

Fine resolution of human sperm nucleoproteins by two-dimensional electrophoresis

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Human sperm nucleoproteins consist of protamines and histones. Changes in composition of these proteins are thought to correlate with spermatogenesis and may be involved in some instances of male infertility. We sought to separate sperm nucleoproteins including variants of protamine using an improved two-dimensional electrophoretic method, with the aim of comprehensively analysing all sperm nucleoprotein constituents. After extracting nuclear basic proteins from the sperm of normal volunteers, we analysed these proteins on a gel sheet by a radical free, highly reducing method based on Kaltschmidt and Whittmann's two-dimensional electrophoresis. Basic proteins from sperm nuclei were separated clearly into 12 spots. By amino acid sequence analysis, these spots corresponded to protamine 1 (P1)- (five spots), protamine 2 (P2)-related proteins (six spots) and testis-specific histone H2B (one spot). The N-terminal amino acid sequences of the six P2-related proteins were compatible with those of HPI1, HPI2, HPS1, HPS2, HP2 and HP3, and quantitative comparison could be performed. In conclusion, human sperm nucleoproteins including all P2-related variants could be analysed quantitatively with high resolution on a single electrophoretic gel.

Key words: histone/human/nucleoprotein/protamine/spermatozoa

Introduction

Several groups of investigators have suggested that a sperm maturation defect resulting from incomplete replacement of nuclear histones might be associated with human infertility. In sperm from fertile men, protamine 1 (P1) and protamine 2 (P2) can be found at a ratio of approximately 1:1 (Balhorn *et al.*, 1988); in contrast, various studies have reported changes in relative amounts of P1 and P2 between fertile and infertile men (Chevaillier *et al.*, 1987; Balhorn *et al.*, 1988; Bach *et al.*, 1990; Blanchard *et al.*, 1990; Belokopytova *et al.*, 1993; de Yebra *et al.*, 1993; Bench *et al.*, 1998; de Yebra *et al.*, 1998) or in patients pursuing IVF programs (Colleu *et al.*, 1997; Khara *et al.*, 1997; Carrell and Liu, 2001; Steger *et al.*, 2001, 2003). Subsequent studies have reported that some infertile men have diminished amounts of P2 in sperm that may reflect incomplete processing of P2 precursors (de Yebra *et al.*, 1998).

In the above studies, separation of sperm nucleoproteins containing protamine had been carried out using one-dimensional (1-D) polyacrylamide gels (de Yebra and Oliva, 1993). However, 1-D electrophoresis has the drawback of overlaps between some protein fractions, resulting in insufficient separation of protamine variants or forms of protamine post-translationally modified by acetylation or phosphorylation. No reports have delineated human sperm nucleoprotein composition including all P2-related variants based upon isolation on a single gel sheet. Non-equivalent two-dimensional (2-D) electrophoresis analysis is appropriate to this task; a single spot corresponds to a single protein. Separating sperm nucleoproteins in this manner would provide a more complete and accurate view of changes in composition of these proteins associated with male infertility. To our knowledge, however, many studies concerning sperm nucleoproteins have not yet made use of 2-D electrophoresis.

Precise separation of sperm nucleoproteins has been difficult because they are highly basic, rich in disulphide bridges, and very small. With an improved method of the radical-free, highly reducing (RFHR) 2-D electrophoresis developed in this laboratory (Wada, 1986a,b), quantitative yield and separation ability for such proteins have improved greatly. Disulphide bridging is blocked by maintaining the gels under highly reducing conditions, whereas artefactual modification of proteins by free radicals in the gels is avoided by thorough scavenging. In this study, we sought to separate sperm nucleoproteins with high resolution on one electrophoretic gel sheet to quantitatively analyse histones, P1, P2 and their precursors.

Materials and methods

Samples

Semen samples were collected from male volunteers demonstrated to be fertile by a history of producing offspring. Semen ejaculates obtained at the hospital by masturbation were kept at 37°C until complete liquefaction had occurred. Standard semen analysis (semen volume, sperm concentration, total sperm count and progressively motile sperm concentration) was performed within 1 h of obtaining the sample. Spermatozoa numbering 50×10^6 per volunteer were washed three times with M199 (Invitrogen, Carlsbad, CA) (using 10 min of centrifugation at 1600 g each time) and twice with phosphate-buffered saline (PBS; using 5 min of centrifugation at 1600 g each time). Pellets were then kept frozen (–80°C) until use.

