

## Sperm Ubiquitination Correlation with Human Semen Quality

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### Abstract

**Background:** Ubiquitin, an 8.5 kDa peptide that marks other proteins for proteasomal degradation, tags defective sperm during epididymal passage. Thus, sperm ubiquitination is a universal marker for sperm defects and can be used as a sperm function test. The objective of the present study was to examine the relationships between sperm ubiquitination and clinical semen parameters, using simplified immunofluorescence assays in order to establish ubiquitin as a biomarker of male infertility.

**Methods:** Semen samples from 100 couples attending Avicenna Infertility Clinic, Tehran, Iran, were collected and analyzed according to WHO criteria. Each sample was washed and adjusted at  $10^6$  sperm/ml concentration. Sperm were coated on slides, using cytospin centrifugation and were fixed in buffered formaldehyde. Subsequently ubiquitinated spermatozoa were evaluated by direct immunofluorescence microscopy using FITC-labeled anti-ubiquitin antibodies. After counting at least 200 sperm per sample, while employing light microscopy, the percentage of ubiquitinated spermatozoa was recorded on the same fields through epifluorescence microscopy.

**Results:** Negative correlations were obtained between sperm ubiquitination and sperm count ( $r=-0.278$ ,  $P< 0.001$ ), sperm concentration ( $r=-0.37$ ,  $P< 0.001$ ), viability ( $r=-0.407$ ,  $P< 0.001$ ), sperm morphology ( $r=-0.509$ ,  $P< 0.001$ ), rapid progressive motility (a) ( $r=-0.246$ ,  $P< 0.001$ ) and slow progressive motility (b) ( $r=-0.474$ ,  $P< 0.001$ ). There was a positive correlation between ubiquitinated sperm and the percentage of immotile spermatozoa ( $r=0.486$ ,  $P< 0.000$ ).

**Conclusion:** Increased sperm ubiquitination is inversely associated with good semen quality parameters, supporting the use of ubiquitin as a biomarker for evaluation of human sperm quality.

**Keywords:** Ubiquitin, Immunofluorescence, Spermatozoa, Male infertility, Oligoasthenoteratozoospermia, Semen quality

### Introduction

Ubiquitin, a small peptide of 8.5 kDa, is a universal marker for proteolysis found in all tissues and organisms (1, 2). Its widespread presence and extremely conserved structure indicate that it may play a vital role in cell metabolism. It has the property of binding covalently to cellular acceptor proteins in the form of mono or poly ubiquitin chains and targets such ubiquitinated substrates for endocytosis and/or proteolytic degradation by the multi-subunit protease, the 26-S proteasome (3, 4).

The ubiquitin system has been implicated in the control of cellular functions as varied as proteolysis, antigen presentation (5), membrane receptor endocytosis (6), transcriptional control (7), apop-

toxis and pathology of various diseases including Alzheimer's (8) and HIV viral infection (9).

The reproductive functions of ubiquitin are just now starting to emerge (10, 11). Recently an important role for sperm protein ubiquitination has been postulated (12). These authors proposed the existence of an ubiquitin-dependent sperm quality control mechanism, acting in the mammalian epididymis, site of sperm maturation and storage. Immunofluorescence assay using anti-ubiquitin antibody, disclosed that defective spermatozoa along with contaminants of cellular origin, including round and elongated spermatids, leukocytes and cellular debris become ubiquitinated on the surface proteins through their epididymal passage (13).

Several independent reports have shown that ubiquitin is secreted by the principal cells of the epididymal epithelium into the epididymal lumen (12, 14). Ubiquitin is also a major protein of human seminal plasma (15). Some spermatozoa seem to be disposed of by the epididymal epithelium, explaining why the percentage of ubiquitinated spermatozoa decreases from the corpus to the cauda of epididymis (12). A portion of abnormal ubiquitinated sperm that are found in the ejaculate are considered to have escaped from epididymal degradation and are characterized by main defects of head and/or axoneme (12, 13). Based upon the above observations, sperm-ubiquitin tag immunoassay (SUTI) was designed as a highly sensitive flow cytometric assay to reveal defective sperm, regardless of whether or not their defects were detectable by light microscopic evaluations.

It has been shown that increased semen ubiquitination is associated with poor sperm quality parameters (16). Moreover, it was found that sperm ubiquitination increases in patients affected by dysplasia of the fibrous sheath (17). It also appears that the ubiquitin-dependent sperm quality control, residing in the epididymal epithelium, has the ability to detect spermatozoa with apoptotic or necrotic DNA (18). All these studies have been performed by employing flow cytometric techniques without distinguishing between spermatozoa and other semen components such as leukocytes and cellular debris. Recently a conflicting result has been reported (19) which indicates a positive correlation between sperm ubiquitination and good quality of semen parameters after excluding the ubiquitinated inclusion of semen components in oligoasthenoteratozoospermia. We aimed to validate further sperm ubiquitination as a suitable marker of sperm quality in infertile men with different etiologies, using ubiquitin immunofluorescence assay by simple microscopic screening of human samples.

