

Ubiquitin-based sperm assay for the diagnosis of male factor infertility

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Sperm morphology is correlated with fertility in men, yet the existing, subjective sperm morphology assays provide only a limited insight into patients' infertility. Here, we provide the experimental background for a new, objective and automated semen assay, based on the cross-reactivity of defective human spermatozoa with antibodies against a proteolytic marker peptide, ubiquitin. Using immunofluorescence and flow cytometry, we screened the spermatozoa from 17 infertility patients and two fertile donors for their cross-reactivity with anti-ubiquitin antibodies. Thirteen out of 17 patients, but neither of two fertile donors, displayed an increased binding of anti-ubiquitin antibodies to sperm surface, that reflected the occurrence of abnormalities in these samples and was corroborated by available clinical data. Highest correlation coefficient ($r = -0.432$) was obtained with the cleavage rate after IVF. The contribution of male factor was revealed in several couples previously diagnosed with idiopathic infertility. Ubiquitin-cross-reactive sperm-surface proteins thus seem to be a universal marker of semen abnormalities, including sperm head and tail defects and semen contaminants such as spermatids, leukocytes and cellular debris. We propose that the sperm-ubiquitin tag immunoassay (SUTI) may be a valuable diagnostic tool in treatment of male factor and idiopathic infertility.

Key words: male factor infertility/SUTI/ubiquitin

Introduction

Male factor contributes to 40% of infertility cases worldwide. While the sperm morphology correlates with the outcome of IVF (Kruger *et al.*, 1987; Donnelly *et al.*, 1998; Coetzee *et al.*, 1999a; Filatov *et al.*, 1999), the semen analysis, on which the diagnosis of male factor infertility relies, is highly subjective (Amann, 1989; Coetzee *et al.*, 1999b; Szczygiel and Kurpisz, 1999). Few objective methods for infertility diagnosis and fertility prediction by sperm screening are currently available, most of them based on the detection of sperm DNA damage by chromatin-based assays (Evenson *et al.*, 1991; Baccetti *et al.*, 1996; Hacker-Klom *et al.*, 1999) or by immunocytochemistry (van der Schans *et al.*, 2000). Other assays detect the components of apoptotic pathways linked to some of the sperm abnormalities (Sakkas *et al.*, 1999). Here, we describe a new method of sperm analysis, termed Sperm-Ubiquitin Tag Immunoassays (SUTI), based on the ubiquitination process taking place in the epididymis of humans and other mammals (Sutovsky *et al.*, 2000b, 2001). This process specifically marks the proteins on the surface of defective spermatozoa as well as on other ejaculated contaminants of cellular origin with the universal proteolytic marker, 8.5 kDa peptide ubiquitin (Ciechanover, 1994), and appears to occur during epididymal passage in ungulates, rodents and primates (Sutovsky *et al.*,

2000b, 2001). Along with abnormal sperm heads and tails, contaminants of cellular origin, including round and elongated spermatids, leukocytes and cellular debris become surface-ubiquitinated and thus are recognizable by anti-ubiquitin antibodies. Ubiquitin-cross-reactive sperm-surface proteins therefore appear to be a universal marker of semen abnormalities and we propose SUTI immunoassay as a valuable new tool for infertility diagnosis and prediction of IVF success in subfertile men and couples diagnosed with idiopathic infertility.

Materials and methods

Sperm samples and corresponding clinical data

Semen samples were obtained from 17 consenting infertility patients (1–17) treated at the Tohoku University Hospital, Sendai, Japan. Samples were coded so that the identity of patients was concealed, and frozen in liquid nitrogen. Appropriate protocols approved by the Institutional Review Board (IRB) of both Tohoku University and Oregon Health Sciences University were strictly followed. Samples from fertile donors (S1 and S2) were purchased from Follas Laboratories (Indianapolis, USA). According to the distributor's data, samples S1 and S2 were standard fertile semen samples with excellent clinical parameters, previously used to obtain numerous pregnancies. Clinical

Table I. Summary of clinical, immunofluorescence and flow cytometry data from one fertile donor and 17 patients examined in this study. Samples from patients 2, 10, 16 and 17 were examined by subjective, immunofluorescence-microscopic analysis only. Average flow cytometry median was $X = 25.3$ for patients and $X = 19.5$ for standard sample S1 in three repeats.

Patient	Count (10 ⁶ /ml)	Motility (%)	Fertility rate (%)	Cleavage rate (%)	Prevailing defect	Median	Histogram shift	Original diagnosis	SUTI diagnosis of male factor	Pregnancy
S1	N/A	N/A	N/A	N/A	Coiled tails	17.15–22.88	NO	N/A	NO	N/A
1	18.3	20.0	0	0	Globozoospermia	15.40/17.14	YES	Male factor	YES	NO
2	136.3	88.3	83.3	83.3	Sperm head malformations	N/A	N/A	Tubal	NO	NO
3	239.0	85.3	25	0	Swollen sperm heads	40.68/22.88	YES	Idiopathic	YES	NO
4	34.0	23.7	45.5	38.5	Lasso tails Residual cytoplasm	27.38/22.88	YES	Tubal and male	YES	YES
5	61.7	59.9	66.7	66.6	Residual bodies	28.39/18.43	YES	Tubal	YES	NO
6	256.0	25.0	42.9	42.9	Cells in ejaculate	29.43/22.88	YES	Male factor	YES	YES
7	222.0	42.3	85.7	85.7	Various defects	33.98/18.43	YES	Tubal	YES	NO
8	173.0	67.7	66.7	27.3	Sperm head malformations	39.24/22.88	YES	Idiopathic	YES	NO
9	70.0	54.2	45.5	27.3	Cells in ejaculate	35.23/18.43	YES	Tubal	YES	YES
10	145.0	25.2	66.7	66.7	Lasso tails, nuclear vacuoles	N/A	N/A	Tubal and male	YES	YES
11	101.0	48	100	100	None	12.41/17.15	NO	Tubal	NO	NO
12	189.0	87.3	50	50	Nuclear vacuoles	21.29/18.43	NO	Idiopathic	NO	NO
13	216.0	47.0	0	0	Two heads, two tails, twin sperm	20.54/18.43	YES	Idiopathic	YES	NO
14	174.0	57.1	57.1	42.9	Lasso tails	11.97/17.15	NO	Tubal	NO	NO
15	140.0	78.0	50	50	Various defects	33.98/22.88	YES	Idiopathic	YES	NO
16	213.0	33.0	0	0	Lasso tails, twin tails	N/A	N/A	Tubal and male	YES	NO
17	184.0	73.6	83.3	25	Globozoospermia, nuclear vacuoles	N/A	N/A	Idiopathic	YES	NO