Extraction of nuclear proteins

Sperm nuclear basic protein was extracted according to the method described by Balhorn *et al.* (1977); with minor modification. Briefly, the sperm pellet

was suspended with minimal sonication in 0.9 ml of 10 mM dithiothreitol (DTT) and 0.05 M trisaminomethane-HCl (Tris-HCl) at pH 8.0 for incubation at 4°C for 15 min. Cetyltrimethylammonium bromide (CTAB) was added to obtain a final concentration of 1%, and the sample was incubated for an additional 30 min at 4°C to allow dissolution of sperm tails. The heads were then pelleted by centrifugation at 3000 g, washed twice in 1% CTAB in 0.01 M Tris-HCl at pH 8.0, and then washed twice in Tris-saline. The purified sperm nuclei were dissolved in 0.1 ml of 5 M guanidine hydrochloride (Gdn-HCl)-0.01 M Tris at pH 8.0, in which the chromatin was allowed to expand at 4°C for 30 min. After sonication, the chromatin was diluted and urea, 2-mercaptoethanol (MSH), and sodium chloride (NaCl) were added to give final concentrations of 0.5 M Gdn-HCl, 3 M urea, 0.5 M MSH and 2 M NaCl. Following dissociation of the proteins at 4°C for 60 min, DNA was precipitated by the addition of HCl to a final concentration of 0.5 N. After precipitation at 4°C for 60 min, the DNA was pelleted by centrifugation at 14 500 g for 10 min. The nucleoproteins were precipitated from the appropriate pooled fractions with 20% trichloroacetic acid at 4°C for 30 min. The precipitate was then pelleted by centrifugation at 14 500 g, washed with acidified acetone, and dried in vacuo and stored at -30°C until 2-D gel analysis. As controls, nucleoproteins of HeLa cells were also extracted in the same manner as above except for the process of dissolution of sperm tails.

Electrophoresis

Extracted nucleoproteins (approximately 0.5 mg/gel) were analysed by the RFHR method of 2-D gel electrophoresis as previously described (Wada, 1986a,b), except that 6 M urea was replaced with 7 M urea, the acrylamide concentrations of the 0-D and 1-D gels were lowered from 8 to 7.2%, and gels 2-mm thick were used in place of 3-mm gels. Apparatus for RFHR 2-D electrophoresis was obtained from Nippon Eido (Tokyo, Japan).

Protein densitometry

Experiments using 2-D electrophoresis for the determination of the relative protamine amounts were carried out in two separate runs using the mixture of five samples. Coomassie Brilliant Blue (CBB) R-250 was used to stain the gel, which then was scanned using a flatbed scanner (Amersham Biosciences, Uppsala, Sweden). Preprogrammed spot quantification was performed using Image Master 2D Elite software (version 2.0, Amersham Biosciences). Specifically, spot detection, spot measurement and background subtraction were performed. Following automatic spot detection, gel images were carefully edited. The quantity represented by a protein spot was expressed as the volume of the spot, which was defined as the sum of the intensities of all pixels making up the spot. To correct for variability of CBB staining and to reflect the quantitative variations in intensity of protein spots, spot volumes were normalized for all spots present in the gel.

Amino acid sequence analysis

Following separation by electrophoresis, proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Pall Bio Support Division, Port Washington, NY) using a semidry transfer blotter (Nippon Eido) and were detected by CBB staining. Stained protein spots were excised from the PVDF membrane and applied to the upper glass block of the reaction chamber in a gas-phase protein sequencer (Procise 494, Applied Biosystems, Foster City, CA, USA). Edman degradation was performed according to the manufacturer's standard program and released phenylthiohydantoin derivatives of amino acid residues were identified by on-line high-performance liquid chromatography (120A; Applied Biosystems). Amino acid sequences obtained were compared with those of known proteins with the web-accessible search program FastA (<http://www.dna.affrc.go.jp>).

Results

In each semen specimen, sperm nuclear basic proteins were separated by RFHR 2-D electrophoresis with high reproducibility. Typical separations of nucleoproteins isolated from human sperm (A) and somatic (HeLa) cells (B) are shown in Figure 1. Based on a comparison of the electrophoretic pattern with that of the somatic cells, which consists of only histone without protamines, one spot was recognized in a position similar to that of the histone of HeLa cells. Several additional

