Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology laboratory clinic population

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BACKGROUND: Ubiquitin, a house-keeping protein that marks other proteins for proteasomal degradation, tags defective sperm during epididymal passage. To establish ubiquitin as a biomarker of human infertility, the present study examines the relationships between sperm ubiquitin content and clinical semen parameters among men from an infertility clinic population with varied aetiologies. METHODS: Anti-ubiquitin immunoreactivity was measured by flow cytometric sperm-ubiquitin tag immunoassay (SUTI) in sperm samples of 28 infertility patients and 15 fertile donors. Semen analyses were performed by computer-assisted semen analysis and World Health Organization morphology. RESULTS: Median values of ubiquitin-induced fluorescence had a strong negative correlation with sperm count ($r = -0.63$, $P = 0.0003$) and a positive correlation with % abnormal morphology ($r = 0.55$, $P = 0.01$). Infertility patients ($n = 28$) had significantly higher levels of sperm ubiquitin. Out of 28 patients, six reported possible occupational exposures to solvents, three were current smokers and six were ex-smokers. Within the patient group, men with known male factor infertility, those with self-reported occupational exposure to solvents and current smokers had the highest sperm ubiquitin levels. When men with jobs involving potential occupational exposure to solvents were combined with current smokers, the highest correlations were found between sperm ubiquitin and motility ($r = -0.74$), count ($r = -0.82$) and % sperm abnormalities ($r = 0.73$). CONCLUSIONS: Increased sperm ubiquitin was inversely associated with sperm count, motility and % normal morphology, supporting the use of ubiquitin as a biomarker of human semen quality. SUTI assay confirmed poor semen quality in all men with poor clinical semen parameters, but also was high in some patients with seemingly good clinical semen parameters. Occupational exposure to solvents and smoking may have contributed to high levels of sperm ubiquitin in some of these patients.

Key words: male infertility/sperm/SUTI assay/toxicology/ubiquitin

Introduction

Semen evaluation for human male infertility and assisted reproduction relies on conventional measures of light microscopic evaluation of sperm count, morphology and motility [Kruger *et al*., 1987; World Health Organization (WHO), 1987, 1992, 1999], and provides useful, yet somewhat limited, clinical information in couples seeking infertility treatment. The correlation between clinical semen parameters and fertility is moderate to low (Guzick *et al*., 2001). For instance, sperm with intrinsic defects may appear morphologically normal upon light microscopic evaluation (Nikolettos *et al*., 1999) and there is substantial inter-laboratory variability in both subjective and automated morphological semen analysis (e.g. Neuwinger *et al*., 1990; Jørgensen *et al*., 1997; Coetzee *et al*.,

1999). Therefore, additional objective techniques for semen evaluation, not based on conventional measures of semen parameters, are sought after (Amann, 1989; Douglas-Hamilton, 1995; Krause, 1995).

Being the universal proteolytic chaperone, the high expression of ubiquitin accompanies many pathological conditions such as apoptosis, Alzheimer's disease and inflammatory disease (reviewed by Glickman and Ciechanover, 2002; Sutovsky, 2003). Ubiquitin, an 8.5 kDa highly conserved protein, forms covalently linked polyubiquitin chains on substrate proteins and targets such 'ubiquitinated' substrates for endocytosis and/or proteolytic degradation by the multi-subunit protease, the 26-S proteasome (reviewed by Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002).

While numerous ubiquitinated proteins could be carried over from the testis (reviewed by Escalier, 2003), the bulk of surface ubiquitination of defective sperm occurs during epididymal passage in both animals (Sutovsky *et al*., 2001a; Sutovsky, 2003) and humans (Dalzell *et al*., 2003). The extracellular ubiquitination pathway in the epididymal lumen is now being deciphered. Several independent reports have shown that ubiquitin is secreted by the principal cells of the epididymal epithelium into the epididymal lumen, where it can mingle with the passing-through sperm (Santamaria *et al*., 1993; Fraile *et al*., 1996; Hermo and Jacks, 2002; Sutovsky, 2003; Sutovsky *et al*., 2001a, 2003). Ubiquitin is also a major protein of human seminal plasma (Lippert *et al*., 1993). Ubiquitin-conjugating enzyme E2 (Sutovsky *et al*., 2000), ubiquitin C-terminal hydrolase PGP9.5 (Sutovsky, 2003) and the valosin-containing protein VCP93 required for the presentation of ubiquitinated proteins to proteasome (Geussova *et al*., 2002) are expressed in mammalian sperm. Furthermore, mRNAs encoding for ubiquitin and various proteasomal subunits are highly abundant in the epididymis (Jervis and Robaire, 2001, 2002). The proteasomal subunits are particularly enriched in the cytoplasm of the endocytotic, clear cells of the epididymal epithelium (P.Sutovsky *et al*., unpublished data), a finding consistent with the proposed role of ubiquitin system in the removal of defective sperm and debris during epididymal passage (Sutovsky *et al*., 2001a, 2003). The clearance of defective sperm and debris in the epididymis is not complete, although the number of defective cells decreases appreciably during epididymal passage. Intraluminal liquefaction, phagocytosis and fragmentation of epididymal sperm have been described (Flickinger, 1982; Barrat and Cohen, 1987; Barth and Oko, 1989). Phagocytosis by resident leukocytes or by specialized epithelial cells, as well as luminal liquefaction and epithelial endocytosis, could contribute to such sperm removal (reviewed by Sutovsky, 2003).

Based upon the above observations, we developed an objective immunoassay (SUTI or sperm-ubiquitin tag immunoassay; Sutovsky *et al*., 2001b), designed to reveal defective sperm regardless of whether or not their defects are detectable by light microscopic evaluation. Increased levels of sperm ubiquitination were found in men with heritable male infertility syndromes (Rawe *et al*., 2002), but also in several idiopathic infertility cases (Sutovsky *et al*., 2001b). In farm animals (Sutovsky *et al*., 2002), ubiquitin immunoreactivity measured by flow cytometric SUTI assay correlated closely with sperm count and % abnormal morphology, but similar correlations have not been examined in humans. Most sperm with DNA fragmentation, a sign of apoptosis or necrotic sperm degeneration (Sun *et al*., 1997; Sakkas *et al*., 2002), are recognized by anti-ubiquitin antibodies in bulls (Sutovsky *et al*., 2002) and humans (P.Sutovsky, unpublished data). The present study aims to further validate sperm ubiquitin as a biomarker of human male infertility in a group of 43 fertile donors and infertility patients with various aetiologies. This study is part of a series of trials that will focus on small, yet statistically informative groups of patients from several collaborating infertility centres.

