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Clinical adaptation of the sperm ubuquitin tag immunoassay (SUTI): relationship of sperm ubiquitylation with sperm quality in gradient-purified semen samples from 93 men from a general infertility clinic population

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BACKGROUND: The proteolytic chaperone peptide ubiquitin accumulates in defective human spermatozoa. Immunodetection of ubiquitin in human sperm samples correlates with semen quality and male fertility. METHODS: Semen samples from 93 men from couples seeking infertility treatment were separated on a PureSperm density gradient and screened by immunofluorescence microscopy with anti-ubiquitin antibodies. The percentage of spermatozoa with head ubiquitylation was recorded and compared with clinical semen evaluation and embryo development data after IVF or ICSI. Subjects were divided into the following four groups based on the initial clinical diagnosis of the couples; group 1, male factor; group 2, idiopathic infertility; group 3, female infertility with neither partner having children previously; and group 4, female infertility with male partners having children from previous relationships. RESULTS: The percentage of sperm with ubiquitylated heads remaining after PureSperm separation in the respective groups was 4.0% (male factor), 2.5% (idiopathic infertility), 0.7% (female infertility and presumed fertile male) and 0.9% (female infertility with established fertile male). Negative correlations between sperm ubiquitin and several parameters reflective of embryo development after assisted fertilization were found within the male factor group. CONCLUSIONS: Use of this simplified ubiquitin-based sperm quality assay is feasible in a clinical environment. Since the gradient separation does not completely deplete the defective spermatozoa, the modified light microscopic sperm ubiquitin tag immunoassay could add a new level of stringency to the selection of human spermatozoa for ICSI.

Key words: andrology/infertility/male factor/sperm/ubiquitin

Introduction

Ubiquitin is a small proteolytic polypeptide that conveys the signal for the degradation of various substrate proteins by the 26S proteasome (Goldstein *et al.*, 1975; Ciechanover, 1994). Ubiquitin binds to lysine residues of a substrate protein and forms long polyubiquitin chains through covalent ligation of additional ubiquitin molecules to one or more of ubiquitin's seven lysines (Pickart, 1998; Peng *et al.*, 2003). The ubiquitin system has been implicated in the control of cellular functions as varied as proteolysis, antigen presentation, membrane receptor endocytosis, transcriptional control, apoptosis and pathology of various diseases including Alzheimer's and HIV viral infection (reviewed by Glickman and Ciechanover, 2002). The reproductive functions of ubiquitin are just now starting to emerge, including the regulation of oogenesis and follicle growth, the menstrual cycle, early pregnancy, spermatogenesis, epididymal sperm maturation, fertilization and zygotic development (reviewed by Bebington *et al.*, 2001; Sutovsky, 2003). Specifically, ubiquitylation facilitates histone–protamine replacement (Chen *et al.*, 1998; Baarends *et al.*, 1999) and the formation of sperm axoneme (Escalier *et al.*, 2003) during spermatogenesis. Apocrine secretion of ubiquitin in the epididymis (Sutovsky *et al.*, 2001a; Hermo and Jacks, 2002) may mark defective spermatozoa for degradation (Sutovsky *et al.*, 2001b, 2004; Rawe *et al.*, 2002). Proteasomal degradation of ubiquitylated proteins is necessary for the penetration of the egg vestments during fertilization (Sawada *et al.*, 2002; Sutovsky *et al.*, 2003), and the ubiquitylation of sperm mitochondrial membrane proteins during spermatogenesis assures their rapid proteolysis after fertilization, promoting maternal inheritance of mtDNA in mammals (Sutovsky *et al.*, 2003).

Male factor represents ~40% of all infertility cases worldwide, with additional male infertility cases possibly being

misdiagnosed as idiopathic infertility (Kim and Lipshutz, 1999; Schultz and Williams, 2002). While conventional semen evaluation by light microscopy provides a good estimate of sperm quality and fertility in most cases, more accurate, biochemical tests are sought in reproductive medicine to better evaluate the sperm quality and to properly diagnose male infertility (reviewed by Eliasson, 2003). Ubiquitin is a suitable marker of male infertility owing to its association with the surface of defective epididymal and ejaculated spermatozoa (reviewed by Sutovsky, 2003). While it is also possible to measure ubiquitin in the human seminal plasma (Lippert *et al.*, 1993), only the sperm-surface ubiquitin correlates with fertility (Sutovsky *et al.*, 2001b, 2004). The sperm ubiquitin tag immunoassay (SUTI) has been developed primarily as a flow cytometric assay that is highly sensitive, but requires expensive equipment and a trained, experienced operator. Here, we adapted the immunofluorescence-based SUTI assay for simple microscopic screening of human semen samples in a clinical setting.

Materials and methods

Sperm samples and clinical data

Semen samples were obtained from 93 consenting infertility patients treated at the Clinique Belledonne, Grenoble, France, in compliance with board-approved human subject protocols. For IVF and ICSI, the separation of the motile spermatozoa from contaminants was performed prior to cryopreservation using discontinuous density gradient (90/70/50% PureSperm® gradient). Cryopreserved samples (90% PureSperm fraction) were coded, and the identity of patients was concealed from investigators.

For all analyses, the gradient-separated spermatozoa were thawed in warm water (37°C) and washed by centrifugation through Universal IVF medium (Medicult, Limonest, France). Available clinical data included original clinical diagnosis of infertility (male factor, idiopathic, female), treatment (IVF or ICSI), number of oocytes retrieved, number of oocytes with the first polar body, number of two-pronuclear zygotes (2PN) with the second polar body (PB+) at 16–18 h after IVF/ICSI, number of cleaved embryos at 44–46 h after insemination, number of transferred and cryopreserved embryos at 72 h and pregnancies. Available semen quality parameters, obtained prior to gradient separation included sperm count, sperm motility, sperm morphology (David's classification; David, 1975; Jouannet *et al.*, 1988), percentage of acrosome-reacted spermatozoa after induced acrosome reaction and percentage of ubiquitin-immunoreactive spermatozoa.