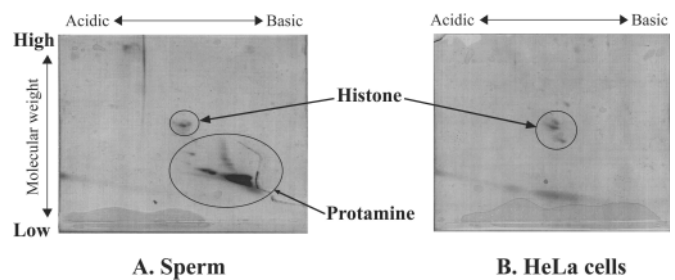


Figure 1. Gel patterns of nucleoproteins compared between sperm and HeLa cells. A typical electrophoretic analysis of the nuclear proteins extracted from human sperm (A) and HeLa cells (B) by the RFHR two-dimensional electrophoresis method is shown. In contrast to the electrophoretic pattern for somatic (HeLa) cells, which includes only histone without protamines, sperm showed spots similar in position to histones of HeLa cells but also smaller, more basic spots thought to be protamines.

spots, which were more basic and of lower molecular weight, appeared in the sperm nucleoprotein pattern; these were thought to represent protamines.

Sperm nucleoproteins were separated into 12 spots. Amino acid sequence analysis showed that these spots corresponded variously to P1-family proteins, P2-related proteins, and testis-specific histone H2B (TH2B; Figure 2, Table I). Protamines in human sperm nucleoproteins resolved well on the gels, located essentially along respective diagonals representing two groups, group P1 and group P2 (Figure 2). Group P1 represented P1-family proteins and was composed of five spots with the same N-terminal amino acid sequence (ARYRXXR; Table I); the amino acid corresponding to 'X' could not be determined by Edman degradation and may represent the cysteine residues of P1. Group P2 consisted of six spots. These proteins corresponded to HPI1, HPI2, HPS1, HPS2, HP2 and HP3 respectively, because the N-terminal amino acid sequences were compatible with differences in N-termini between the reported testicular P2 variants (Table I). All P2-related molecular forms including P2 and its precursors were separated clearly from one another. Such resolution was highly reproducible under the conditions of electrophoresis described here.

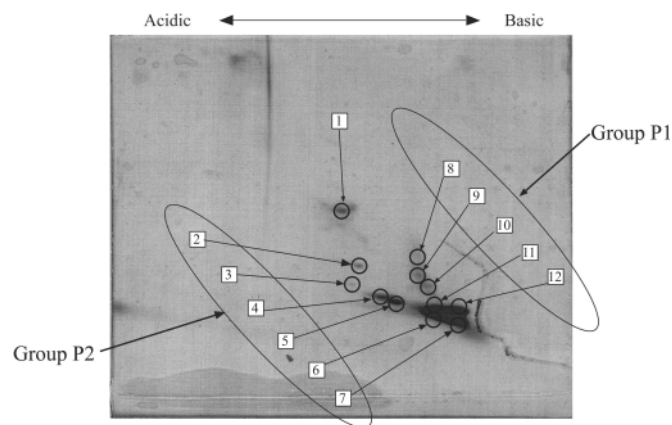


Figure 2. Sperm nucleoproteins spots analysed by radical-free, highly reducing (RFHR) two-dimensional electrophoresis. Human sperm nucleoproteins were separated into 12 spots with high reproducibility. Protamines in human sperm nucleoproteins were resolved distinctly, located essentially along two diagonals representing protamine 1 (P1) and protamine 2 (P2) groups. P1 consisted of five P1 forms, whereas group P2 consisted of six P2-related proteins (HPI1, HPI2, HPS1, HPS2, HP2 and HP3). All P2-related proteins, representing HP2, HP3 and their precursors, were separated clearly from each other.

Table 1. Amino acid sequences for each spot

Spot	N-terminal amino acid sequence	Protein
1	XEVSSKGATISXKGF	TH2B (testis-specific histone H2B)
2	VRYRVRSLERSHEV	HPI1 (human protamine 2)
3	HGQEQ	HPI2 (human protamine 2)
4	QGLSP	HPS1 (human protamine 2)
5	SPEHVEVYERTHGQ	HPS2 (human protamine 2)
6	RTHG	HP2 (human protamine 2)
7	GQSHYRRR	HP3 (human protamine 2)
8	ARYR...	Human protamine 1
9	ARYR...	Human protamine 1
10	ARYR...	Human protamine 1
11	ARYRXXR...	Human protamine 1
12	ARYR...	Human protamine 1

The five protamine 1 (P1) spots were found to have the same N-terminal amino acid sequence (ARYRXXR). The amino acid 'X' could not be determined by Edman degradation and may correspond to the cysteine residue of P1. Differing locations of these five spots presumably represent differences in posttranslational modification

Human protamine 1 (50 aa)