parameters and treatment results from these trials were not disclosed and are thus not included in Table I. For all analyses, frozen ejaculates were thawed in warm water and washed by centrifugation through Tyrode's albumin lactate pyruvate (TALP)-HEPES medium. Repeated freezing and thawing did not affect the intensity and topology of labelling and anti-ubiquitin antibodies also bind to live sperm (P.Sutovsky, unpublished data). To ensure unbiased analysis of experimental data, corresponding clinical data including sperm counts, motility, fertilization and cleavage rates, original clinical diagnosis of infertility (male factor, tubal, combined or idiopathic) and pregnancy outcome, provided by the staff of Tohoku University Hospital, were requested and obtained by the authors after SUTI.

Immunofluorescence

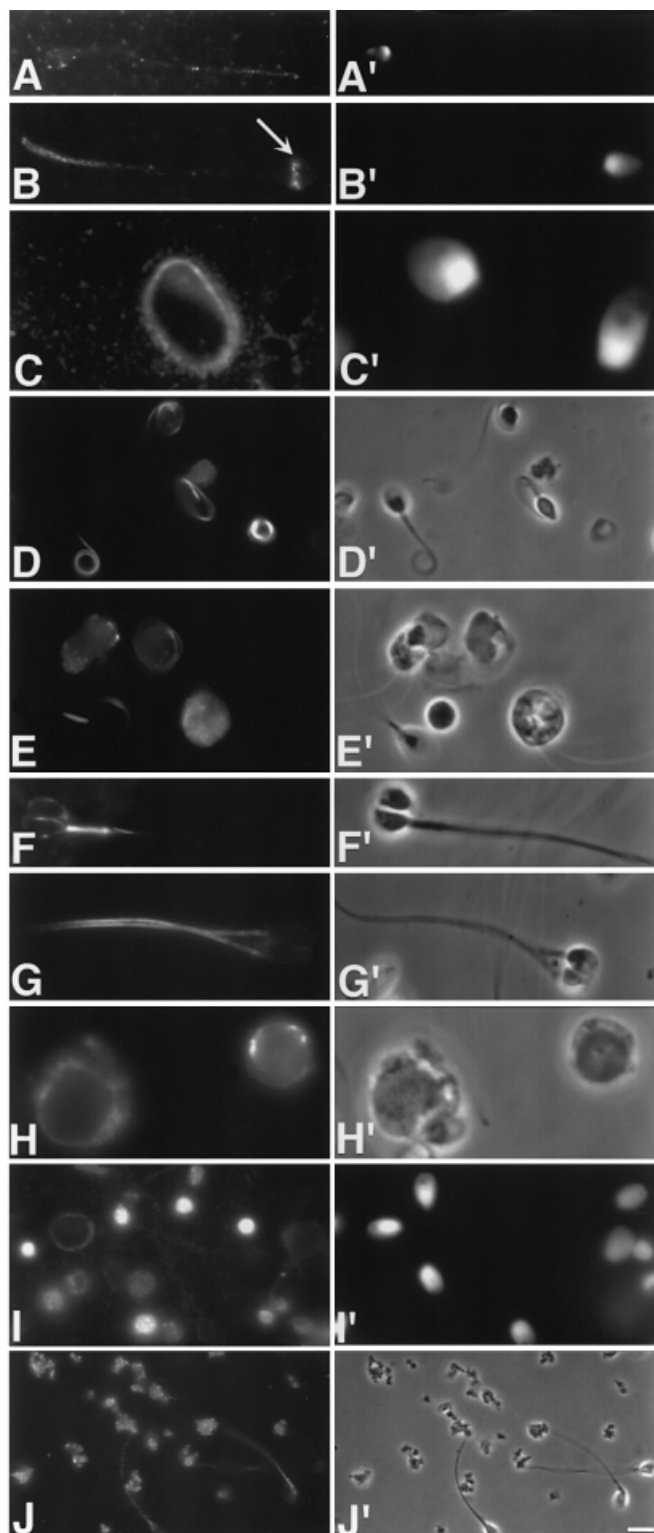
Two microlitres of sperm pellets from each man were resuspended in 500 μ l drops of 37°C warm KMT medium (Sutovsky *et al.*, 1999b) on the poly-L-lysine coated microscopy coverslips and allowed to attach for 5 min on a slide warmer. Coverslips were submerged in 2% formaldehyde in PBS and fixed for 40 min. No permeabilization was performed to avoid the contribution of constitutively ubiquitinated sperm substrates to the signal. Samples were blocked for 25 min in 5% normal goat serum (NGS; Sigma, St Louis, MO, USA) in phosphate buffered saline (PBS) and incubated for 40 min with the monoclonal antibody KM 691 raised against the recombinant human ubiquitin (dilution 1/100; Kamyia Biomedical Company, Seattle, WA, USA). PBS with 1% NGS was used for washing and dilution of primary and secondary antibodies. After washing, samples were incubated for 40 min with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgM (dilution 1/80; Zymed) and DNA-stain DAPI (Molecular Probes, Eugene, OR, USA) was added to this solution 10 min before the end of incubation. Samples were washed and mounted on microscopy slides in Vectashield (Vector

Labs, Burlingame, CA, USA) medium. Detection of perinuclear theca (PT) proteins and acrosin in globozoospermic patients 1 and 17 was performed as described previously (Sutovsky *et al.*, 1999b). Samples were examined under a Zeiss Axiophot microscope. Images were captured by a Princeton Digital camera using MetaMorph software, edited by Adobe Photoshop 4.0 (Adobe Systems Inc., Mountain View, CA, USA) and printed by a Sony UP-D 8800 dye sublimation printer.