The sperm parameters of the male patients were analysed by conventional light microscopic semen evaluation using WHO criteria for sperm count and motility, and WHO morphology criteria adapted according to David (1975). By David's classification, a semen sample was considered fertile at a minimum of 30% normal spermatozoa of Test-Simplet staining (Boehringer, Mannheim). Patients were divided into four groups as follows.

Group 1 ($n = 28$): male factor infertility. The sperm parameters showed one or more anomalies of the spermogram, as defined by WHO/David's criteria. These patients were included in an ICSI protocol, except for two patients whose spouses underwent intrauterine insemination. Female partners of all 28 men had normal clinical fertility profile.

Group 2 ($n = 27$): idiopathic, unexplained infertility. The clinical parameters of the men and women in this group did not reveal any reproductive problems, and clinical sperm parameters appeared normal by WHO/David's criteria. None of the patients had children previously.

Group 3 ($n = 21$): female infertility with unknown male fertility. Female patients from these couples were diagnosed with one or more types of reproductive dysfunction, including endometriosis, tubal factor, anovulatory infertility, polycystic ovary syndrome and uterine malformations. Male clinical parameters were normal, but no previous pregnancies were reported for the partners of these men.

Group 4 ($n = 17$): female infertility with male patients with history of proven fertility. This group was similar to group 3, but all male partners had children in their previous relationships. Nevertheless, it is possible that sperm quality in some of these subjects declined since the time they fathered their children.

Evaluation of acrosome reaction, acrosomal integrity and sperm viability

Spermatozoa were separated on discontinuous density gradient as described above, washed in IVF medium, adjusted to final concentration of $5–20 \times 10^6$/ml and incubated for 4 h at 37°C under 5% CO_2. To induce acrosome reaction, 100 ml of sperm in IVF medium were incubated with 100 ml of calcium ionophore A23187 solution (2 mmol/l final concentration; Sigma–Aldrich, Saint-Quentin Fallavier, France) for 30 min at 37°C under 5% CO_2. To assess sperm viability, spermatozoa were washed in phosphate-buffered saline (PBS), centrifuged and the pellet was mixed with 200 ml of PBS containing 1 mg/ml Hoechst 33258 (DNA stain; Hoechst/Aventis, Strasbourg, France) and incubated in the dark for 10 min. Spermatozoa were washed with PBS + 2% PVP and resuspended in 100 ml of PBS. After washing, 500 ml of 1% formol solution was added to each tube, incubated for 5 min at room temperature, centrifuged, resuspended in 100 ml of PBS, smeared on a microscopy slide, air-dried and fixed with acetone for 30 min at 4°C. Acrosomal integrity was determined by incubation with FITC-conjugated peanut agglutinin (PNA)-lectin (lectin from *Arachis hypogaea* conjugated with FITC, 1 mg/ml in deionized water; Sigma) as described by Cross *et al.* (1986), Kallajoki *et al.* (1986) and Mortimer *et al.* (1987). Briefly, a 2 ml stock solution of PNA lectin was mixed with 50 ml of ultrapure water, and 20 ml of this solution was added on a slide with fixed spermatozoa and incubated for 15 min at 37°C in a humidified chamber. Slides were rinsed in PBS, air-dried and covered with a coverslip in a drop of mounting medium. Sample evaluation was performed under an epifluorescence microscope with a 100× lens using 340 nm wavelength filter for Hoechst 33258 (blue fluoresce of dead spermatozoa) and a 490 nm filter for PNA-FITC (green fluorescence of intact sperm acrosomes). In each sample, 200 live, Hoechst-negative spermatozoa were evaluated for percentage of acrosome reaction with and without induction.

Immunofluorescence SUTI assay

Two microlitres of gradient-purified sperm pellets from each subject were resuspended in a 500 µl drop of 37°C warm KMT medium on a poly-L-lysine coated microscopy coverslip and allowed to attach for 5 min on a slide warmer (Sutovsky, 2004). Coverslips were submerged in 2% formaldehyde in PBS and fixed for 40 min. Samples were blocked for 25 min in 5% normal goat serum (NGS; Sigma) in PBS and incubated for 40 min with the monoclonal antibody KM-691 raised against the recombinant human ubiquitin (dilution 1/100; Kamiya 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 FITC-conjugated goat anti-mouse IgM (dilution 1/80; Sigma Diagnostics) and the DNA-stain DAPI (Sigma Diagnostics) was added to

this solution 10 min before the end of incubation. Samples were washed and mounted on microscopy slides. Negative controls included omission of the anti-ubiquitin antibody and incubation with KM-691 immunosaturated with purified erythrocyte ubiquitin (Sigma). In both cases, microscopy and image acquisition setting comparable to parameters for ubiquitin image acquisition were used. Clinical sample analyses were performed by using a Leitz Laborlux 11 microscope with a fluorescent module (Ploemopak 2.5) using a 100× immersion lens. Additional analyses were carried out using a Nikon Eclipse 800 microscope with epifluorescence and differential interference contrast (DIC) optics, and a CoolSnap HQ CCD camera. All analyses (93 subjects) were carried out by the same evaluator, an experienced andrologist-MD. Using the available clinical grade equipment, it was difficult to categorize the levels of anti-ubiquitin fluorescence on the sperm tails. Therefore, the analysis was focused on the presence or absence of the ubiquitin signal on the sperm heads. Since the sperm samples were not permeabilized, it was presumed that the observed staining was due to anti-ubiquitin-immunoreactive proteins on the sperm head surface.

Flow cytometric SUTI assay

To rule out an effect of freeze-thawing on semen quality, sperm samples from two patients were washed by centrifugation through Universal IVF medium (Medicult) as described above (less the PureSperm gradient separation) and screened by flow cytometric SUTI as described previously (Sutovsky *et al.*, 2001b, 2004). Briefly, samples were fixed in 2% formaldehyde in PBS, blocked with 5% NGS and incubated sequentially in suspension with anti-ubiquitin KM-691 and goat-anti-mouse IgM-FITC. Ubiquitin levels in 10 000 cells per sample were evaluated as described previously (Sutovsky *et al.*, 2004; see Figure 4).