Materials and methods

Semen samples

The study was approved by the Harvard School of Public Health and Massachusetts General Hospital (MGH) Human Subjects Committees, and by the University of Missouri–Columbia Institutional Review Board. All subjects signed an informed consent. Subjects were male partners of subfertile couples who presented to the Vincent Burnham Andrology Laboratory at MGH for semen analysis as part of an infertility work-up. Men presenting for post-vasectomy semen analysis were excluded. Sixty-six per cent of eligible men between 20 and 54 years of age agreed to participate. A questionnaire was used to collect information on medical history and lifestyle habits. The medical history collected information on risk factors that may contribute to poor semen quality. This included questions on testicular torsion surgery, vasectomy and vasectomy reversal, prostatectomy, varicocelectomy, orchiodopexy, cystic fibrosis, diabetes, varicocelectomy, infection of seminal vesicles, prostatitis, epididymitis, cancer, radiation therapy, chemotherapy, injured testicles with/without accompanying increase or decrease of testis size, and mumps before or after puberty. A self-administered detailed occupational history was used to collect information on current and past jobs, as well as exposures at each job. Individuals currently employed in the following jobs were considered to have solvent exposure: mechanic, printer and chemist. Information on female partners of these men was not available.

A semen sample was produced on-site by masturbation into a sterile plastic specimen cup. The sample was allowed to liquefy at 37°C for 20 min prior to analysis. Subjects were instructed to abstain from ejaculation for 48 h prior to producing the semen sample.

We analysed samples for sperm concentration and motion parameters by computer-aided semen analysis (CASA; Hamilton-Thorne Version 10HTM-IVOS). Setting parameters and the definition of measured sperm motion parameters for the CASA were established by Hamilton-Thorne Co. (frames acquired: 30; frame rate: 60 Hz; straightness (STR) threshold: 80.0%; medium VAP threshold: 25.0 µm/s; duration of the tracking time: 0.5 s). To measure both sperm concentration and motility, aliquots of semen samples (5 µl) were placed into a pre-warmed (37°C) Makler counting chamber (Sefi Medical Instruments, Israel). A minimum of 200 sperm from at least four different fields were analysed from each specimen. We defined % motile sperm as WHO grade 'a' sperm (rapidly progressive with a velocity ≥25 µm/s at 37°C) plus 'b' grade sperm (slow/sluggish progressive with a velocity ≥5 µm/s but <25 µm/s).

Sperm morphology was determined by utilizing the WHO (1987) criteria and expressed as % abnormal sperm. Samples were coded so that the donor remained anonymous to the authors of this study.

Semen samples from 15 fertile donors were purchased from Fairfax Cryobank (USA). Fertile donors were non-smoking men with no known history of occupational exposure to toxic substances. All samples, from the fertile and infertile clinic patients, were cryopreserved using conventional techniques and kept in liquid nitrogen until examination.

Flow cytometric SUTI assay

Semen samples from 28 men from couples seeking infertility treatment (nos. 1–30; further 'patients') and 15 fertile donors (further 'donors') recruited and screened by a major sperm bank, were evaluated by the flow cytometric sperm-ubiquitin tag immunoassay (SUTI; Figure 1A–C; Sutovsky *et al*., 2001b). Samples from two remaining patients (nos. 5, 24) were eliminated from the trial due to extremely low sperm count, which did not allow us to perform flow cytometric processing (no visible sperm pellet after centrifugation).

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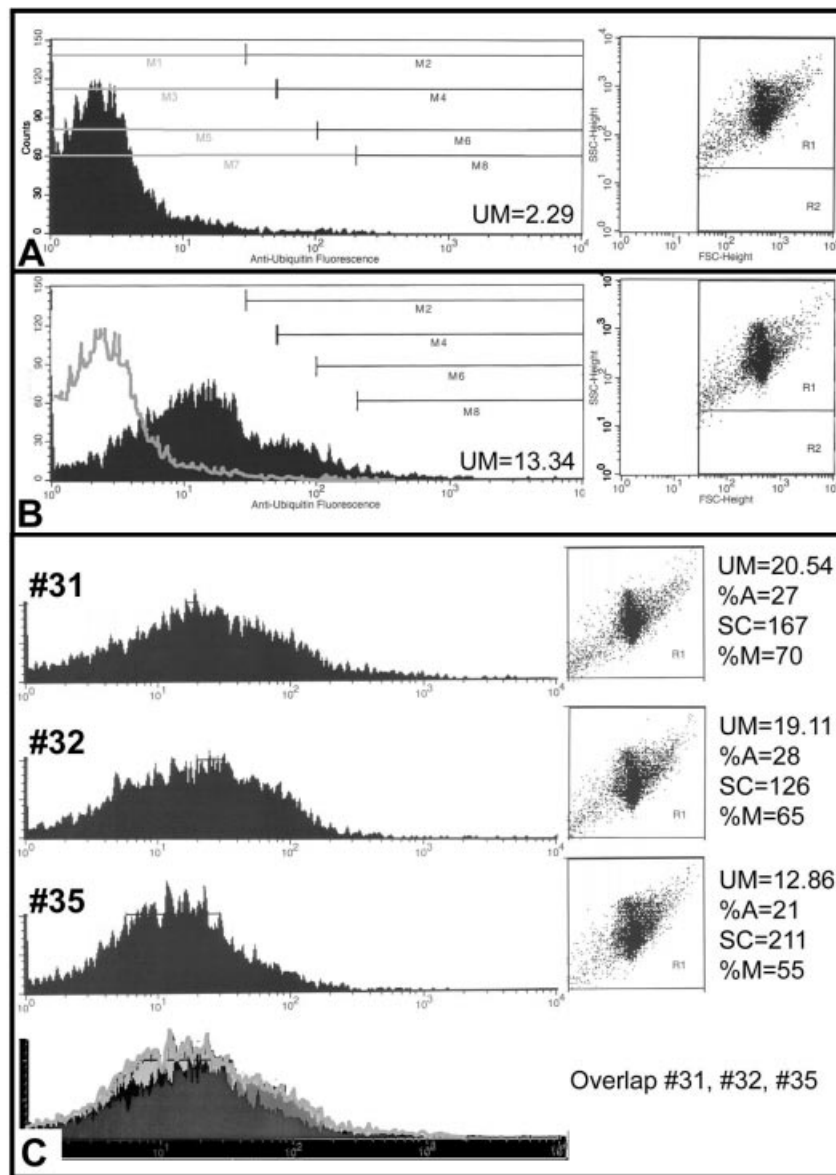
Figure 1. Flow cytometric evaluation of human sperm by sperm-ubiquitin tag immunoassay (SUTI). Sperm samples were processed with anti-ubiquitin antibodies and appropriate green-fluorescent, secondary antibodies, and the relative fluorescence was measured by flow cytometer in 10 000 cells per sample. (**A**) Blank, negative control sample from a fertile donor processed with secondary antibody only. Quartile markers M1–M8 subdivide the screened cell populations in this and all other histograms into high (even marker numbers) and low (odd marker numbers) fluorescent cells. (**B**) Positive sample from the same fertile donor shown in **A**, the histogram of the blank sample (empty curve; corresponds to histogram in **A**) is superimposed here. Note that the scatter diagrams of visible light, indicative of cell size distribution (right box in **A**, **B**), are similar, while the ubiquitin median value (UM) is six times higher in the positive sample. Markers M2, M4, M6 and M8 correspond with the values shown in Figure 3 and Table II. (**C**) Distribution of fluorescently labelled cells in three fertile donors. Donors nos. 31 and 32 were among the highest in % sperm abnormalities by WHO standards (%A) and UM values. Note that visible light-scatter diagrams (box on right) in all three samples are similar, with a tight focus of presumably normal-sized and -shaped sperm in the centre. In scatter diagrams, the dots in the lower left corner represent small cells and cellular fragments/debris, the dots in the upper right corner correspond to very large cells. SC = sperm count ($\times 10^6$); %M = % motile sperm. Each histogram represents 10 000 measured cells. Histograms of these three donors were merged (overlap; bottom of the **C**) to create the standard curve of fertile sperm sample superimposed on patients' histograms in Figure 2.