Flow cytometry

Sperm suspensions were fixed, blocked and incubated with antibody KM-691, washed by resuspension in PBS and centrifugation, then incubated with FITC-conjugated goat anti-mouse IgM (dilution 1/80; Zymed), washed and resuspended in 500 μ l of pure PBS without serum. Blank, negative control samples were prepared for each experimental sample by omitting the primary antibody. Typically, samples from five patients and one fertile donor were prepared and analysed in each session. Data from three representative sessions are shown. Samples were analysed using FACS Calibur Analyzer (Becton Dickinson, San Diego, CA, USA) at 488 nm. Relative levels of fluorescence in individual cells were recorded. A sample of PBS buffer used for labelling was used to eliminate non-specific fluorescence and to calibrate the cytometer; a blank sample of corresponding patient/donor was run before each anti-ubiquitin-labelled sample. Five thousand cells (5000 gated events) were recorded for each sample. Analysis threshold was set at channel zero. The median value, representing the channel number about which half the cells are dimmer and half the cells are brighter and increases with the increase in the number of brighter cells, was recorded. Other parameters taken into consideration were: (i) shape of the fluorescence histogram, typically with a sharp peak in fertile men and with flat shape in subfertile men; (ii) scattering pattern reflecting cell shape, typically with a single focus in fertile men with uniform shape and with two

foci or elongated large single focus in subfertile men with misshapen spermatozoa; (iii) shift of the curve towards high relative fluorescence in subfertile men; and (iv) position of the peak of relative fluorescence curve on the x -axis, typically close to 10^1 in fertile men and close to 10^2 in patients with elevated ubiquitin levels. Flow cytometry data in subfertile men typically displayed the combination of two or more of the above characteristics as described for patients, in addition to a high median value. After each repeat, leftover samples were stored overnight at 4°C and re-run the next day. No significant differences were found between such re-runs and original, first run data.



Electron microscopy

Sperm were isolated, fixed in formaldehyde and processed with antibody KM-693 without permeabilization as described for immunofluorescence, except that the fluorescently conjugated secondary antibodies were replaced by a 10 nm-gold-conjugated goat anti-mouse IgM (Jackson Immunochemicals) and the sperm were handled by centrifugation instead of being attached to microscopy coverslips. The labelled cells were pelleted by centrifugation, fixed for transmission electron microscopy (TEM) and embedded in Epon 812 as described previously (Sutovsky *et al.*, 1999b). Ultrathin sections were cut on a Sorvall MT-5000 ultramicrotome (Ivan Sorvall Inc., Norwalk, CT, USA), stained by uranyl acetate and lead citrate, and examined in Philips EM 300 electron microscope. Negatives were scanned by Umax Powerlook 3000 flat bed scanner (Umax Technologies Inc., Fremont, CA, USA) and printed on a Sony UP-D 8800 dye sublimation printer using Adobe Photoshop 4.0 software.

Statistical analysis

Flow cytometry medians are shown for each of the three, non-overlapping groups in which the samples were processed for flow cytometry. Scatter diagrams and predicted linear regression lines were drawn based on sample regression coefficients ($b_{yx} < -\infty; \infty >$), calculated using flow cytometry medians and clinical data (sperm count, sperm motility, fertilization rate, cleavage rate) provided by the clinic (Snedecor and Cochran, 1973; Sokal and Rohlf, 1981). Correlation between flow cytometry medians and clinical parameters was evaluated by calculating sample correlation coefficients ($r = < -1; 1 >$) as described (Snedecor and Cochran, 1973). Median values for standard samples were not included in these calculations since the clinical data were not made available by the distributor of those samples.

Results

We assayed spermatozoa from 17 infertility patients (1 through 17) and standard sperm samples (S1 and S2) from two fertile donors by immunofluorescence (all patients) and flow cytometry (all men except S2 and patients 2, 10, 16, 17), using antibodies against the recombinant human ubiquitin that bind to fixed, as well as to live, unfixed spermatozoa, and appropriate fluorescent conjugates of secondary antibodies. Ubiquitin data are summarized and compared with clinical data in Table I.

By immunofluorescence, most spermatozoa from fertile donors displayed typical ellipsoid shape of the sperm head and a straight sperm tail with a mitochondrial sheath of even diameter. Weak ubiquitin labelling was found on the surface

Figure 1. (A–C) Ubiquitin presentation on the principal piece and end piece (A, B) of the sperm tail, and on the equatorial segment of the sperm head (B; arrow) in morphologically normal sperm from standard sample S2. (C) A ubiquitinated, coiled tail of a defective spermatozoon in the sample from standard sample S2. (A'–C') Corresponding images of sperm-nuclear DNA staining. (D–J) Common anomalies recognized by anti-ubiquitin antibodies in the sperm of infertility patients, including coiled and lasso tails (D; patient 10), round-headed sperm (E; 1), twin heads (F; 13), twin heads and tails (G, 13), somatic (H; left cell) and immature spermatogenic cells (H; right cell, 6), unresorbed residual bodies (I; 5) and fragmented or sperm associated residual cytoplasm (J; 4). (D'–H'; J) Corresponding phase-contrast images. (I') DNA staining showing no DNA in the unresorbed residual cytoplasmic bodies. Scale bar = 10 μm .