Statistical analysis

Two hundred spermatozoa were evaluated randomly in each sample and data were entered into Microsoft Excel tables together with available clinical data. Correlation (Pearson's) and variance (single factor ANOVA) analyses were performed using SAS 8.2 and statistical tools in Microsoft Excel.

Results

Semen samples of 93 patients were screened during the IVF/ICSI treatment cycles over the course of 8 months. Well-characterized monoclonal antibodies against the recombinant human ubiquitin and appropriate fluorescent conjugates of secondary antibodies were used. Ubiquitin data were compared with the results of clinical semen analyses and with the data summarizing the clinical outcomes of IVF and ICSI. Clinical profiles of all couples and ubiquitin measures are summarized in Table I. Statistically significant differences were recorded for most sperm parameters including, but not limited to, sperm motility, count and morphology in the initial clinical analysis prior to gradient separation (Table IC). After gradient separation, the four groups did not differ significantly in acrosome reaction measured by PNA fluorescence after induction of acrosomal exocytosis (Table IC; Figure 1), but the male infertility and idiopathic infertility groups showed a statistically significant increase in the percentage of ubiquitylated sperm heads after gradient separation (Table ID).

Using the available instrument for immunofluorescence, most spermatozoa showed relatively little intensity of ubiquitin signal on the tail. Therefore, only the fluorescence of the sperm head was taken into account. The most common patterns of ubiquitin localization included even ubiquitylation of the whole sperm head (Figure 2A and D–F) and ubiquitylation of the acrosomal region (Figure 2B and C). Occasionally,

Table I. Clinical profiles (**A**), treatment outcomes (**B**), clinical semen quality (**C**) and sperm ubiquitin (**D**) data from 93 couples included in the present study

Group/clinical characteristic	Group 1: male factor	Group 2: idiopathic	Group 3: female infertility and unknown male fertility	Group 4: female infertility and proven fertile male
(A) General data				
Number of couples	28	27	21	17
Age male[a]	35.2 ± 1.1	34.8 ± 1.6	34.5 ± 1.5	37.4 ± 1.9
Age female[a]	33.6 ± 0.9	34.9 ± 0.8	34.0 ± 1.9	34.8 ± 1.2
IVF/ICSI	2/26	24/3	20/1	14/3
(B) Oocyte/embryo data and outcomes				
PB +[a]	8.8 ± 1.2	9.4 ± 1.9	8.0 ± 1.4	8.2 ± 1.2
2PN[a]	5.2 ± 0.8	5.7 ± 0.9	6.4 ± 1.2	5.8 ± 0.9
Cleaved[a]	4.4 ± 0.7	5.4 ± 0.9	6.1 ± 1.2	6.1 ± 1.0
Good embryos[a]	2.2 ± 0.4	2.0 ± 0.4	3.2 ± 0.8	2.9 ± 0.7
Number pregnant	7	6	7	2
(C) Semen quality				
Volume[a]	3.6 ± 0.3	3.2 ± 0.2	3.7 ± 0.4	2.5 ± 0.4
Count[a]	16.5 ± 3.4	78.8 ± 9.6	79.7 ± 7.6	84.6 ± 9.7
% dead[a]	47.8 ± 3.0	29.1 ± 2.0	23.2 ± 0.9	26.2 ± 1.8
% motile[a]	38.4 ± 3.2	53.7 ± 2.5	62.1 ± 2.4	60.3 ± 3.5
% progressive motility[a]	18.0 ± 2.0	32.0 ± 2.3	39.0 ± 2.1	36.5 ± 3.2
% normal morphology[a]	38.6 ± 3.6	51.6 ± 1.9	53.0 ± 2.8	54.8 ± 2.1
Acrosome reaction	13.9 ± 3.0	13.1 ± 0.8	13.4 ± 0.9	15.0 ± 0.9
Sperm survival after 24 h	64.8 ± 3.6	75.4 ± 2.2	79.3 ± 0.7	78.1 ± 1.0
(D) Sperm ubiquitin				
% ubiquitin[a]	4.0 ± 0.8	2.5 ± 0.7	0.7 ± 0.3	0.9 ± 0.6
% ubiquitin pregnant/non pregnant	5.0/3.7	0.001/3.2	0.1/0.9	0/1.1

[a]These parameters are an average ± SD.
PB + = second polar body present after fertilization; 2PN = two pronuclei present after fertilization.

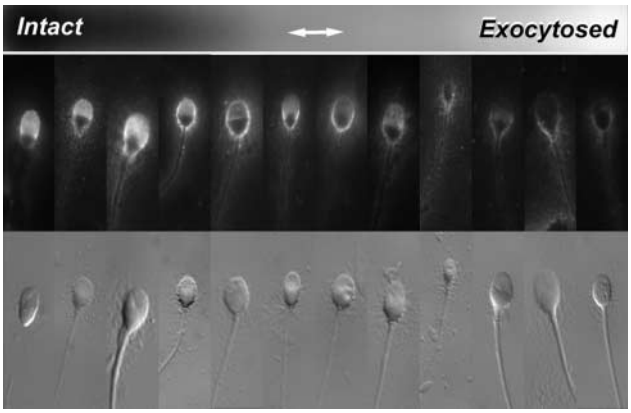

Figure 1. Representative patterns of acrosomal status as evaluated by epifluorescence microscopy of spermatozoa labelled with FITC-conjugated PNA after induction of acrosomal exocytosis.

ubiquitylation was restricted to the postacrosomal sheath (Figure 2G) or equatorial segment (Figure 2H). Negative control slides obtained using KM-691 antibody saturated with purified erythrocyte ubiquitin (Figure 2I) showed low levels of sperm fluorescence not comparable to samples labelled with non-saturated antibody. Since the normal spermatozoa in the screened samples were previously purified on a discontinuous gradient, the overall percentage of ubiquitin-positive spermatozoa was low in all screened groups. Undoubtedly, many more defective spermatozoa with increased surface ubiquitin were present in the purified sperm pellets, but not clearly detectable at the given level of resolution. However, even with relatively low percentage of spermatozoa with clearly ubiquitylated sperm heads, the differences between four screened groups were of significant clinical value. The highest percentage of ubiquitylated spermatozoa was present in group 1 (male factor; average 4.0%), and in the idiopathic infertility patients (group 2; average 2.5%), with low average rates in group 3 (0.7%) and group 4 (0.9%).