ARYRCRSQSRSRYRQRQRSSRRRRSSCQTRRRAMRCCRPRYRPCRHH
 ARYRXXR

Human protamine 2 (102 aa)

SPEHVEVYERTHGQ
 #5, 37-, HPS2
 MVRYRVRSLERSHEVYRQQLHGQEQGHGQEEQGLSPEHVEVYERTHGQSHYRRRHC...
 #2, 2-, HPI1 | #3, 22-, HPI2 | #4, 34-, HPS1 | #6, 46-, HP2
 VRYRVRSLERSHEV HGQEQ QGLSP RTHG
 #7, 49-, HP3
 GQSHYRRR

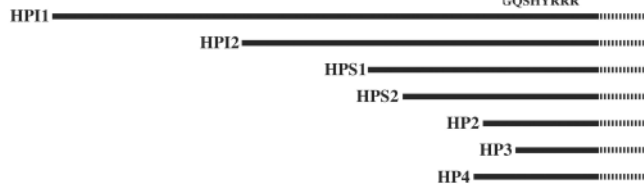


Figure 3. Amino acid sequence of each spot separated by RFHR two-dimensional electrophoresis in comparison with reported human protamine sequences. Amino acid sequences of protamines reported in previous studies and protamines analysed in this study are shown. Amino acid sequences of protamine 2 (P2)-related proteins corresponded to portions of the *prm2* gene (accession number p04554) sequence. HPI1, HPI2, HPS1, HPS2, HP2 and HP3 corresponded to segments 2-, 22-, 34-, 37-, 46-, and 49- of *prm2*. HP4 (45- of *prm2*), not detected in this study, was thought to be extremely low in quantity.

Amino acid sequences of human protamines reported in the UniProt database (<http://www.ebi.uniprot.org/index.shtml>) and previous studies (McKay *et al.*, 1985, 1986; Ammer *et al.*, 1986; Gusse *et al.*, 1986) and the protamines that we analysed in this study are shown in Figure 3. The amino acids of these P2-related proteins corresponded well in sequence to the gene *prm2* (accession number p04554). Specifically, N-terminal amino acid sequence of HPI1, VRYRVRSLERSHEV, corresponded to residues 2- of *prm2*, that of HPI2, HGQEQ, corresponded to residues 22- of *prm2*, that of HPS1, QGLSP, corresponded to residues 34- of *prm2*, that of HPS2, SPEHVEVYERTHGQ, corresponded to residues 37- of *prm2*, that of HP2, RTHG, corresponded to residues 46- of *prm2*, and that of HP3, GQSHYRRR, corresponded to residues 49- of *prm2*. HP4 (45- of *prm2*), not detected in this study, is thought to be present only in extremely small quantities.

Densitometry results obtained by averaging the quantitation from two gel samples are shown in Figure 4. Relative amount of TH2B extracted in this study was 282.6 as compared to the total amount of analysed nucleoprotein spots, 7364.8. Even considering the amount of

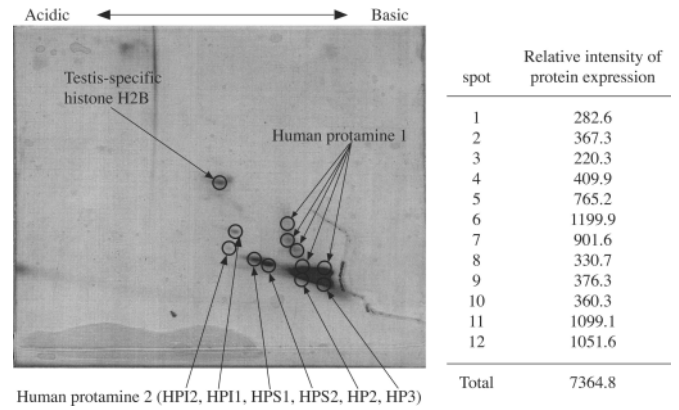


Figure 4. Protein expression level of protamine 1 (P1) and protamine 2 (P2). After Coomassie Brilliant Blue (CBB) staining, the gel was scanned and pre-programmed spot quantification was performed using Image Master 2D Elite software. Relative quantities averaged from two gel samples were calculated. The ratio P1/P2, measured as in previous studies, was 1.02, representing good agreement. In addition, the relative quantity of all P2-related forms (HPI1, HPI2, HPS1, HPS2, HP2 and HP3) was determined as 1.000, 0.6000, 1.116, 2.083, 3.267 and 2.455, respectively.