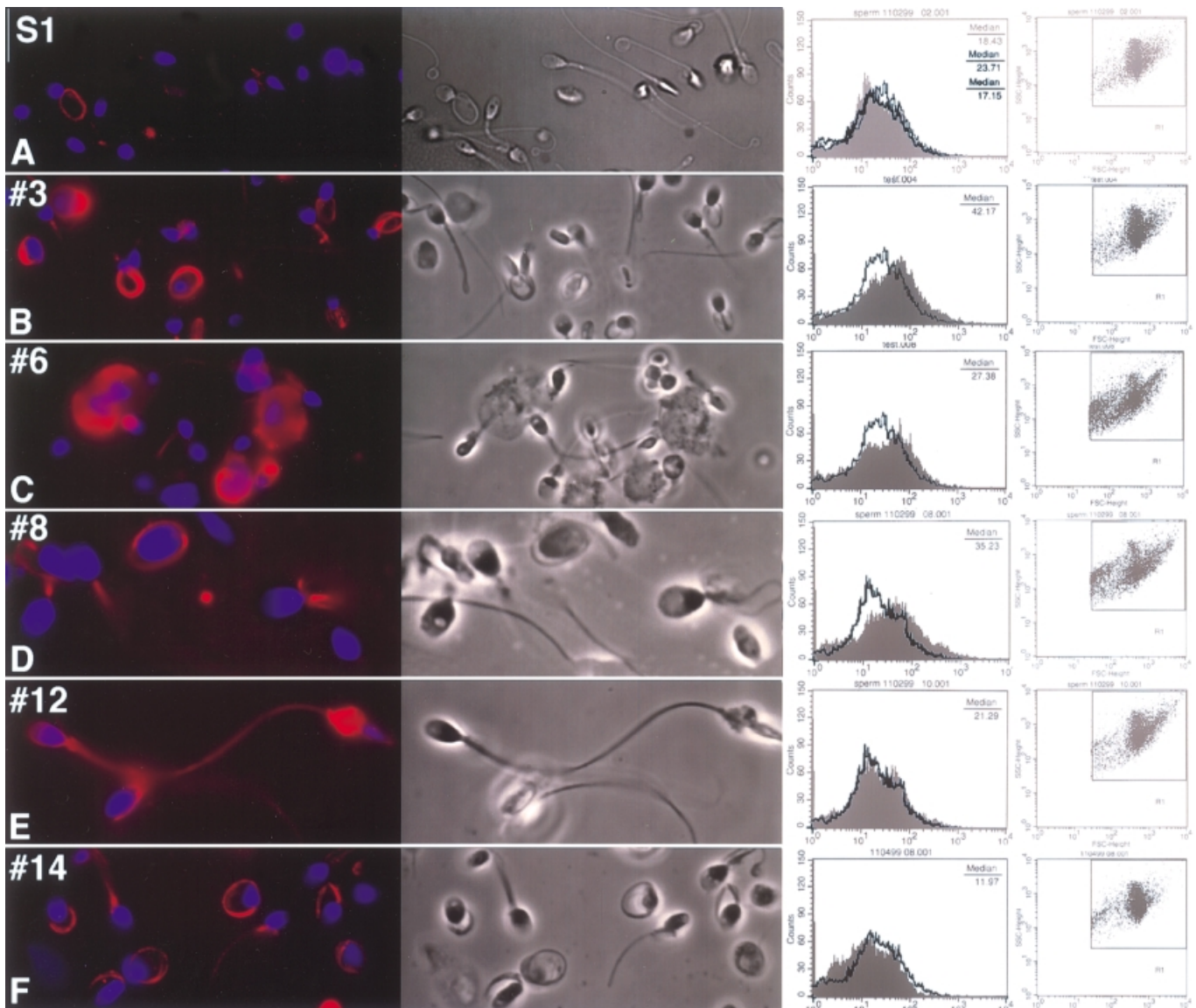


Figure 2. Ubiquitin profiles from representative samples as shown by immunofluorescence (left column), phase contrast microscopy (centre), and flow cytometry (right column; relative fluorescence curves on the left, x = relative fluorescence, y = number of cells; scattering patterns on the right; empty black curve in **B–F** represents the standard curve for standard S1). (**A**) Sample S1 used as a standard for flow cytometry; three different runs are shown. (**B**) Patient 3 from a couple whose infertility was previously pronounced idiopathic, while SUTI data suggest male factor contribution. (**C**) Patient 6 in whom SUTI assays confirmed previous diagnosis of male factor. (**D**) Patient 9 from a couple with previously diagnosed tubal infertility; SUTI data suggest male factor contribution. (**E**) Donor 12; the contribution of male factor to this unexplained infertility is not likely. (**F**) Donor 14 is not likely to contribute this previously diagnosed tubal infertility case. Note the flat shape of the curve (**C**, **D**), high median values (**B–D**) and visible shift in the number of highly fluorescent cells (**B–D**) in men diagnosed as subfertile by SUTI, as opposed to a sharper curve with lower numbers of highly fluorescent cells in men with low sperm-surface ubiquitination (**A**, **D**, **E**). These patients (**B–D**) often display aberrant sperm cell shapes, evidenced by enlarged scattering patterns (**B–D**) and/or two distinct scattering foci (**C**, **D**). Scale bar = 10 μ m.

of the sperm tail's principal and end pieces in such sperm (Figure 1A), and, in some cases, on the equatorial segment of the sperm head (Figure 1B). No permeabilization was used to avoid the contribution of constitutively ubiquitinated sperm substrates such as mitochondrial membrane proteins (Sutovsky *et al.*, 1999a, 2000a) and nuclear histones (Chen *et al.*, 1998; Baarends *et al.*, 1999) to the fluorescent signal. Abnormal spermatozoa with strongly ubiquitinated, coiled or lasso tails were also found in the spermatozoa from both fertile donors (Figures 1C, 2A), while their abundance, as judged subjectively

by fluorescence microscopy was diminished in comparison with patient's samples. Abnormalities in the defective sperm (Figures 1, 2) included coiled or lasso tails (Figure 1D), misshaped and globozoospermic sperm heads (Figure 1E), twin tails and/or twin heads (Figure 1F,G), and spermatogenic and somatic cells (Figure 1H), residual bodies (Figure 1I) and fragmented residual cytoplasm (Figure 1J) present in the ejaculates. Semen from donor S1 was used as a standard sample for flow cytometry, where its median channel for fluorescence peak (further 'median'), reflecting relative number