ANOVA showed that there was a highly significant difference in percentage of ubiquitylated spermatozoa (Table II) between male factor infertility (group 1), and female infertility (group 3; $P = 0.002$) and group 4 ($P = 0.01$), but not between male factor and idiopathic infertility ($P = 0.18$), suggesting that some of the idiopathic cases were contributed to by an undetected male factor. The idiopathic group was significantly different from the female infertility group ($P = 0.03$), but the difference was less significant between idiopathic group 2 and fertile male group 4 ($P = 0.12$). The ANOVA further demonstrated that there were significant differences in the percentage of cleaved versus ovulated ova, cleaved versus PB + ova, and percentage cleaved versus 2PN ova between men with high and low ubiquitin ($P < 0.01$) within the male factor infertility (group 1; Figure 3A–C). To avoid possible statistical flaws, we reanalysed the whole data set after eliminating five 'outlier' samples with percentage of ubiquitylated sperm heads ≥ 10. After this reanalysis, we observed even more statistically significant differences between individual groups (Table II), including a statistically more significant difference in percentage ubiquitylated sperm heads between the male factor and idiopathic

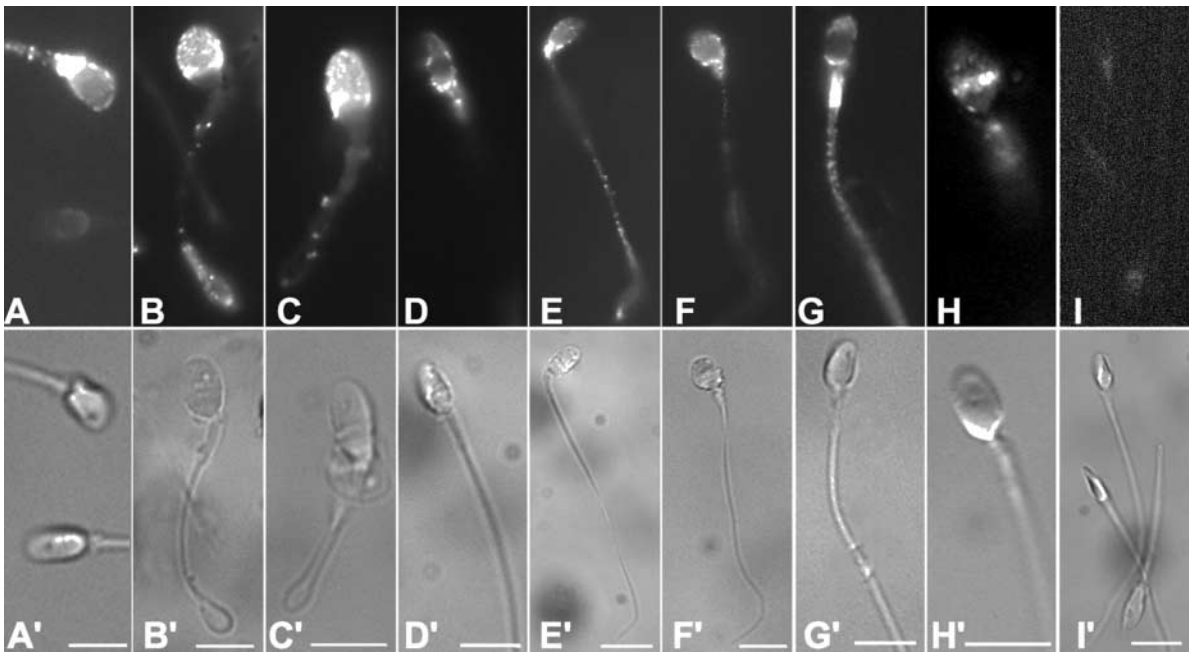

Figure 2. Representative patterns of sperm immunofluorescence labelling with monoclonal anti-ubiquitin antibody KM-691 (**A**–**H**), and corresponding transmitted light micrographs (**A′**–**H′**). (**A**) The fluorescence in a presumed defective spermatozoon (top) compared with that of a morphologically normal one (bottom). Commonly observed sperm head ubiquitylation patterns included predominant ubiquitylation in the acrosomal region (**B**, **C**), and even ubiquitylation of the whole sperm head and midpiece (**D**–**F**). Occasionally, we observed ubiquitylation restricted to the post-acrosomal sheath (**G**; note also signal at the apex of the acrosome) and equatorial segment (**H**). Negative control image was obtained by using KM-691 antibody saturated with purified erythrocyte ubiqutin (**I**) and a corresponding transmitted light image (**I′**) acquired by using comparable acquisition settings. Scale bars = 5 μm.

Table II. Single factor ANOVA comparing ubiquitin levels in the four groups of patients in this study[a]

	Group 1: male factor	Group 2: idiopathic	Group 3: female infertility and unknown male fertility	Group 4: female infertility and proven fertile male
Group 1: male factor	–	0.18/0.06	0.002/0.0002	0.01/0.0001
Group 2: idiopathic	0.18/0.06	–	0.03/0.06	0.12/0.03
Group 3: female infertility and unknown male fertility	0.002/0.0002	0.03/0.06	–	0.67/0.48
Group 4: female infertility and proven fertile male	0.01/0.0001	0.12/0.03	0.67/0.48	–

[a]The first number in each cell is the *P*-value based on all data collected. Second number represents the *P*-value after the removal of five outlier samples from data set ('outlier' samples with the percentage of ubiquitylated spermatozoa ≥ 10 were removed from analysis).

group ($P = 0.18$ in the original analysis, $P = 0.06$ after 'outlier' elimination).