several small spots near the TH2B spot, the total amount of histone analysed in this study accounted for less than 15%, of the previously reported quantity (Gatewood *et al.*, 1987). In addition, the ratio of P1 to P2, measured in the same manner as in the previous 1-D electrophoresis study that seemed not to recognize spot 8, spot 9 or spot 10 in our study, was $HPI1/(HP2+HP3) = (\text{spot } 11 + \text{spot } 12)/(\text{spot } 6 + \text{spot } 7) = (1099.1 + 1051.6)/(1199.9 + 901.6) = 1.02$. The value of P1/P2 (1.02) overlapped greatly with ratios reported in a study concerning fertile men (Balhorn *et al.*, 1988). Relative quantification of all P2-related forms, HPI1:HPI2:HPS1:HPS2:HP2:HP3 resulted in 367.3/367.3:220.3/367.3:409.9/367.3:765.2/367.3:1199.9/367.3:901.6/367.3 = 1.000:0.6000:1.116:2.083:3.267:2.455.

Discussion

Approximately 85% of human sperm nuclear histones are replaced by protamines during late spermatogenesis (Barone *et al.*, 1994) accompanied by a dramatic change in the structure of chromatin (Fuentes-Mascorro *et al.*, 2000; Brewer *et al.*, 2002). In humans, sperm nuclear proteins include two families of protamines, P1 and P2, which differ in molecular mass, amino acid composition and sequence. The P1 family is represented by HPI1 with different degrees of phosphorylation and is related to other mammalian P1 protamines. The P2 family includes HP2, HP3 and HP4 proteins, which differ only by an amino-terminal extension of one to four residues (Gusse *et al.*, 1986; McKay *et al.*, 1986; Sautiere *et al.*, 1988; Arkhis *et al.*, 1991; Oliva and Dixon, 1991; Chauviere *et al.*, 1992; Bianchi *et al.*, 1994). HPI1, HP2, HP3 and HP4 have 50, 57, 54 and 58 amino acids, respectively (Figure 3). HPI1 is synthesized as a mature protein product. In contrast, P2 family proteins are synthesized initially as a precursor protein of 102 amino acids (Balhorn, 1989; Alimi *et al.*, 1993a) directed by a single-copy gene located on chromosome 16 in humans (Domenjoud *et al.*, 1990). P2 family proteins attain mature form by proteolytic cleavage of the amino terminus (Balhorn, 1989). 1-D polyacrylamide gel electrophoresis has detected minor components of human sperm nucleoproteins showing a rate of migration that is intermediate between those of histones and protamines (Sautiere *et al.*, 1988). These proteins therefore have been called intermediate basic proteins, designated HPI1, HPI2, HPS1 and HPS2 (Gusse *et al.* 1986). Because intermediate

basic proteins, which have lengths of 66–100 residues, share a common C-terminal domain identical to the amino acid sequence of HP2, HP3 and HP4 (Sautiere *et al.*, 1988; Martinage *et al.*, 1990; Alimi *et al.*, 1993a), these four intermediate basic proteins are thought to be the immediate precursors of HP2, HP3 and HP4.

Precise separation and quantification of nucleoproteins in mature sperm, including minor P2 precursors, is considered important for understanding spermatogenesis as well as pathogenesis and diagnosis in many cases of male infertility. However, sperm nucleoproteins are highly basic and have such a small molecular size that fine separation and precise quantification are difficult even using an acid-urea-Triton gel or a pH-gradient gel. For this reason, no reports have described the precise quantitative composition of all human sperm nucleoproteins including all HP2 precursors based on analysis of one gel. The RFHR 2-D electrophoresis method in this study was developed (Wada, 1986a,b) as an improvement of the Kaltschmidt and Wittmann method (1970) with the aim of separating and quantifying proteins that are small, basic and rich in disulphide bridges. Thus, the method is well suited to the analysis of human sperm nucleoproteins.

Extremely small basic proteins are difficult to separate because their mobility is exceedingly high. In RFHR 2-D electrophoresis, the proteins are separated mainly by their charge in acid-urea gels in the first dimension, whereas in the second dimension separation is based mainly on mobility rate (Wada, 1986a,b). Preruns using radical scavengers were carried out to eliminate residual free radicals from gels and thereby decrease loss of proteins caused by such radicals. The sample charging electrophoresis, before the first dimensional electrophoresis, also was designed to limit protein loss and, in addition, to concentrate a dilute sample solution as a sharp band. The second dimensional electrophoresis was carried out at a more acidic pH, 3.6, to obtain better separation of extremely small basic proteins. With these modifications, quantitative yield and reproducibility became much better. In addition, protamines rich in cysteine residues migrated together with charged reductants, which served in this RFHR method to avoid formation of artefactual disulphide bridges during migration, whereas protamines were separated after modifying cysteine residues with iodoacetate in the previous study (de Yebra and Oliva, 1993). Because human nucleoproteins could be separated and purified without molecular modification by our method, these purified protamine variants should contribute importantly to analysis of protamine function.