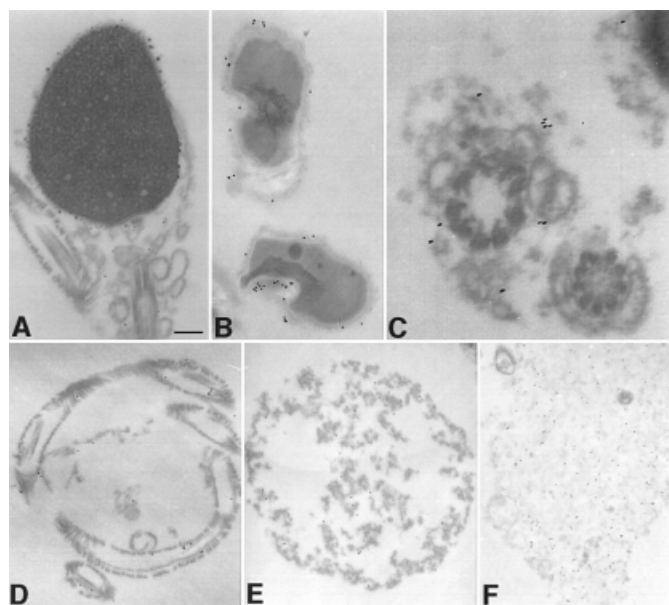


Figure 3. Colloidal gold labelling of ubiquitin on the surface of defective human sperm from patients 1 (A, B), 3 (C–E) and 4 (F). (A) Round headed spermatozoon. (B) Misshaped sperm heads. (C) Abnormal mitochondrial sheath. (D) Lasso tail, labelling on fibrous sheath. (E) Residual cytoplasmic droplet. (F) Fragmented residual cytoplasm. Scale bar = 200 nm.

of sperm with average relative fluorescence, fluctuated between 17.15 to 22.88 (Figure 2A). The presence of ubiquitin-cross-reactive proteins on the surface of defective spermatozoa was confirmed by ultrastructural immunocytochemistry (Figure 3A–F).

Patient 1 had a high proportion of ubiquitinated, round-headed spermatozoa, reminiscent of globozoospermia, a rare spermatogenic disorder arising from the failure of sperm nuclear condensation and aberrant differentiation of sperm head perinuclear theca (Escalier, 1990). This diagnosis was also supported by the partial or complete absence of acrosome and perinuclear theca in the individual spermatozoa from this sample (not shown). Other abnormalities included lasso tails and round and elongated spermatids present in the ejaculate. This case was previously diagnosed as male factor infertility, which was supported by immunofluorescence data. While the median channel of fluorescence peak obtained by flow cytometry was low (15.4 versus 17.15 in S1), the distribution curve was unusually flat and scatter pattern was enlarged substantially. No fertilization, cleavage or pregnancy was obtained; sperm count and motility were low ($18.3 \times 10^6/\text{ml}$ and 20% respectively).

Malformations of the sperm heads were the major anomaly in sperm of patient 2, while the sample contained mostly normal sperm. Accordingly, the infertility was previously diagnosed as tubal, with no pregnancy but excellent fertilization and cleavage rates (both 83.3%) and sperm characteristics ($136.3 \times 10^6/\text{ml}$; 88.3%). Flow cytometry was not performed.

Patient 3 displayed significantly higher median (40.68 versus 22.88 in S1) and a shift in number of highly fluorescent cells (Figure 2B) by flow cytometry. By immunofluorescence, the major defect revealed was swollen sperm heads and lasso tails. Heads separated from tails, nuclear vacuoles and cytoplasmic

droplets were also frequent. Infertility was deemed idiopathic with good sperm motility (88.3%) and indication for primary sterility. No pregnancy was obtained. SUTI indicates the diagnosis of male factor infertility in this previously unexplained case.

In patient 4, the major defect was large amounts of cellular debris in the form of irregularly shaped clusters (see Figure 1J), while most spermatozoa did not display major defects. Flow cytometry median channel was 27.38 as opposed to 22.88 in S1, and a significant shift in fluorescence distribution was observed. Both tubal and male factor infertility were previously diagnosed, with slightly reduced sperm count ($34 \times 10^6/\text{ml}$) and low motility (23.7%). Male factor infertility was confirmed by SUTI.

The major defect in patient 5 was the presence of residual cytoplasmic bodies, normally removed by Sertoli cells in the testis, in the ejaculate (see Figure 1I). Nuclear vacuoles and swollen sperm heads were also frequent. Flow cytometry median of fluorescence peak was elevated to 28.39 versus 18.43 in S1. Sterility was previously diagnosed as tubal, with average sperm count of $61.7 \times 10^6/\text{ml}$ and motility of 59.9%. No pregnancy was obtained despite the treatment for tubal infertility. SUTI suggests the contribution of previously undiagnosed male factor.

The prevailing anomaly in patient 6 was the presence of small cells with nuclei, probably leukocytes and/or immature spermatogenic cells (Figures 1H, 2C). Swollen sperm heads were also frequent. Median channel of fluorescence peak was 29.43 versus 22.88 in S1 with a shift towards highly fluorescent cells. Sperm count was high ($256 \times 10^6/\text{ml}$), while only 25% of sperm were motile. Ubiquitin data confirm male factor infertility, diagnosed previously.