With regard to pregnancy rates, there was a significant difference in the percentage of ubiquitylated spermatozoa between pregnant and non-pregnant couples within the idiopathic group (group 2; $P = 0.06$) and when all 93 couples were analysed together (groups 1–4; $P = 0.04$), but not within the remaining groups alone. The average percentage of ubiquitylated spermatozoa was high for both pregnant and non-pregnant couples in group 1 (Table I), while the remaining three groups showed significantly lower ubiquitin averages in pregnant couples compared with non-pregnant ones.

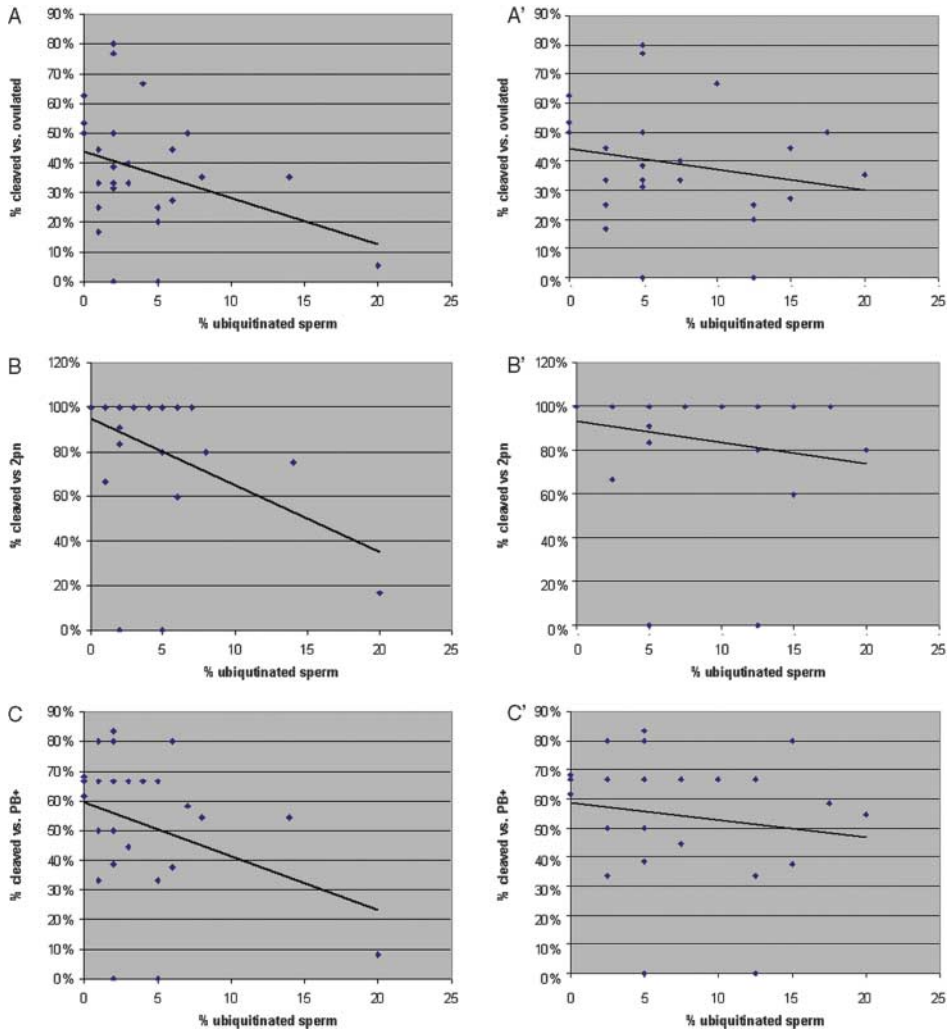

Figure 3. Sperm ubiquitin levels correlated negatively with early embryonic development in couples from group 1 (male factor). Diagrams depict relationship between the presence of ubiquitin-immunoreactive spermatozoa (percentage ubiquitylated sperm) and parameters of early embryo development including percentage of cleaved embryos out of all ovulated/isolated ova (**A**), percentage of cleaved embryos out of all embryos reaching 2PN after IVF or ICSI (**B**) and percentage of cleaved embryos out of all zygotes displaying second polar body (PB+) after fertilization (**C**). (**A′–C′**) Similar distribution was observed when the 'outlier' samples with percentage of ubiquitylated sperm heads ≥ 10 were eliminated from the dataset.

Within the male factor group (Figure 3; Table I), in which all but two couples were treated by ICSI, percentage ubiquitylated spermatozoa correlated negatively with percentage cleaved/2PN embryos ($r = -0.42$), percentage cleaved/PB+ ($r = -0.39$) embryos and percentage cleaved/ovulated ova ($r = -0.37$). Interestingly, there were strong negative correlations between the percentage of acrosome-reacted spermatozoa after induced acrosomal reaction and several embryo cleavage parameters within this group ($r = -0.36$ to -0.78). Negative correlations between the percentage of ubiquitylated spermatozoa and individual embryo development characteristics were also found in group 3. Ubiquitin showed moderate levels of positive correlation with percentage dead/necrotic spermatozoa in all four groups combined ($r = 0.38$).