The ratio of P1 to P2 in mature sperm varies between species; in humans it is approximately 1.0 (Balhorn *et al.*, 1988). In addition, previous studies have reported differences in amounts of P1 and P2 between fertile and infertile men (Chevaillier *et al.*, 1987; Balhorn *et al.*, 1988; Bach *et al.*, 1990; Blanchard *et al.*, 1990; Belokopytova *et al.*, 1993; de Yebra *et al.*, 1993, 1998; Bench *et al.*, 1998) or patients undergoing IVF (Colleu *et al.*, 1997; Khara *et al.*, 1997; Carrell and Liu, 2001; Steger *et al.*, 2001, 2003). Further, some groups have reported a relationship between abnormal amounts of protamines and late spermiogenetic defects (Barone *et al.*, 1994; Khara *et al.*, 1997; Bench *et al.*, 1998; de Yebra *et al.*, 1998; Carrell and Liu, 2001). Although the P1/P2 ratio had been measured by analysing spots separated by 1-D electrophoresis in these previous studies, 1-D electrophoresis does not always completely eliminate overlap between protein fractions. Our RFHR 2-D electrophoresis eliminated this drawback; separation and quantitative yield were greatly improved. Nucleoproteins in sperm from fertile men were separated into 12 spots on a single gel; these spots were shown to be HPI1, HPI2, HPS1, HPS2, HP2, HP3 and TH2B, with P1 being separated into five spots with the same N-terminal amino acid sequence (ARYRR). This might suggest that multiple forms of P1 with different degrees of C-terminal phosphorylation were separated sensitively or differing formation of intermolecular disulphide bridges occurred. We have tried to analyse

these five spots by mass spectrometry or Western blotting, but have not yet succeeded, probably because of the high basicity or small molecular size of protamine. P2-related proteins were separated into six spots; their N-terminal amino acid sequences were compatible with those of HP2, HP3 and four reported basic proteins of size intermediate between histones and protamines (HPI1, HPI2, HPS1 and HPS2) with different N-termini (Roux *et al.*, 1988; Sautiere *et al.*, 1988; Alimi *et al.*, 1993a,b) (Table I). Quantification of protein spots was performed automatically using Image Master 2D Elite software. As a result, total human sperm nucleoprotein analysed was found to include 4% TH2B, a major histone in spermatozoa. This relative amount was less than in a previous report (Gatewood *et al.*, 1987), in which approximately 15% of histone remained unreplaced in mature sperm nuclei; we suspect that our methodology for nuclear protein extraction was not optimal for analysis of histone, or that relative collection of protamine was increased by our method. On the other hand, the P1/P2 ratio, eliminating the small amounts of spots 2, 3, 4, 5, 8, 9, 10 in this study which might not be recognized by the previous 1-D electrophoretic method, was 1.02, which corresponded to the ratio reported by other studies in fertile men (Balhorn *et al.*, 1988).

Concerning the relationship between male infertility and changes in composition of sperm nucleoproteins resulting from late spermatogenic defects, de Yebra *et al.* (1998) reported that some infertile men have diminished amounts of P2 in their sperm possibly resulting from incomplete processing of P2 precursors, and they have an increased amount of putative P2 precursors. Accordingly, the composition of P2 precursors, such as HPI1, HPI2, HPS1 and HPS2 in mature sperm is thought to be important for analysis of spermatogenic mechanisms. Previous sperm nucleoprotein quantification by 1-D electrophoresis analysis in previous studies has failed to determine the entire protein complement of mature sperm including all protamine precursors, presumably because of overlap of proteins; 2-D electrophoresis eliminated this problem. Our investigation of P2 precursor content of mature sperm of fertile men showed a relative quantification of HPI1:HPI2:HPS1:HPS2:HP2:HP3 of 1.000:0.6000:1.116:2.083:3.267:2.455. To the best of our knowledge, this report is the first to quantify sperm nuclear basic proteins, especially P2 variants, in mature human sperm on one gel. In the future, pathogenesis of spermiogenetic defects may be better understood by specimens from infertile men.