Patient 7 had a combination of swollen sperm heads, abnormal mitochondrial sheaths and residual cytoplasmic bodies. Other defects included nuclear vacuoles, cytoplasmic droplets still attached to the sperm mid piece, abnormal mitochondrial sheaths and large somatic cells present in the sample. Median reached 33.98 versus 18.43 in S1, with histogram shifted towards highly fluorescent cells. Previously diagnosed as a primary, tubal infertility, sperm count was good ($222 \times 10^6/\text{ml}$), whereas motility was average (42.3%). SUTI suggests male factor contribution to this case previously diagnosed as maternal infertility.

Patient 8 carried a high number of misshapen sperm heads, suggesting a defect of the sperm nuclear condensation or differentiation of the perinuclear theca. Some abnormal mitochondrial sheaths and somatic cells were also observed. Median was high (39.24 versus 22.88 in S1) with a curve shift and a bifocal scatter pattern. This was an unexplained infertility with high sperm count ($173 \times 10^6/\text{ml}$) and motility (67.6%), good fertilization rate (66.7%), but a low cleavage rate (27.3%). No pregnancy was obtained and SUTI indicated male factor.

Ubiquitinated somatic cells, probably leukocytes, were detected in the sample from patient 9 (Figure 2D). Other abnormalities included round and elongated spermatids and globozoospermic sperm present in the ejaculate. High median (35.23 versus 18.43 in S1), flat and shifted flow cytometry histogram, and multi-focal scattering patterns were recorded.

Infertility was previously diagnosed as tubal, with average sperm count ($70 \times 10^6/\text{l}$) and motility (54.2%). SUTI suggests male factor contribution.

Sample 10 contained ubiquitinated sperm with coiled tails, nuclear vacuoles and abundant residual cytoplasmic bodies. Both male factor and tubal infertility were diagnosed previously and are corroborated by subjective ubiquitin assay (flow cytometry was not performed). While the sperm count was good ($145 \times 10^6/\text{ml}$), motility was only 25.2%. The remaining motile spermatozoa yielded identical cleavage and fertilization rates (66.7%).

Patient 11 had a good sperm sample with few ubiquitinated spermatozoa. No predominant abnormality was detected, although the abnormalities described in other cases were occasionally found. While the flow cytometry histogram was atypically flat and the sample displayed enlarged scatter pattern, the median was only 12.41 versus 17.15 in S1, and there was no shift. This case was previously diagnosed as tubal infertility, fertilization and cleavage rates were excellent (both 100%). Accordingly, SUTI did not indicate male factor.

The sample from patient 12 contained relatively few ubiquitinated sperm cells. The major defect observed was nuclear vacuoles. Median (21.29) was close to that of S1 (18.43) and the histogram was neither shifted nor flat (Figure 2E). Sperm parameters were excellent ($189 \times 10^6/\text{ml}$; 87.3% motility). Considering the excellent results of SUTI and good cleavage rates (50%), male factor is not likely to contribute to this case of unexplained infertility.

The prevalent defect in patient 13 was the presence of twin heads and/or twin tails. Median channel of fluorescence peak was only 20.54, as compared to 18.43 in S1, while the histogram was flat and clearly shifted to the right, with a bifocal scattering pattern. The presence of ubiquitinated twin sperm may account for relatively low motility (47%) and 0% fertilization and cleavage rates. The case was pronounced idiopathic with no pregnancy. With the exception of a good median, ubiquitin data suggest male factor.

Patient 14 had a good sample with a major defect being broken and coiled tails (Figure 2F). This man had the lowest median value in sampled group (11.97 versus 17.15 in S1). The couple was diagnosed with tubal infertility, fertilization and cleavage rates were good (57.1 and 42.9% respectively). Motility was average (45.6%) with a good sperm count of $174 \times 10^6/\text{ml}$. There was no pregnancy and SUTI suggests that this infertility was not contributed by male factor.

Patient 15 carried a mixture of various defects, including lasso tails, swollen heads, nuclear vacuoles, cells, residual cytoplasm and abnormal mitochondrial sheaths. Median reached 33.98 versus 22.88 in S1, with a shift towards highly fluorescent cells. This case was previously diagnosed as idiopathic with good sperm parameters ($140 \times 10^6/\text{ml}$; 78% motility), while SUTI points to a male factor infertility.

Abnormal, lasso and twin sperm tails were the prevailing defects in patient 16, other defects included malformed sperm heads and cells present in ejaculate. Both tubal and male factor infertility were diagnosed previously and corroborated by immunofluorescence data and low motility (33% at 213×10^6

sperm/ml), and 0% fertilization and cleavage rates. No pregnancy was obtained.

The major defect in patient 17 was nuclear vacuoles, while round sperm heads and a residual cytoplasmic droplet were also observed. The case was previously pronounced unexplained and a low cleavage rate (25%) was contradicted by a good IVF rate (83.3%) and sperm parameters ($184 \times 10^6/\text{ml}$; 73.6% motility). Ubiquitin data suggest male factor contribution, while maternal contribution cannot be ruled out.

Discussion

Ubiquitination is a universal mechanism for protein recycling, by which the obsolete substrates become marked by the covalent attachment of one or more molecules of ubiquitin, a small 8.5 Da peptide that targets such substrates for proteolysis in the lysosome or proteasome. Each molecule of ubiquitin attached to a molecule of a substrate thus increases its molecular weight by 8.5 kDa (Ciechanover, 1994). Our recent studies (Sutovsky *et al.*, 2000b, 2001) showed that defective sperm in the semen of domestic bulls, wild cattle (gaur and buffalo), rhesus monkeys, humans and mice were strongly ubiquitinated on the surface. This step in sperm maturation occurs in the epididymis, as judged by the abrupt increase in the number of ubiquitinated sperm between rete testis and caput epididymis in bulls. Ubiquitin is expressed by epididymal epithelium and accumulated in its apical microvilli, a known site of apocrine protein secretion. It is thus conceivable that sperm-surface ubiquitination may be the means for the immobilization and/or resorption of defective sperm during epididymal passage. As the proposed mechanism allows a certain portion of ubiquitinated, defective spermatozoa to be discharged during ejaculation, ubiquitin on the sperm surface could be a good marker of semen quality in men and animals.