To ascertain that increased rates of sperm ubiquitylation were not due to freeze-thawing of semen samples, we performed flow cytometric analyses (SUTI assay; Sutovsky *et al.*, 2001b, 2004) on semen samples from two donors before and after semen preparation and freeze-thawing (Figure 4). As expected, these analyses did not reveal a significant change in the pattern of sperm ubiquitylation before and after freeze-thawing (Figure 4). This is likely due to the fact that substrate ubiquitylation occurs through a stable covalent bond and specific deubiquitinating enzymes are required for the removal of polyubiquitin chains from the ubiquitylated substrates. In addition to flow cytometric analysis, we performed the simplified immunofluorescence SUTI as described above, and found that the percentage of

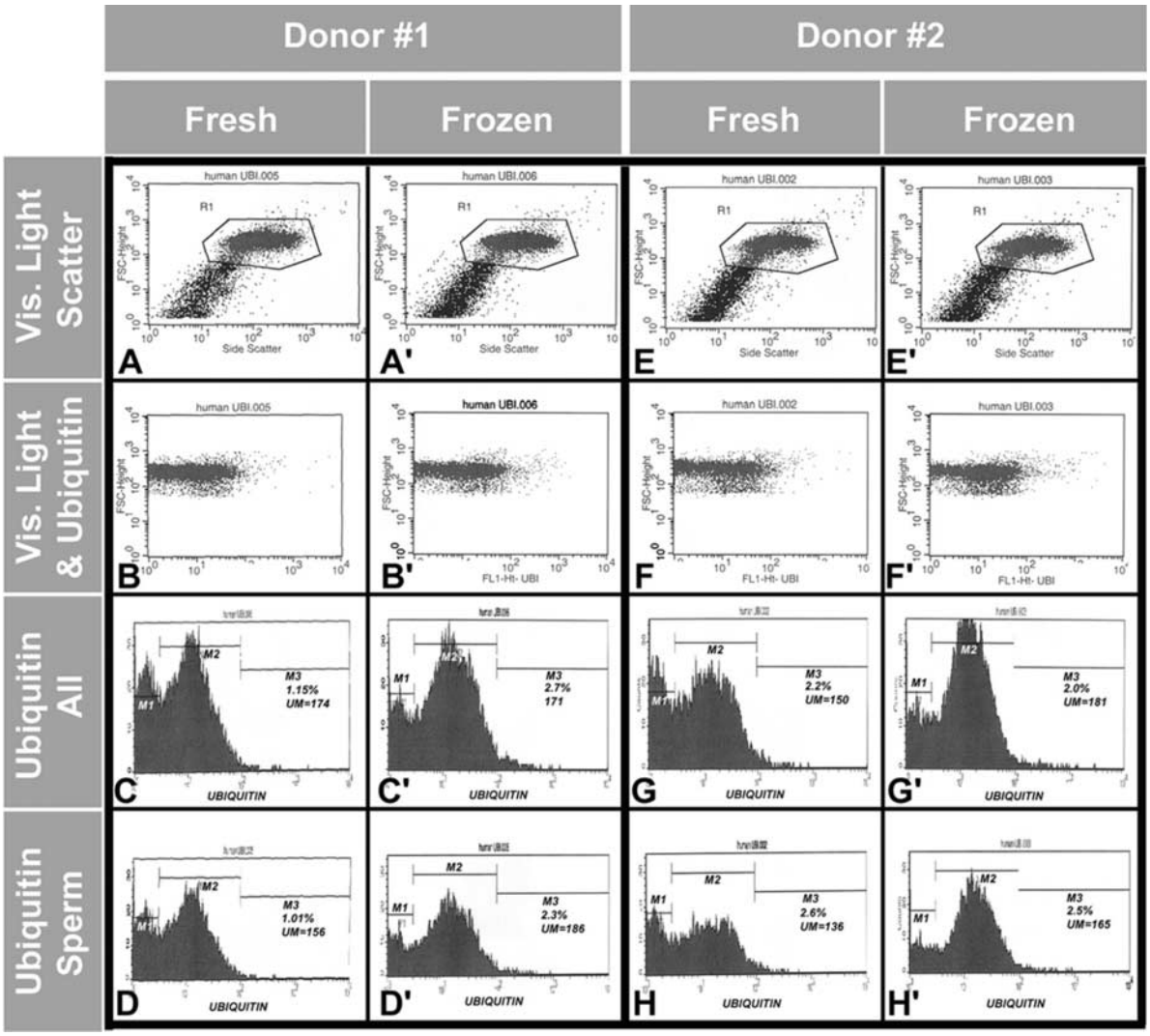

Figure 4. Ubiquitin levels in 10 000 cells per sample as evaluated by flow cytometric SUTI assay in samples from two donors (donor 1, **A′**–**D′**; donor 2, **E′**–**H′**) before and after freeze-thawing. No significant differences in the pattern of visible light scatter were found between fresh (**A**–**H**) and frozen-thawed (**A′**–**H′**) sperm samples when the scatter diagrams of visible light were evaluated alone (**A**, **A′**, **E**, **E′**), or in combination with relative fluorescence induced by anti-ubiquitin antibody binding to presumed defective spermatozoa (**B**, **B′**, **F**, **F′**). Ubiquitin-induced fluorescence histograms were evaluated for all cells in the sperm samples (**C**, **C′**, **G**, **G′**) and for the presumed spermatozoa only (**D**, **D′**, **H**, **H′**), as gated based on the visible light scatter pattern (gate R1 in **A** and **E**). In all cases, the relative levels of sperm surface ubiquitin did not differ significantly before freezing (**C**, **D**, **G**, **H**) and after thawing (**C′**, **D′**, **G′**, **H′**) within the subpopulations divided into small debris (marker M1), normal size spermatozoa with basal, background levels of ubiquitin-induced fluorescence (marker M2) and highly ubiquitylated, presumably defective spermatozoa (marker M3). % = % of cell within marker M3; UM = ubiquitin median values, i.e. the median values of fluorescence measured by flow cytometer, induced by fluorescently conjugated anti-ubiquitin antibody bound to the surface of defective spermatozoa.

spermatozoa with ubiquitylated sperm heads was 42 before and 34 after freeze-thawing in the first donor, and 38 before and 31 after freeze-thawing in the second donor (samples were not purified on PureSperm gradient).