All patients were of similar age (29–43 years), except for patients no. 18 (48 years) and no. 25 (47 years). The sperm samples from 15 fertile donors (nos. 31–45; aged 20–42 years) were used as standards.

Semen samples were processed with anti-ubiquitin antibody KM 691 (mouse IgM; Kamiya Biomedical, USA) and appropriate secondary antibodies as described previously (SUTI assay; Sutovsky et al., 2001b). Briefly, the samples were thawed and 300 µl of semen was washed by a 5 min centrifugation at 350 g in 10 ml of Sperm TL medium. Sperm pellets were collected and fixed for 40 min in 2% formaldehyde in phosphate-buffered saline (PBS). Fixative was removed, and the pellet was washed by centrifugation in 10 ml PBS and resuspended for 30 min in blocking solution composed of 0.1 mol/l PBS and 5% normal goat serum (NGS; Sigma). All subsequent steps were performed using PBS with 1% NGS as a washing solution and antibody diluent. Samples were incubated with 1/100 solution of KM 691, washed by resuspension and centrifugation 40 min later, then
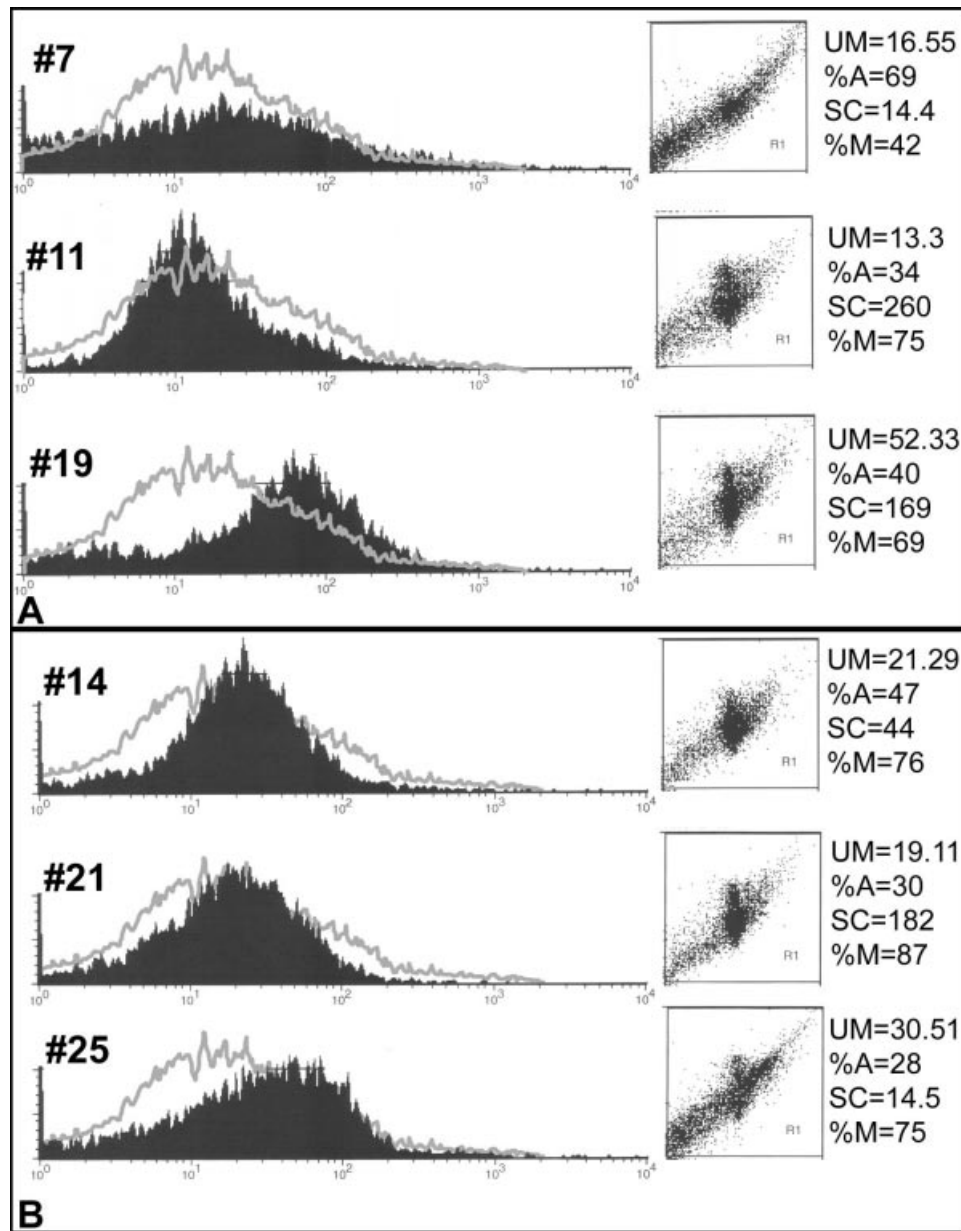
Figure 2. Representative histograms of sperm samples from infertility patients with self-reported history of occupational exposures (**A**) and those who were active smokers at the time of sample donation (**B**). Merged histogram curve from three fertile donors (see Figure 1C) was superimposed on these histograms. Patient no. 7 has low ubiquitin median value (UM), but the histogram (left) is unusually flat and the scatter diagram (right) indicates the prevalence of small cells and cellular debris over the normal-sized sperm. This corresponds with low sperm count (SC; 10⁶/ml semen) and motility (%M), and high % abnormal sperm (%A). Patient no. 11 shows nearly normal parameters, while the UM and %A are high and the fluorescence histogram is shifted to the right in patient no. 19, despite relatively good sperm count and motility. Similar parameters, i.e. increased UM and %A, accompanied by relatively good sperm count and motility, are seen in two current smokers (**B**; patients 14 and 21). The tendency towards high percentage of cellular debris and high UM in smoker no. 25 was accompanied by low sperm count, but not captured by subjective morphological analysis (only 28% of morphologically abnormal sperm by subjective evaluation) performed at the clinic prior to sperm-ubiquitin tag immunoassay evaluation.