The present data show that the levels of sperm-surface ubiquitination corroborate available clinical profiles in 17 patients studied, in six of them confirming the previously diagnosed male factor infertility (patients 1, 4, 6, 10, 16), in five cases revealing possible reason for infertility that was previously assessed as idiopathic (patients 3, 8, 13, 15, 17), in three cases (patients 2, 11, 14) confirming the diagnosis of maternal only infertility and in three cases (patients 5, 7, 9) suggesting the contribution of previously overlooked male factor to the previously diagnosed maternal infertility. In patient 12, ubiquitin assays suggested that male factor is not likely to contribute to this case of idiopathic infertility. As expected, ubiquitin data were not correlated with treatment outcome, as pregnancy was not obtained in some couples with good sperm-ubiquitin parameters (2, 11, 12, 14; most of them tubal infertility cases) and some couples with increased sperm ubiquitination did achieve pregnancy after IVF (4, 9, 10).

Both immunofluorescence and flow cytometry assays provided valuable information about each given sperm sample. The main value of the subjective immunofluorescence screening is in its ability to reveal which particular types of sperm defects prevail in a given sample. This could be useful in planning the treatment strategy. For example, immunofluorescence assay revealed sperm head malformations reminiscent

of globozoospermia, a clinical indication for intracytoplasmic sperm injection (ICSI) combined with artificial oocyte activation (Rybouchkin *et al.*, 1997), in patient 1. Both fertilization and cleavage rates were zero and pregnancy was not obtained. Similar defects were overlooked in patient 17 during clinical semen evaluation and the infertility was originally diagnosed as idiopathic. Other easy-to-overlook causes of presumed idiopathic infertility, such as the easily decapacitated spermatozoa defect (Kamal *et al.*, 1999), Kartagener's syndrome (Baccetti *et al.*, 1980) and dysplasia of fibrous sheath (Chemes *et al.*, 1987), can be recognized by epididymal ubiquitination machinery and properly diagnosed by SUTI (V.Rawe, P.Sutovsky and E.Neuber, unpublished data). Similarly, spermatozoa with head defects in *azh/azh* mutant mice (Cole *et al.*, 1988; Meistrich *et al.*, 1990), display strong surface-ubiquitination (P.Sutovsky and R.Moreno, unpublished data). Subjective immunofluorescence examination readily detects the elevated number of ubiquitinated spermatozoa, somatic and spermatogenic cells, and residual cytoplasm, and after appropriate training, the evaluators could also generate quantitative data by counting the number of highly ubiquitinated spermatozoa per unit of spermatozoa screened (e.g. per 1000 spermatozoa in 10 randomly selected optical fields).

Ideally, the subjective immunofluorescence assay should be complemented by flow cytometry trial providing the means for objective, unbiased quantification of ubiquitin titre in a sperm sample. Such analysis could be performed using conventional flow cytometers or dedicated, automated semen analysers (Coetzee *et al.*, 1999a,b). Other immunological titration assays, such as enzyme linked immunoabsorbent assay (ELISA) could be used to objectively evaluate the ubiquitination levels of human sperm samples and eventually developed into simple titration test kits detecting increased ubiquitin levels in fresh semen samples. All fluorescence based assays could be streamlined by using anti-ubiquitin antibodies conjugated with suitable fluorochromes. Alternative flow cytometric assays of sperm morphology were proposed, including the sorting of semen samples stained with DNA stains (Evenson and Melamed, 1983; Filatov *et al.*, 1999; Hacker-Klom *et al.*, 1999), or specific antibodies against single-stranded DNA (van der Schans *et al.*, 2000), apoptotic markers (Sakkas *et al.*, 1999) and leukocyte contaminants (Moilanen *et al.*, 1999). Other objective assays of sperm quality are based on the detection of sperm DNA damage resulting from apoptosis, oxidation or necrosis (Baccetti *et al.*, 1996; Shen *et al.*, 1999; Irvine *et al.*, 2000). Most abnormal sperm cells revealed by such assays will likely be surface ubiquitinated and thus also detectable by SUTI, while our method will also detect abnormalities of the sperm tail and cellular contaminants other than defective sperm, including leukocytes, residual bodies, cellular detritus, persisting cytoplasmic droplets, and immature spermatogenic cells present in patients' ejaculates. Finally, sperm cell fragments generated during sample processing (e.g. tails and heads separated by pipetting and centrifugation) will not be detected as defective by SUTI, unless they originate from abnormal sperm.

In patients 3, 13, 15 and 17, relatively good sperm count, motility, fertilization and cleavage parameters did not suggest