Discussion

Using a simplified immunofluorescence analysis technique in clinical settings, we examined the frequency of human sperm ubiquitylation in human spermatozoa purified on a PureSperm gradient. We were particularly interested in a possible relationship of sperm ubiquitin levels with sperm parameters and embryo development after IVF and ICSI. The presence of ubiquitin in human spermatozoa was detected by subjective immunofluorescence analysis focused specifically on sperm head ubiquitylation. It is conceivable that the defective, ubiquitylated spermatozoa may interfere with the success of IVF and ICSI treatments, because ubiquitylation was previously demonstrated to accompany various types of sperm head and sperm tail anomalies in human and animal spermatozoa (Sutovsky *et al.*, 2001a,b; 2002; 2004; Rawe *et al.*, 2002). Accordingly, the present study found increased sperm ubiquitylation in gradient-purified semen samples from men previously diagnosed with male factor infertility, who are expected to have both an increased incidence of defective spermatozoa and a reduced fertility. In addition, we also found increased sperm ubiquitin levels in approximately half of cases of unexplained infertility, wherein this finding could, at least in some cases, explain the difficulties of these couples to conceive.

The above observations were made despite the purification of spermatozoa on PureSperm gradient, which preceded the evaluation of ubiquitin content and presumably removed most of the defective, ubiquitylated spermatozoa found in raw semen samples. It follows that gradient separation alone may not be sufficient to completely deplete defective spermatozoa from a semen sample used for assisted reproduction. This has particular implications for ICSI, wherein one spermatozoon is chosen from such partially purified population based on morphological appearance and motility (if present). The pre-selection of spermatozoa for low ubiquitin could add a higher level of stringency to sperm selection for ICSI even after sperm separation on a density gradient. Such added level of stringency could be especially important in cases with very low sperm count and motility, wherein the gradient separation is not possible. Two such patients were initially present in group 1 in the present trial, but were eliminated from all statistical analyses since their sperm samples were not treated equally to the remaining 93 patients' samples. Both samples showed high percentages of ubiquitin-positive sperm heads (data not shown). The present data further support the requirement for a more stringent sperm selection by showing better IVF and ICSI fertilization rates in men with low sperm ubiquitin levels. It remains to be resolved how to avoid a potentially harmful fluorescence exposure of spermatozoa in such protocols. Since the ubiquitin binding occurs mainly on the surface of defective spermatozoa, a good alternative to fluorescent labelling of ubiquitylated spermatozoa could be the immunodepletion of gradient-separated sperm samples with matrix-bound anti-ubiquitin antibodies or the detection of defective spermatozoa by microspheres conjugated with anti-ubiquitin antibody or a ubiquitin-binding protein.

Higher levels of sperm ubiquitylation also corroborate poor sperm quality and fertility in the group of men diagnosed with male factor infertility (group 1), wherein sperm ubiquitin correlated negatively with the percentage of cleaved embryos, and percentage of embryos with two pronuclei after IVF or ICSI. As mentioned above, it was not possible to perform gradient separation in several additional patients originally included with this group, owing to low sperm concentration and poor motility. Therefore, it is possible that in such patients, some spermatozoa with a high proportion of surface ubiquitin could be selected during ICSI procedure, and this could adversely affect PN development and embryo cleavage rates after ICSI. This does not rule out a possibility of obtaining a pregnancy in such patients. Indeed, the average percentage of ubiquitylated spermatozoa was high for both pregnant and non-pregnant male factor couples, while the remaining three groups, predominantly treated by IVF, showed significantly lower ubiquitin averages in pregnant couples compared with non-pregnant ones. This could be reconciled by the use of ICSI in 26 of 28 male factor couples. High percentage of ubiquitylated spermatozoa could thus be an indication for the use of ICSI in idiopathic couples, whenever IVF is also considered. Therefore, it could be attempted to use the simplified ubiquitin-based semen evaluation to reduce the number of unsuccessful IVF cycles by redirecting the treatment towards ICSI.

In group 2 (idiopathic infertility), we found increased sperm ubiquitin levels after gradient separation in some of the subjects (eight out of 27 had $>3\%$ ubiquitylated sperm), but no significant correlation between sperm ubiquitin and embryo cleavage rates. This can be explained by two factors. First, at least half of these couples could have experienced infertility due to an undiagnosed female factor. Secondly, in couples with an undiagnosed male factor, just as in properly diagnosed male factor patients, the fertilization and development rates could have been improved by gradient separation of spermatozoa, which appears to be a fairly effective method for selecting the fittest cells for IVF in cases with acceptable sperm morphology and motility.

In groups 3 and 4, where the sperm parameters were expected to be normal by WHO/David's criteria, we found a significantly lower proportion of sperm ubiquitylation compared with groups 1 and 2. Similarly, there were no correlations between sperm ubiquitin and embryo development parameters. However, at least one patient in group 4 showed a high degree of sperm ubiquitylation.

Recent studies showed that a portion of spermatozoa in both fertile and infertile subjects contain a partially degraded nuclear DNA, probably a result of apoptosis or necrosis. Such DNA fragmentation can be detected by various techniques including SCSA, COMET or TUNEL. Late, but not early, paternal influence on embryo development is thought to be related to sperm DNA fragmentation

(Benchaib *et al.*, 2003; Henkel *et al.*, 2003; Larson-Cook *et al.*, 2003; Tesarik *et al.*, 2004). The DNA damage can be also detected by the flow cytometric sperm ubiquitin assays, in which a significant positive correlation between ubiquitin median values and median values of TUNEL-induced fluorescence is observed (Sutovsky *et al.*, 2002). In contrast to the above DNA/chromatin integrity assays, SUTI detects a variety of other defects, not only those related to DNA damage. Ubiquitin detection may thus be a reliable marker of overall sperm quality, including, but not limited to, DNA damage. The level of the sperm-surface ubiquitylation could be informative in the sperm evaluation using highly sensitive, objective flow cytometric or biochemical techniques, but also by using simple epifluorescence microscopy such as the method described here. This is useful even after sperm gradient separation/purification. Further studies of sperm ubiquitin and related infertility markers could lead to the development of new techniques for individual sperm selection for ICSI, which in turn could improve the rate of success in most couples with male factor infertility and in some couples with unexplained infertility.