incubated for 40 min with green-fluorescent goat anti-mouse IgM–FITC (Zymed Laboratories). After second antibody, samples were washed again and resuspended in 500 µl of ultrafiltrated PBS without serum. Blank, negative control samples were prepared by omitting anti-ubiquitin antibody.

Samples were screened on the day of processing using FACS Calibur flow cytometer (Becton Dickinson, USA), set at 488 nm wavelength. Ten thousand cells were measured in each sample and the overall ubiquitin median values (the value of ubiquitin-induced fluorescence at which half the cells are dimmer and half the cells are brighter) were recorded. To compare fertile men with patients, histograms of ubiquitin-induced fluorescence from three donors, one representing the low and two representing the high ubiquitin median values within the fertile group, were superimposed. The resulting combination curve (Figure 1C) was used as a standard for visual comparison with the patients' samples (Figure 2). In addition to overall Ubi-median values, the histograms were divided into low fluorescent and highly fluorescent, 'positive' cells (Figure 1 B) by

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Table I. Clinical semen parameters for 43 subjects categorized by occupational job title with solvent exposure and smoking habits

	Count ($\times 10^6$/ml)	Motility (%)	Morphology (% abnormal)	Age (years)
1. All patients ($n = 28$)	122.2 ± 89.0	63.7 ± 15.4	42.0 ± 11.4	35.0 ± 5.3
2. Male factor ($n = 5$)	10.3 ± 6.0*	49.8 ± 17.3*	51.0 ± 18.3	37.6 ± 6.4
3. Self-reported solvent exposure ($n = 6$)	113.1 ± 101.7	58.8 ± 19.0	**51.8** ± 15.2	35.5 ± 3.7
4. No reported solvent exposure ($n = 22$)	124.6 ± 87.0	62.2 ± 14.7	39.4 ± 8.1	34.9 ± 5.8
5. Current smokers ($n = 3$)	80.3 ± 89.4	**79.3** ± 6.7	35.0 ± 10.4	36.6 ± 9.3
6. Former smokers ($n = 6$)	**152.5** ± 99.0	65.2 ± 13.0	37.2 ± 4.9	**37.0** ± 7.6
7. Never smoked ($n = 19$)	111.7 ± 86.2	61.2 ± 16.2	45.3 ± 12.4	34.1 ± 3.6
8. Current smokers and solvent exposure ($n = 9$)	102.2 ± 93.5	65.7 ± 18.5	46.2 ± 15.6	35.9 ± 5.5
9. Fertile donors ($n = 15$)	141.0 ± 40.8	62.7 ± 5.3	24.3 ± 3.3*	30.3 ± 6.8*

Values are mean ± SD.
All fertile donors ($n = 15$) were non-smokers with no history of toxic chemical exposure. Patients reporting possible occupational exposures ($n = 6$) are identified as 'solvent-exposed'. Patients with abnormally low sperm counts and/or motility are identified as 'male factor'. Rows 1–8 refer to infertility patients and patients may contribute data to more than one group. Asterisk denotes lowest value in column. The highest value in each column is in bold type.

quartile markers M2, M4, M6 and M8. For each of those markers/ subpopulations, values in a complementary, remaining subpopulation of low fluorescent cells (markers M1, M3, M5 and M7) were also measured. Therefore, the M1 population represented the cells on the left of the threshold, complementary to M2-cell population to the right of the same threshold. M3 was then complementary to M4, M5 to M6 and M7 to M8. Blank, negative control samples from fertile donors and patients were processed with secondary antibodies only and showed low levels of fluorescent signal (Figure 1A, B). Blank samples were measured in the same runs under identical conditions. Scatter diagrams of visible light, indicative of cell size distribution (Figures 1 and 2) were also evaluated.

Statistical analysis

The data were entered into Microsoft Excel tables and analysed by statistical analysis tools and SAS 8.2 statistical package. Pearson's correlation coefficients (r) and the P-values were computed for ubiquitin medians, % ubiquitin-immunoreactive cells and clinical data (sperm count, motility % abnormalities, age; Tables I and II).

Results

Subject demographics

The study population consisted of 43 men, 28 of whom were partners in an infertile relationship and 15 were fertile donors. The age of the 28 male partners of an infertile relationship ranged from 29 to 48 years (mean 35 ± 5.3), while the age of the 15 fertile donors ranged from 20 to 42 years (mean 30.3 ± 6.8). Nine male partners in infertile relationships had medical histories for risk factors for infertility. By WHO criteria, only five patients had abnormally low sperm counts ($<20 \times 10^6$/ml). Only one patient had poor motility by WHO standards (i.e. <40% motile sperm), though we considered three other patients to have marginal, yet acceptable, sperm motility.

Flow cytometric evaluation of sperm ubiquitin

With one exception (donor no. 44; high ubiquitin medians, shifted histogram), sperm samples from fertile donors invariably showed normal, Gaussian distribution of fluorescent cells (Figure 1A–C), low ubiquitin medians (Table I) and normal clinical semen parameters (Table I). Visible light-scatter diagrams (Figures 1 and 2) in donors invariably showed a