Abnormal amounts of protamine were found to be common in sperm with diminished penetration capacity, whereas patients lacking P2 but having increased P2 precursors showed significantly diminished sperm penetration (Carrell and Liu, 2001). Intriguingly, however, altered amounts of protamine did not affect sperm decondensation following ICSI or subsequent embryo growth, implantation rates, or pregnancy rates. Thus, decondensation and pronuclear formation can proceed adequately when P2 is not detectable (Carrell and Liu, 2001). Because replacement of protamine by histone occurs late in spermiogenesis along with acrosome formation, membrane remodelling, and other functionally important morphologic and biochemical events, altered protamine expression is thought to reflect a wide range of spermiogenetic defects. Specifically, altered protamine expression appears not to be directly related to or causative of diminished fertilization ability but represents an indication of abnormal late-stage spermiogenesis in general (Carrell and Liu, 2001). Although the mechanism of histone replacement by protamine is not clear, a strong relationship is apparent between abnormal amounts of protamine and late spermiogenetic defects including diminished fertilization capacity. In addition, posttranslational modification or posttranslational cleavage events may be responsible for the observed variation in protamine expression. Using our sensitive RFHR 2-D electrophoretic method, even protein alterations such as posttranslational forms can be clearly separated and quantified. Thus, ejaculated semen specimens could be used to predict fertilization ability.

In conclusion, this study demonstrated that sperm nucleoproteins, especially all P2-related proteins from ejaculated semen specimen could be separated clearly with high resolution and relatively quantified on one electrophoretic gel by RFHR 2-D electrophoresis. Application of this method to specimens from infertile men might prove useful in determining pathogenesis and specific diagnosis in male infertility.

