SPA.

expression is particularly variable. In humans, P1 and P2 are found in roughly equal quantities in mature sperm. Both protamines are transcribed in the round spermatid stage, then undergo polyadenylation and storage for 1 week before translation. Both P1 and P2 bind to chromatin in their initially translated protein form, however, following binding to the DNA, the 103-amino acid precursor of P2 protein undergoes modification to a 63-amino acid protein. Some P2 undergoes a further cleavage to a 57-amino acid protein. While protamines appear to be necessary for the increased packaging of chromatin required in sperm, the wide variations of protamine expression seen throughout nature indicates that the packaging can be accomplished in numerous manners.

Previous studies have shown that the mean P1:P2 ratio in humans is approximately 1.0 (Balhorn et al, 1999). However, some infertile men have been shown to have increased levels of histones or nondetectable P2 in mature sperm. De Yerba et al (1998) have reported increased protamine precursor in some men who lack P2. Khara et al (1997) have reported that a negative correlation was observed between P1:P2 ratios and IVF rates in 12 patients analyzed, and a negative relationship of P1:P2 ratios and sperm motility in a second group of patients. Our

Table 1. *Semen quality of patients with and without measurable P2 and low-mobility P2 precursor bands*

Group*	n	SPA Rate, %	Normal Morphology, %	Progressive Motility, %	Concentration, Million/mL
1 (No P2)	13	4.6 ± 1.2†	22.4 ± 3.6‡	22.3 ± 2.5‡	45.8 ± 7.4
2 (P2)	62	32.8 ± 2.9	48.7 ± 4.2	35.4 ± 2.1	51.6 ± 4.9
2A	14	18.4 ± 2.8§	44.6 ± 4.1	33.6 ± 2.8	53.7 ± 6.8
2B	48	36.7 ± 3.0	49.9 ± 5.1	35.9 ± 1.9	50.8 ± 4.7

* Group 1 is patients without a measurable P2 band. Group 2 patients have measurable P2. Group 2A and 2B patients have measurable P2, but increased (2A) and no (2B) low-mobility bands immunoreactive for protamines. All data are expressed as Mean ± SE. P2 indicates protamine 2; SPA, sperm penetration assay.

† $P < .005$ compared to group 2.

‡ $P < .05$ compared to group 2.

§ $P < .05$ compared to group 2B.

Table 2. *Fertilization, cleavage, and pregnancy during IVF**

Group	n	% Fertilization (Mean ± SE)	% Cleavage (Mean ± SE)	% Pregnant
No P2 (ICSI)	12	74.8 ± 4.8 (29–100)	97.4 ± 1.7 (87–100)	50 (6/12)
Measurable P2 (All)	63	69.4 ± 3.8 (40–100)	98.9 ± 1.0 (83–100)	49.2 (31/63)
Measurable P2 (ICSI)	9	71.1 ± 4.9 (60–100)	98.6 ± 0.9 (90–100)	55.5 (5/9)

* IVF indicates in vitro fertilization; ICSI, intracytoplasmic sperm injection.

study has demonstrated that abnormal P1:P2 ratios are relatively common in patients undergoing IVF, but they were not observed in 50 men of known fertility. In addition, the mean P1:P2 ratio was significantly increased in patients compared with donors of known fertility, with a ratio of less than 1.0 (0.83 ± 0.05) in fertile men and greater than 1.0 (1.12 ± 0.04) in the patient group.

Protamine defects were most common in patients with diminished penetration capacity. The SPA rate was abnormal in 12 of 13 patients without measurable P2. The 13th patient with no measurable P2 had a normal SPA, but low fertilization using microdrop insemination. Second, those patients with no P2 but increased P2 precursors had a significantly diminished SPA rate. It is unlikely that the altered protamine expression is directly related to, or causative of, diminished fertilization ability. Rather, it is likely that it is indicative of abnormal, late-stage spermiogenesis in general. Histone/protamine replacement is a late-spermiogenesis event, along with acrosome formation, membrane remodeling, and other significant morphological and biochemical events that are necessary for normal sperm function. It is likely that altered protamine expression is associated with a wide range of spermiogenesis defects. Further studies will define the accuracy of protamine defects in predicting abnormal penetration capacity, but it is intriguing that in this study, all patients with normal P2 bands and no increase of P2 precursors had a normal SPA.

Altered protamine levels did not affect sperm decondensation following ICSI, or subsequent embryo growth, implantation rates, or pregnancy rates. The mechanism of decondensation and protamine replacement with histones is not well understood, but it appears that decondensation and pronuclear formation proceed adequately when P2 is not present. Second, replacement of sperm histones with protamines has been hypothesized as a possible mechanism of ''resetting'' the paternal genome for embryogenesis (Balhorn et al, 1999). This study indicates that P2 is not necessary if a ''resetting'' does occur.

Previous studies have not shown any mutations in the P1 and P2 genes of patients with abnormal expression of protamines (Balhorn et al, 1999). It is possible that the variations in expression observed in infertility patients may be due to ''uncoupling'' of the expression of P1 and P2 genes (Balhorn et al, 1988; Hecht, 1999). It is also possible that the posttranslation events, such as polyadenylation, or posttranslational cleavage events may be responsible for the variations of protamine expressions observed.

In conclusion, this study has demonstrated that abnormal protamine levels in sperm are a relatively common event in infertile patients, but it has never been observed in a control group of fertile donors. P1:P2 ratios are generally increased in infertile patients compared with fertile controls, and decreased sperm penetration capacity is associated with altered P2 expression. There appears to be a strong relationship between abnormal protamine levels and late spermiogenesis defects, particularly diminished fertilization ability.

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