tight focus of presumably normal-sized and -shaped sperm in the centre of the diagram. Within the patient group, the five patients with known male factor (abnormally low sperm count and motility by WHO criteria) had the highest average of ubiquitin median values for M2, M4, M6 and M8. Six patients with a self-reported history of occupational exposure had the highest overall median values of ubiquitin-induced fluorescence (Table I), followed by male factor patients, active smokers and ex-smokers. Male factor patients had highest ubiquitin medians M2, M4, M6 and M8. High ubiquitin levels were also recorded when the group of men with possible occupational exposures was combined with current smokers. Four of the six patients with self-reported occupational exposures had high ubiquitin medians and high % sperm abnormalities, though none of them was aged >40 years. In such samples, the histograms of ubiquitin-induced fluorescence were shifted to the right, reflecting an increase in the number of highly fluorescent, highly ubiquitinated sperm. In many of those patients, high proportion of cellular debris, documented by a large number of dots in the lower left corner of light-scatter diagrams (Figure 2; patients nos. 7 and 25), and lower sperm count were found. Importantly, in some patients with high sperm ubiquitin, the implied poor sperm quality was not revealed by subjective morphological analysis (e.g. only 28% of abnormal sperm, comparable with most fertile donors, were reported in patient no. 25; Figure 2B). Some of the patients (e.g. patient no. 7; Figure 2A) had low overall ubiquitin medians, but their histograms were unusually flat and the scatter diagrams indicated the prevalence of small cells and cellular debris over the normal-sized sperm. This corresponded with low sperm count and motility, and high % abnormal sperm. Some other patients showed atypical distribution of fluorescent cells with a plateau and a distinct peak in their histogram (e.g. Figure 2A; patient no. 19). Finally, some patients (e.g. Figure 2A; patient no. 11) had nearly normal clinical semen parameters and low ubiquitin medians, suggesting that they were fertile men from infertile couples with female factor infertility. We were unable to confirm this assumption since we did not collect data on the female partners of our patients, or on the outcome of infertility treatment.
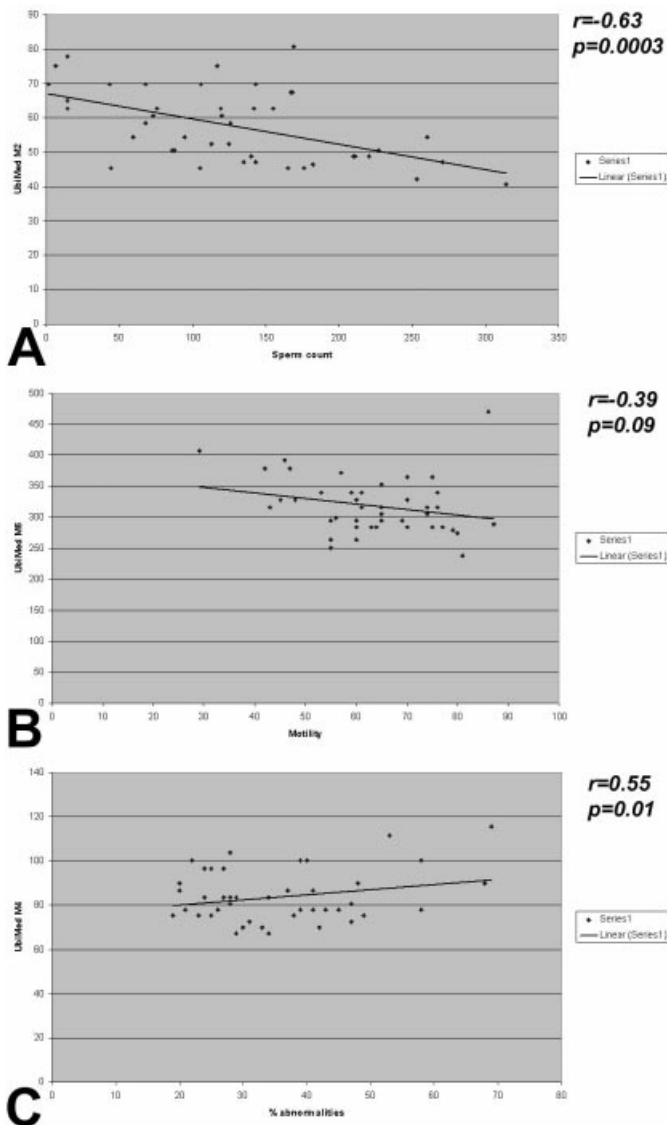
Figure 3. Distribution of ubiquitin median values in 28 men from couples seeking infertility treatment, and 15 fertile donors. Sperm count (**A**) and motility (**B**) decline proportionally with the increased ubiquitin medians, reflecting the increased expression of ubiquitin protein in defective sperm. Ubiquitin medians grow proportionally with the increased percentage of abnormal sperm (**C**), evaluated subjectively by WHO standards. Sample linear regression lines are shown; r = Pearson's correlation coefficient. Values M2, M4 and M6 correspond with markers in Figure 1A and Table II.

Relationship of the sperm ubiquitin values and clinical semen parameters

In general, the ubiquitin median values showed strongest correlations with clinical data when they were taken from cell subpopulations determined by the quartile markers M2, M4, M6 and M8 rather than from the overall sample median measured in all screened cells without arbitrary subdivision. In the pooled analysis of all 43 subjects, ubiquitin median values increased with the declining sperm count (Figure 3A) and motility (Figure 3B), and increased proportionally with an increase in the percentage of abnormal sperm, evaluated by

WHO criteria (Figure 3C), showing moderate-to-stronger correlations with sperm count ($r = -0.63$), % abnormalities ($r = +0.55$) and motility ($r = -0.39$). There was no correlation between donor age and either ubiquitin medians or % abnormalities. When fertile donors (men nos. 29–43) were evaluated separately from patients, ubiquitin medians correlated well with sperm count ($r = -0.41$) and motility ($r = -52$). In the patient subgroup (men nos. 1–28), the highest correlations were found between ubiquitin medians and sperm count ($r = -0.52$). Similarly, ubiquitin medians correlated moderately with % abnormalities ($r = +0.37$) and to a lesser extent with motility ($r = -0.26$) and age ($r = -0.28$). Clinical sperm morphology (% abnormal sperm by WHO criteria) correlated moderately with sperm count ($r = -0.47$) and motility ($r = -0.33$) in all subjects. The correlation between sperm count and motility was also moderate ($r = 0.40$). Overall, the correlation coefficients for clinical semen parameters with ubiquitin were higher than those among individual clinical semen parameters. Smaller correlations between individual clinical parameters were found in subgroups of fertile men and infertility patients.

The strongest correlations between ubiquitin medians and clinical sperm parameters were found when the six men with job titles that indicated possible solvent exposure were combined into one group with three currently smoking patients. These were two subgroups of patients with aetiologies known to contribute to human male infertility and to reduce sperm quality. Strong correlations were found between sperm ubiquitin and sperm count ($r = -0.82$), motility ($r = -0.74$) and % sperm abnormalities ($r = 0.73$). Ubiquitin median M6 also seemed to increase with age in this subgroup of patients ($r = 0.55$). Similar to the comparison of ubiquitin median values with sperm parameters, the correlations between individual sperm parameters were strongest within this subgroup of patients. The absolutely highest correlation coefficient in this whole study was found between sperm motility and % abnormalities within this group ($r = -0.89$).