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References

- Alimi E, Martinage A, Arkhis A, Belaiche D, Sautiere P and Chevaillier P (1993a) Amino acid sequence of the human intermediate basic protein 2 (HPI2) from sperm nuclei. Structural relationship with protamine P2. *Eur J Biochem* 214,445–450.
- Alimi E, Martinage A, Sautiere P and Chevaillier P (1993b) Electroblotting proteins onto carboxymethylcellulose membranes for sequencing. *Biotechniques* 15,912–917.
- Ammer H, Henschen A and Lee CH (1986) Isolation and amino-acid sequence analysis of human sperm protamines P1 and P2. Occurrence of two forms of protamine P2. *Biol Chem Hoppe Seyler* 367,515–522.
- Arkhis A, Martinage A, Sautiere P and Chevaillier P (1991) Molecular structure of human protamine P4 (HP4), a minor basic protein of human sperm nuclei. *Eur J Biochem* 200,387–392.
- Bach O, Glander HJ, Scholz G and Schwarz J (1990) Electrophoretic patterns of spermatozoal nucleoproteins (NP) in fertile men and infertility patients and comparison with NP of somatic cells. *Andrologia* 22,217–224.
- Balhorn R (1989) Mammalian protamines: structure and molecular interactions. In Adolph KW (ed.), *Molecular Biology of Chromosome Function*. Springer-Verlag, New York, pp. 366–395.
- Balhorn R, Gledhill BL and Wyrobek AJ (1977) Mouse sperm chromatin proteins: quantitative isolation and partial characterization. *Biochemistry* 16,4074–4080.
- Balhorn R, Reed S and Tanphaichitr N (1988) Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. *Experientia* 44,52–55.
- Barone JG, de Lara J, Cummings KB and Ward WS (1994) DNA organization in human spermatozoa. *J Androl* 15,139–144.
- Belokopytova IA, Kostyleva EI, Tomilin AN and Vorob'ev VI (1993) Human male infertility may be due to a decrease of the protamine P2 content in sperm chromatin. *Mol Reprod Dev* 34,53–57.
- Bench G, Corzett MH, De Yebra L, Oliva R and Balhorn R (1998) Protein and DNA contents in sperm from an infertile human male possessing protamine defects that vary over time. *Mol Reprod Dev* 50,345–353.
- Bianchi F, Rousseaux-Prevost R, Bailly C and Rousseaux J (1994) Interaction of human P1 and P2 protamines with DNA. *Biochem Biophys Res Commun* 204,1197–1204.
- Blanchard Y, Lescoat D and Le Lannou D (1990) Anomalous distribution of nuclear basic proteins in round-headed human spermatozoa. *Andrologia* 22,549–555.
- Brewer L, Corzett M and Balhorn R (2002) Condensation of DNA by spermatid basic proteins. *J Biol Chem* 277,38895–38900.
- Carrell DT and Liu L (2001) 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. *J Androl* 22,604–610.
- Chauviere M, Martinage A, Debarle M, Sautiere P and Chevaillier P (1992) Molecular characterization of six intermediate proteins in the processing of mouse protamine P2 precursor. *Eur J Biochem* 204,759–765.
- Chevaillier P, Mauro N, Feneux D, Jouannet P and David G (1987) Anomalous protein complement of sperm nuclei in some infertile men. *Lancet* 2,806–807.
- Colleu D, Lescoat D, Thomas D and Gouranton J (1997) Changes in protamine 1 distribution in human sperm nucleus during in vitro sperm–oocyte interaction: an immunoelectron microscopic study. *Fertil Steril* 67,123–128.
- de Yebra L and Oliva R (1993) Rapid analysis of mammalian sperm nuclear proteins. *Anal Biochem* 209,201–203.
- de Yebra L, Balleasca JL, Vanrell JA, Bassas L and Oliva R (1993) Complete selective absence of protamine P2 in humans. *J Biol Chem* 268,10553–10557.
- de Yebra L, Balleasca JL, Vanrell JA, Corzett M, Balhorn R and Oliva R (1998) Detection of P2 precursors in the sperm cells of infertile patients who have reduced protamine P2 levels. *Fertil Steril* 69,755–759.
- Domenjoud L, Nussbaum G, Adham IM, Greeske G and Engel W (1990) Genomic sequences of human protamines whose genes, PRM1 and PRM2, are clustered. *Genomics* 8,127–133.
- Fuentes-Mascorro G, Serrano H and Rosado A (2000) Sperm chromatin. *Arch Androl* 45,215–225.
- Gatewood JM, Cook GR, Balhorn R, Bradbury EM and Schmid CW (1987) Sequence-specific packaging of DNA in human sperm chromatin. *Science* 236,962–964.
- Gusse M, Sautiere P, Belaiche D, Martinage A, Roux C, Dadoune JP and Chevaillier P (1986) Purification and characterization of nuclear basic proteins of human sperm. *Biochim Biophys Acta* 884,124–134.
- Kaltschmidt E and Wittmann HG (1970) Ribosomal proteins. VII. Two-dimensional polyacrylamide gel electrophoresis for fingerprinting of ribosomal proteins. *Anal Biochem* 36,401–412.
- Khara KK, Vlad M, Griffiths M and Kennedy CR (1997) Human protamines and male infertility. *J Assist Reprod Genet* 14,282–290.
- Martinage A, Arkhis A, Alimi E, Sautiere P and Chevaillier P (1990) Molecular characterization of nuclear basic protein HPI1, a putative precursor of human sperm protamines HP2 and HP3. *Eur J Biochem* 191,449–451.
- McKay DJ, Renaux BS and Dixon GH (1985) The amino acid sequences of human sperm protamine P1. *Biosci Rep* 5,383–391.
- McKay DJ, Renaux BS and Dixon GH (1986) Human sperm protamines. Amino-acid sequences of two forms of protamine P2. *Eur J Biochem* 156,5–8.
- Oliva R and Dixon GH (1991) Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Prog Nucleic Acid Res Mol Biol* 40,25–94.
- Roux C, Gusse M, Chevaillier P and Dadoune JP (1988) An antiserum against protamines for immunohistochemical studies of histone to protamine transition during human spermiogenesis. *J Reprod Fertil* 82,35–42.
- Sautiere P, Martinage A, Belaiche D, Arkhis A and Chevaillier P (1988) Comparison of the amino acid sequence of human protamines HP2 and HP3 and of intermediate basic nuclear proteins HPS1 and HPS2. *J Biol Chem* 263,11059–11062.
- Steger K, Failing K, Klonisch T, Behre HM, Manning M, Weidner W, Hertle L, Bergmann M and Kliesch S (2001) Round spermatids from infertile men exhibit decreased protamine-1 and -2 mRNA. *Hum Reprod* 16, 709–716.
- Steger K, Fink L, Failing K, Bohle RM, Kliesch S, Weidner W and Bergmann M (2003) Decreased protamine-1 transcript levels in testis from infertile men. *Mol Hum Reprod* 9, 331–336.
- Wada A (1986a) Analysis of *Escherichia coli* ribosomal proteins by an improved two dimensional gel electrophoresis. I. Detection of four new proteins. *J Biochem* 100, 1583–1594.
- Wada A (1986b) Analysis of *Escherichia coli* ribosomal proteins by an improved two dimensional gel electrophoresis. II. Characterization of four new proteins. *J Biochem* 100, 1595–1605.

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