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References

Baarends WM, Hoogerbrugge JW, Roest HP, Ooms M, Vreeburg J, Hoeijmakers JH and Grootegoed JA (1999) Histone ubiquitination and chromatin remodeling in mouse spermatogenesis. Dev Biol 207,322–333.

Bebington C, Doherty FJ and Fleming SD (2001) The possible biological and reproductive functions of ubiquitin. Hum Reprod Update 7,102–111.

Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H and Guerin JF (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum Reprod 18,1023–1028.

Chen HY, Sun JM, Zhang Y, Davie JR and Meistrich ML (1998) Ubiquitination of histone H3 in elongating spermatids of rat testes. J Biol Chem 273,13165–13269.

Ciechanover A (1994) The ubiquitin–proteasome proteolytic pathway. Cell 79,13–21.

Cross NL, Morales P, Overstreet JW and Hanson FW (1986) Two simple methods for detecting acrosome-reacted human sperm. Gamete Res 15, 213–226.

David G (1975) Anomalies morphologiques du spermatozoïde humain. Propositions pour un système de classification. J Gyn Obst Biol Reprod 4 (Suppl 1),7–36.

Eliasson R (2003) Basic semen analysis. In Matson P (ed.) Current Topics in Andrology. Ladybrook Publishing, Perth, Australia, pp. 35–89.

Escalier D, Bai XY, Silvius D, Xu PX and Xu X (2003) Spermatid nuclear and sperm periaxonemal anomalies in the mouse Ube2b null mutant. Mol Reprod Dev 65,298–308.

Glickman MH and Ciechanover A (2002) The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 282, 373–428.

Goldstein G, Scheid M, Hammerling U, Schlesinger DH, Niall HD and Boyse EA (1975) Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc Natl Acad Sci USA 72,11–15.

Henkel R, Kierspel E, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Schill WB and Kruger TF (2003) DNA fragmentation of spermatozoa and assisted reproduction technology. Reprod Biomed Online 7,477–484.

Hermo L and Jacks D (2002) Nature's ingenuity: Bypassing the classical secretory route via apocrine secretion. Mol Reprod Dev 63,394–410.

Jouannet P, Ducot B, Feneux D and Spira A (1988) Male factors and the likelihood of pregnancy in infertile couples. I. Study of sperm characteristics. Int J Androl 11,379–394.

Kallajoki M, Virtanen I and Suominen J (1986) The fate of acrosomal staining during the acrosome reaction of human spermatozoa as revealed by a monoclonal antibody and PNA-lectin. Int J Androl 9,181–194.

Kim ED and Lipshutz LI (1999) Evaluation and imaging of the infertile male. In Diamond MP and DeCherney AH (eds) Infertility and Reproductive Medicine; Clinics of North America, Vol. 10/3. WB Saunders Company, Philadelphia, PA, USA, pp. 377–409.

Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET and Evenson DP (2003) Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertil Steril 380,895–902.

Lippert TH, Seeger H, Schieferstein G and Voelter W (1993) Immunoreactive ubiquitin in human seminal plasma. J Androl 14,130–131.

Mortimer D, Curtis EF and Miller RG (1987) Specific labelling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. J Reprod Fertil 81,127–135.

Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D and Gygi SP (2003) A proteomics approach to understanding protein ubiquitination. Nat Biotechnol 32,921–926.

Pickart CM (1998) Polyubiquitin chains. In Peters J-M, Harris JR, and Finley D (eds) Ubiquitin and the Biology of the Cell. Plenum Press, New York, NY, USA, pp. 19–63.

Rawe VY, Brugo Olmedo S, Benmusa A, Shiigi SM, Chemes HE and Sutovsky P (2002) Sperm ubiquitination in patients with dysplasia of the fibrous sheath. Hum Reprod 17,2119–2127.

Sawada H, Sakai N, Abe Y, Tanaka E, Takahashi Y, Fujino J, Kodama E, Takizawa S and Yokosawa H (2002) Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. Proc Natl Acad Sci USA, 99,1223–1228.

Schultz RM and Williams CJ (2002) The science of ART. Science 296, 2188–2190.

Sutovsky P (2003) Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization, and sperm quality control: killing three birds with one stone. Microsc Res Tech 61,88–102.

Sutovsky P (2004) Visualization of sperm accessory structures in the mammalian spermatids, spermatozoa, and zygotes by immunofluorescence, confocal, and immunoelectron microscopy. Methods Mol Biol 253,59–78.

Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE and Schatten G (2001a) A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. J Cell Sci 114,1665–1675.

Sutovsky P, Terada Y and Schatten G (2001b) Ubiquitin-based sperm assay for the diagnosis of male factor infertility. Hum Reprod 16,250–258.

Sutovsky P, Neuber E and Schatten G (2002) Ubiquitin-dependent, sperm quality control mechanism recognizes spermatozoa with DNA defects, as revealed by dual ubiquitin-TUNEL assay. Mol Reprod Dev 61,406–413.

Sutovsky P, McCauley TC, Sutovsky M and Day BN (2003) Early degradation of paternal mitochondria in domestic pig (Sus scrofa) is prevented by selective proteasomal inhibitors lactacystin and MG132. Biol Reprod 68,1793–1800.

Sutovsky P, Hauser R and Sutovsky M (2004) Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology laboratory clinic population. Hum Reprod 19,628–638.

Takizawa S and Yokosawa H (2002) Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. Proc Natl Acad Sci USA 99,1223–1228.

Tesarik J, Greco E and Mendoza C (2004) Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. Hum Reprod 19,611–615.

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