Evaluation of the relationship between medical history, clinical semen analysis and SUTI results

Diagrams summarizing clinical and SUTI measurements in 10 selected patients and five donors are shown in Figure 4. Average ubiquitin median M8 of male factor patients, defined as having abnormally low sperm counts and/or motility, was 383.0, compared to 331.0 for current smokers, 327.0 for men with self-reported solvent exposures and 301.9 for fertile donors (Table II). The P-values were $P = 0.01$ for ubiquitin comparison between exposed and non-exposed patients, $P = 0.05$ between non-exposed patients and donors, and $P = 0.05$ between exposed patients and donors. According to clinical semen analysis that accompanied the samples (Table III), none of the patients would be considered infertile due to poor sperm morphology, expected to be >20% of normal sperm in a normal, fertile semen sample. It should also be considered that many sperm abnormalities revealed by SUTI analysis are not readily detectable by subjective morphology evaluation under the light microscope (Sutovsky et al., 2001b, 2002). Five patients had a history of testicular injury. Of those five patients, two also indicated a change in testicular size following the

Table II. Average sperm ubiquitin median values for 43 subjects categorized by occupational job title with solvent exposure and smoking habits

	Median overall	M2	M4	M6	M8
1. All patients ($n = 28$)	20.0	56.3	82.6	149.6	327.0
2. Male factor ($n = 5$)	21.9	**70.0**	**97.4**	**169.0**	**383.0**
3. Self reported solvent exposure ($n = 6$)	**25.6**	66.2	93.1	156.6	325.5
4. No reported solvent exposure ($n = 22$)	18.5	53.6	76.3	147.7	327.4
5. Current smokers ($n = 3$)	23.6	52.2	75.2	140.5	331.3
6. Former smokers ($n = 6$)	19.7	51.5*	75.2*	131.8*	302.0
7. Never smoked ($n = 19$)	20.2	59.4	87.2	158.1	333.9
8. Current smokers and solvent exposure ($n = 9$)	24.9	61.6	87.0	151.2	327.4
9. Fertile donors ($n = 15$)	18.0*	59.7	86.0	145.1	302.0*

Overall ubiquitin medians (Median overall) of the whole sperm samples, and the flow cytometric ubiquitin medians of cell subpopulations divided by quartile markers M2–M8 (see Figure 1), are shown.

All fertile donors ($n = 15$) were non-smokers with no known history of toxic chemical exposure, as specified by the distributors of samples. The highest value in each column is in bold type. Patients reporting possible occupational exposures ($n = 6$) are identified briefly as 'exposure'. Patients with abnormally low sperm counts and/or motility are identified as 'male factor'. Lines 1–8 refer to infertility patients and patients may contribute data to more than one group. Asterisk denotes lowest value in column. The highest value in each column is in bold type.

injury. Four patients had mumps before puberty. All other patients had normal clinical semen parameters by WHO criteria and no indication of medical history with potential to affect reproductive functions.

Discussion

In the present study, the sperm ubiquitin median values from 28 infertility patients and from 15 fertile donors, correlated negatively with sperm count, motility, and normal morphology values obtained by conventional semen analysis. High sperm ubiquitin levels were found in all five patients with sperm count and/or motility below WHO standards, in four of the six men with self-reported workplace exposure to solvents, and in several of the current smokers. Finally, a number of patients with acceptable semen characteristics by WHO standards showed increased sperm ubiquitin levels potentially indicative of poor semen quality/male infertility. Reduced sperm count, reduced motility, increased % abnormal sperm, smoking habits and self-reported workplace solvent exposure were among the factors that corresponded to increased sperm ubiquitin levels.

Reduced sperm count of high ubiquitin sperm samples could be a result of increased sperm degradation in the epididymis, as described previously (e.g. Flickinger, 1982; Barrat and Cohen, 1987; Barth and Oko, 1989). We have shown in animal models that the overall proportion of the surface-ubiquitinated, defective sperm decreases during epididymal passage, suggesting that some defective sperm undergo passive breakdown and/or active proteolysis during their descent from caput to cauda epididymis (Sutovsky et al., 2001a). Alternatively, the reduction of the sperm count in the infertility patients could be a result of an overall reduction in sperm output by apoptosis of germ cells in the testis (Maeda et al., 2002), or a combination of testicular and epididymal factors. In the present study, we found an increase in the proportion of small cells/cellular debris in the samples with high ubiquitin levels. Our previous studies indicate that such debris is mostly of spermatogenic origin (cytoplasmic droplets, residual bodies and fragments thereof; Sutovsky et al., 2001b). It is thus possible that instead of reduced testicular output, there is an increase in sperm degradation rates in the epididymis, where the binding of

ubiquitin to defective sperm occurs. Recent studies of the apoptotic process in the testis and epididymis suggest that the bulk of DNA fragmentation, suggestive of apoptosis, is seen in the epididymal sperm, rather than in the testis (Sakkas et al., 2002). A feasible explanation is that the apoptosis could be induced during the final steps of spermiogenesis, while the fragmentation of DNA, a late/terminal apoptotic event, only occurs once the cells are relocated to the epididymis. Alternatively, necrosis, rather than caspase-mediated apoptosis, could be occurring in the epididymal sperm.

Motility is a highly variable semen characteristic that may reflect the sample quality/fertility to some extent. However, sperm motility declines significantly with time between sample donation and evaluation, and within the length of time for evaluation of motility (Elliason, 1981; Drobnis, 1992; Jørgensen et al., 2001). It is therefore not surprising that both clinical sperm morphology values and ubiquitin values show smaller correlations with motility than with sperm count or sperm morphology. Others noted that while there is significant overlap between clinical semen parameters of fertile and infertile men, poor sperm morphology is a stronger infertility predictor than poor motility (Guzick et al., 2001).

Increased sperm abnormalities, assessed by subjective light microscopic analysis, should and do correlate positively with ubiquitin median values. However, the anti-ubiquitin antibodies bind to many sperm with intrinsic, hidden defects that would not be detected by light microscopic evaluation (Sutovsky et al., 2001b, 2002). Morphology in the present study showed slightly better correlation with the sperm motility than with the sperm count, which can be expected because of two reasons. First, secondary sperm defects such as sperm coiling occur in some samples concomitantly with the decline in sperm motility in the period between sample donation and clinical evaluation. Such defects are not a result of abnormal spermatogenesis and thus are not recognized by SUTI assays. Second, sperm motility is less reflective of fertility than sperm count or sperm morphology (Guzick et al., 2001).