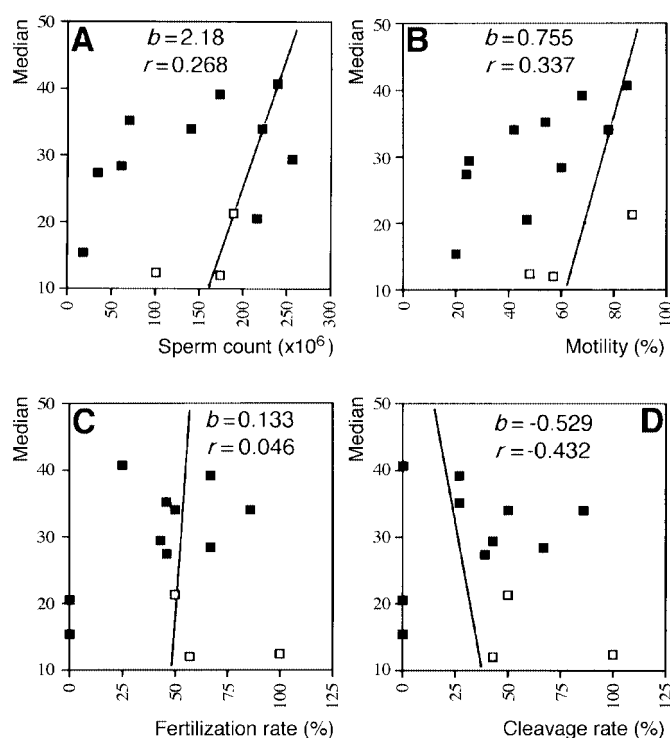


Figure 4. Scatter diagrams and sample regression lines reflecting the relationship of sperm ubiquitination, as determined by flow cytometry median, to sperm count (A), sperm motility (B), fertilization rates (C) and cleavage rates (D), respectively. Only those patients in whom flow cytometry analysis was performed are included. Note that three patients, in whose samples SUTI ruled out the contribution of male factor (empty squares), have low flow cytometry medians and good-excellent clinical parameters. Sample regression coefficients (b) determine the shape of regression line and sample correlation coefficients (r) reflect the degree of correlation between a given clinical parameter and flow cytometry median of ubiquitin.

male factor infertility, while ubiquitin data suggested male factor contribution to these cases of unexplained infertility. This is probably due to the fact that defective and immotile spermatozoa were removed from such ejaculates prior to IVF (but not before clinical semen analysis and SUTI) by gradient centrifugation or swim-up, thus yielding good fertilization rates. The surface-ubiquitinated spermatozoa may interfere with motility and/or fertilizing ability of such spermatozoa after coitus, but can be eliminated by motile sperm separation prior to IVF. It follows that the fertilization rate *in vitro* may not be a reliable parameter for the diagnosis of male factor in such unexplained cases. The predictive and diagnostic value of other traditional sperm parameters, such as sperm concentration, motility and morphology has been disputed (Tomlinson *et al.*, 1999). Accordingly, the analysis of regression and correlation in the subgroup of patients tested by flow cytometry (Figure 4) showed low-to-moderate positive correlation of sperm ubiquitination to sperm count, sperm motility and fertilization rates (motility > count > fertilization; sample correlation coefficients $r = 0.337, 0.268$ and 0.046 respectively), while there was a substantial negative correlation ($r = -0.432$) between ubiquitin, flow cytometry median and cleavage rates. Three patients, in whose samples SUTI ruled

out the contribution of male factor (11, 12, 14), had low flow cytometry medians, and average-to-excellent sperm count, sperm motility, fertilization rates and cleavage rates. As a method reflecting the occurrence of morphological abnormalities in individual sperm samples, SUTI may have predictive value for the outcome of IVF treatment, as measured by embryo cleavage rates. Other studies showed that sperm morphology has higher predictive value than other clinical sperm parameters, when strict criteria are applied (Lim *et al.*, 1998; Zinaman *et al.*, 2000). Donnelly *et al.* (1998) reported correlation coefficients between strict morphology and IVF outcome, that are similar to our data on the correlation between ubiquitin medians and cleavage rates. Therefore, ubiquitin data may be closely correlated with abnormal morphology, thus providing a new tool for automated semen analysis. Further tests on larger groups of samples from subfertile patients with known clinical history and previous strict morphology assessments will be performed in order to determine the exact correlation between these parameters. It is not likely that SUTI analysis will be highly predictive of pregnancy rates after IVF, since these are affected by maternal factors independent of sperm quality (e.g. tubal infertility, implantation rate). The tendency towards the increased sperm count and motility in patients with increased sperm ubiquitination could be due to the saturation of the epididymal mechanism responsible for the removal of defective, ubiquitinated spermatozoa.

We have shown previously that sperm accessory structures such as the perinuclear theca, mitochondrial sheath and fibrous sheath are incorporated by the oocyte cytoplasm at fertilization (Sutovsky *et al.*, 1996, 1997). At least in the case of the sperm mitochondria, the constitutional ubiquitination targets them for the destruction by the oocyte's lysosomes (Sutovsky *et al.*, 1999a, 2000a) as soon as they fulfil their fertilization-related functions and become obsolete. It is possible that after natural fertilization and ICSI, the superfluous ubiquitin species present on the surface of both motile and immotile spermatozoa from some subfertile men can be carried over to the oocyte cytoplasm, where they can target the vital structures of such sperm cells towards the oocyte's proteolytic machinery and effectively prevent further embryonic development by decomposing the paternally-contributed zygotic components such as centrosome and male pronucleus. The presence of basal ubiquitin levels on the surface of normal sperm's equatorial segment and sperm tail end piece may be a potential target for immuno-contraceptives. The manipulation of epididymal ubiquitination may provide the means for inducing contraceptive epididymal infertility. It was proposed that the induction of sperm tail coiling, the so called 'Dag defect' originally described in infertile bulls (Blom, 1966), during epididymal passage, may lead to a new contraceptive approach (Cooper *et al.*, 1988). The present study suggests that coiled tails are the primary target of epididymal ubiquitination and previous studies showed that the increased percentage of sperm tail abnormalities correlates negatively with fertilization rates (Lim *et al.*, 1998). Ubiquitination may also play an important role in the disposal of spermatozoa and spermatid granula (Flickinger, 1982) after vasectomy.

In summary, the objective immunoassay of sperm-surface

ubiquitination provides a promising new approach to semen evaluation in humans. In contrast to the existing, subjective methods of semen evaluation discussed by others (Amann, 1989; Coetzee *et al.*, 1999a,b), SUTI assay may provide a reliable, objective and measurable parameter of abnormal spermatogenesis and suboptimal semen quality acquired prior to sperm discharge from urogenital tract. Of particular importance is the ability of the SUTI assay to detect male factor in some cases of infertility, previously pronounced idiopathic.

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