The increased ratio of defective sperm in some patients could also be explained by the reduced capacity of the epididymis for their removal. Epigenetic factors and ageing
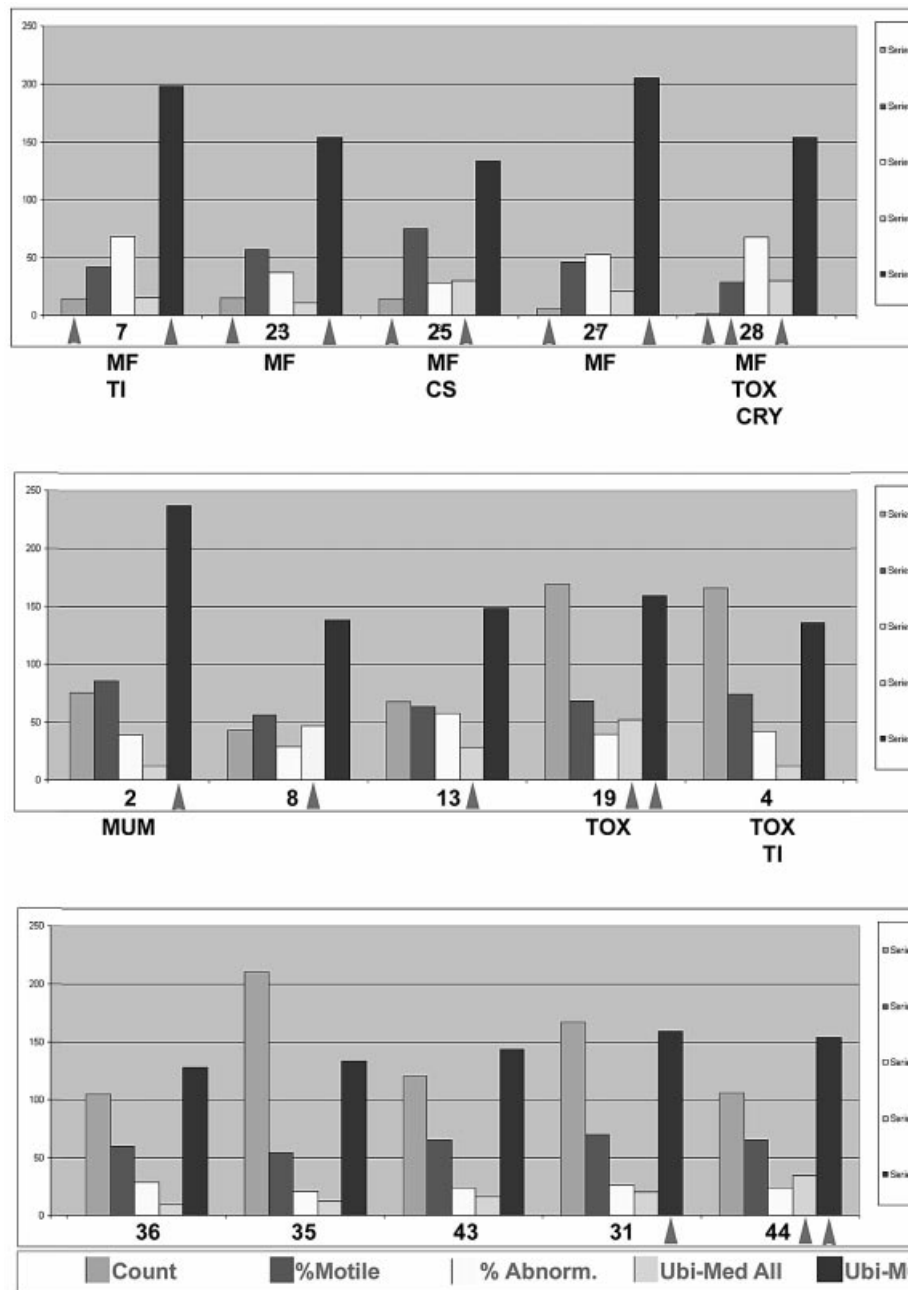
Figure 4. Diagrams summarizing clinical semen parameters (sperm count, motility and % abnormalities) and ubiquitin median values (overall ubiquitin median = Ubi-Med All) in five presumed male factor patients with inferior sperm count by WHO criteria (top row), five patients with acceptable clinical parameters (middle row; only patient no. 4 shows low ubiquitin values), and in five fertile donors (bottom row; donor no. 44 was the only man in the fertile group showing high ubiquitin levels; donor no. 31 had somewhat elevated ubiquitin median M6, all other donors showed low ubiquitin medians). MF = male infertility; TI = testicular injury; CS = current smoker; TOX = self-reported solvent exposure; CRY = unilateral cryptorchidism; MUM = mumps prior to puberty.

can influence gene expression in the epididymal epithelium and the maturation of epididymal sperm (Robaire and Serre, 2000; Jervis and Robaire, 2002) by reducing transcription of specific genes in the epididymal cells (Pera *et al.*, 1996). For instance, the transcription of genes related to ubiquitin-proteasome pathway, mainly those encoding for various proteasomal subunits, is markedly reduced in the rat epididymis during ageing (Jervis and Robaire, 2002). Our new data demonstrate that the proteasomal subunits are most prominently expressed

in the epididymal clear cells, responsible for the removal and degradation of proteins and cellular debris from the epididymal epithelium (P.Sutovsky *et al.*, unpublished data). Thus the effect of occupational exposure and age on the epididymal sperm could be mediated by the down-regulation of specific mRNA for proteasomal subunits in the epididymal epithelium.

We have found some of the strongest correlations of ubiquitin levels and clinical sperm parameters in the respective subgroups of patients that were current smokers and patients

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Table III. Clinical and sperm-ubiquitin tag immunoassay (SUTI) evaluation of 28 men from infertile couples

Pat#/MF	SC	%M	%A	S	Tox	Medical History/ Indications of Infertility.	Ubi Med			SUTI		SUTI diagnosis/notes*
							All	M6	M8	HIS	SCA	
1	94.6	63	38	F	No	None	14.86	128.64	283.87	M+S	S	C (multipeak histogram; cells)
2	75.5	86	39	N	No	Mumps before puberty	12.19	237.14	469.76	F	S	C (high M6, M8)
3	270.7	80	31	N	No	None	16.55	133.35	273.84	G	M	F (all parameters satisfactory)
4	165.2	74	42	F	Y	Test. injury w. change in test. size	12.41	135.77	305.5	G	M	F (all parameters satisfactory)
6	72.8	48	48	N	No	None	22.07	159.63	327.81	S	S+L	C (cells present, high M8)
7MF	14.4	42	69	N	Y	Low sperm quality in prev .inf. exam; test injury w. change in test. size.	15.96	198.1	378.55	F	S+L	C (high M6, M8)
8	43.5	56	29	F	No	None	46.98	138.24	299.61	G+S	M	C (high M-All)
9	119.6	76	41	N	No	None	25.48	153.99	316.23	G+S	M	C (high M-All)
10	209.8	79	39	F	No	Mumps before puberty	15.4	128.64	278.81	G	T	F (all parameters satisfactory)
11	260.3	75	34	N	Y	Test. injury	13.34	143.3	283.87	G	M	F (all parameters satisfactory)
12	176.1	81	33	N	No	None	9.31	130.97	237.14	G	M	F (all parameters satisfactory)
13	67.9	64	58	N	Y	None	28.39	148.55	283.87	F	S+L	C (high M-All, flat histogr.)
14	44.4	76	47	C	No	Low sperm quality in prev. inf. exam	21.29	159.63	339.82	G	S	C (high M6; cells)
15	113	77	58	N	No	Low sperm quality in prev. inf. exam	14.86	133.35	283.87	F	S	C (flat histogram, small cells)
16	87.7	45	41	F	No	None	17.78	128.64	327.81	G/F	M	C (high M6, flat histogram)
17	314.1	74	34	F	No	None	10	130.97	316.23	G	M	F (all parameters satisfactory)
18	59.2	59	47	N	No	None	26.42	171.54	339.82	G+S	M	C (high M-All, M6, M8)
19	169.3	69	40	N	Y	None	52.33	159.63	294.27	G+S	S+L	C (high M-All; cells present)
20	142.9	43	45	N	No	None	14.33	133.35	316.23	G	M	F (all parameters satisfactory)
21	182.1	87	30	C	No	Test. injury; Mumps before puberty; Low sperm quality in prev. inf. exam	19.11	128.64	289.03	G	M	F (all parameters satisfactory)
22	86	47	43	N	No	None	12.86	138.24	378.55	F	S	C (high M6)
23 MF	14.8	57	37	N	No	None	10.75	153.99	371.8	F	S	C (flat histogr.; small cells)
25	14.5	75	28	C	No	None	30.51	133.35	365.17	S	S	C (high M6, shifted)
26	253.3	53	34	M	No	None	8.06	130.97	339.82	G	M	C (high M6)
27 MF	6.3	46	53	N	No	None	21.29	205.35	392.42	F	S	C (high M6)
28 MF	1.7	29	68	N	Y	unilat. cryptorchism at age 7	30.51	153.99	406.79	F+S	S	C (high M-All, M8)
29	139.9	61	49	N	No	Test. injury; Mumps before puberty	21.29	143.3	316.23	G	M	F (somewhat elevated M8)
30	220.7	61	23	N	No	None	13.82	148.55	339.82	G	M	C (elevated M8)

Sperm count values that are defined as infertility by WHO ($>20\times10^6$/ml) are underlined along with sperm morphology and motility values that are normal by WHO criteria (>40% motile; >20% normal morphology), yet considered marginal (<50% motile; <60% normal morphology), in the present study.
MF = indication of male infertility based on clinical semen analysis (sperm count $<20\times10^6$/ml and or motility <40%); SC = sperm count ($\times10^6$/ml); %M = % motile sperm; %A = % abnormal sperm by WHO standards; S = smoking (C = current smoker; F = former smoker; N = never smoked); Tox = self-reported toxic exposure (Yes/No) SUTI HIS = SUTI histogram (G = Gaussian distribution; F = flat; S = shifted; M = multi-peak); SUTI SCA = SUTI scatter diagram (T = tight focus; M = marginally tight; L = large cells present in semen; S = small cells/debris present in semen; M = missing information).
*F = fertile; C = compromised sperm quality

with job titles with suggested occupational exposure to solvents. When evaluated separately, the highest average ubiquitin median values, highest % of ubiquitin-positive cells, the highest % of morphological abnormalities by WHO standards, lowest motility and low sperm counts were recorded in the group of patients with job titles with solvent exposure.

Current smokers ranked second highest on ubiquitin median, had lowest sperm count, and high % ubiquitin-positive cells. Pooled evaluation of current smokers and potentially exposed workers yielded the overall highest correlation coefficients between ubiquitin median values and clinical sperm parameters. The link between male infertility and smoking or occupational exposure has been established by a number of studies (e.g. Rubes *et al.*, 1998; Moline *et al.*, 2000). Using TUNEL assay, Sun *et al.* (1997) reported higher incidence of DNA fragmentation in smokers. Surprisingly, this study and others (e.g. Wallock *et al.*, 2001) showed higher sperm motility in smokers. Adelusi *et al.* (1998) even suggested that smoking may actually increase sperm motility in subfertile men. This could potentially lead to misdiagnosis if clinical motility data are taken into account as a determinant of fertility. Ubiquitin assay, in contrast, clearly recognized semen abnormalities in several patients who had high ubiquitin levels despite having good sperm motility.

In addition to epigenetic factors, the pre-existing conditions possibly affecting the male reproductive system could also affect sperm quality and sperm ubiquitin levels. Two 'high ubiquitin' patients with normal or poor clinical semen parameters reported testicular injury (no. 7), pre-pubertal mumps (no. 2) and one of the five low count/high ubiquitin patients suffered from unilateral cryptorchidism (no. 28). Other patients reporting testicular injury (nos. 4, 11, 21) and mumps (nos. 10, 21) had low ubiquitin levels and good clinical semen parameters. We have observed increased ubiquitin levels in all five patients with previous indication of poor sperm quality (nos. 7, 23, 25, 27 and 28; low count in all five patients, combined with low motility in patient no. 28). Thus, SUTI assay clearly identified men with obvious male factor. Overall, neither the subjective morphology nor ubiquitin values showed strong correlations with age. However, most subjects of this study were in their prime reproductive age (average age 35 years for patients, 30 years for donors). Some of the fertile donors were in their low 20s, thus complicating the evaluation of the effect of age on ubiquitin in this group. Count, motility and % abnormal sperm did not correlate with age in any of the evaluated populations (patients, fertile donors, patients and donors together).

Our previous studies showed increased sperm ubiquitin levels in infertility patients with male factor and idiopathic infertility (Sutovsky *et al.*, 2001b) and in teratospermic men suffering of heritable infertility syndrome (FSD; Rawe *et al.*, 2002). In the present study, the relative measures of sperm ubiquitin content correlated closely with sperm count and motility in a mixed population of infertility patients with varied aetiologies. While some of the correlations and averages in the present study are limited by unbalanced and small numbers of subjects in subgroups, they provide useful preliminary data for possible studies in reproductive toxicology and epidemiology. Even more importantly, measures of relative sperm ubiquitin levels appear to be more informative than the evaluation of sperm morphology by WHO criteria, as they correlate better with sperm count and motility.

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