## Materials and Methods

**Reagents** Polyclonal anti-ubiquitin antibodies were developed in rabbit, using polyubiquitin as

an immunogen. polyubiquitin was prepared by glutaraldehyde polymerization of ubiquitin (Sigma-Aldrich, Germany) according to Deutsch's procedure (20). Anti-ubiquitin antibodies were purified from rabbit serum using affinity chromatography. Purified antibodies were conjugated with Fluorescein Isothiocyanate, isomer I (FITC)(Sigma-Aldrich, Germany). Bovine serum albumin (BSA) and formaldehyde were purchased from USB (USB, Ohio) and BDH Chemicals (BDH, England), respectively.

**Semen collection and preparation** Semen samples were collected from 100 infertile men undergoing routine semen analyses in the andrology laboratory of Avicenna Infertility Clinic (AIC), Tehran, Iran, in compliance with board-approved human subject protocols. Briefly, a sample was produced on-site by masturbation into a sterile plastic specimen cup. The samples were allowed to liquefy at 37° C for 20 min prior to analysis. Subjects were instructed to abstain from ejaculation for at least 48 h prior to producing the semen. The sperm parameters were analyzed prior to immunofluorescence assay by conventional light microscopic evaluation, using WHO criteria (21) for sperm counts, motility and morphology. All semen evaluations were performed only by one expert laboratory technician.

**Immunofluorescence assay** Raw semen samples were washed three times by centrifugation through PBS. One hundred microliters of washed spermatozoa containing 10<sup>6</sup> sperm were attached to microscopy slides, using cytospin centrifuge (Thermo, Shandon, PA), 300 g for 5 min and allowed to air dry for 15 min in room temperature. Slides were submerged in 2% formaldehyde in PBS and fixed for 40 min. Slides were blocked for 15 min in 5% normal rabbit serum in phosphate buffered saline. One percent BSA in PBS was used for washing and diluting the antibodies. After washing, the samples were incubated for 1 h with 50 microliter of FITC-conjugated affinity purified rabbit anti-ubiquitin antibody (15µg/ml). Finally, slides were washed twice for 5 min with washing buffer and mounted in 50% Glycerol/PBS. Counts of 200

spermatozoa were made to get an estimate of ubiquitinated spermatozoa. For each sample, sperm were counted through light microscope and the percentage of ubiquitinated sperm was recorded on the same fields using epi-fluorescent microscope (Zeiss, Germany). All the images were captured by a digital camera (1200 DXM Nikon, Japan). In order to ascertain that the sperm immunofluorescence signals were indeed due to surface sperm ubiquitination and were not related to constitutively sperm internal proteins, coated slides were treated with 1% Triton X-100 in PBS for 60 min, prior to incubation stage. Finally, ubiquitinated sperm were compared in permeabilized and non-permeabilized spermatozoa.

Negative controls of immunofluorescence assay included the following: a) Sperm coated slides treated with affinity purified IgG, labeled with FITC. The purified IgG was prepared from normal rabbit serum b) Two types of anti-ubiquitin antibodies (FITC-labeled and unlabeled anti-ubiquitin antibodies) were used. In the first step, we used unlabeled anti-ubiquitin antibodies to mask ubiquitinated proteins on the sperm surface. In the next step, FITC labeled anti-ubiquitin antibodies were applied to detect cross reactivity of affinity purified anti-ubiquitin antibody with other protein.

**Statistical analysis** The significance level (*P*) of the difference in the percentage of ubiquitinated spermatozoa between the two groups was determined by Mann-Whitney test. Correlations between the percentages of ubiquitin immunoreactive sperm and semen analysis parameters were evaluated by calculating Pearson's correlation coefficient (*r*). A backward multiple linear regression analysis was performed to identify which independent variables (among semen parameters) could explain a significant amount of variation in the sperm ubiquitination. All statistical analyses were carried out using SPSS, version 11.5 (SPSS Inc, USA, IL).

## **Results**

The studied population consisted of 100 men from infertile couples, 42 of whom were oligoasthenoteratozoospermic (sperm concentra-

tion less than  $20 \times 10^6$  sperm/ml, sperm motility less than 50% and normal sperm morphology less than 30%). The rest of the cases (58 cases) had acceptable normal semen parameters according to the WHO criteria. The age of oligoasthenoteratozoospermic males ranged between 23-52 yr (mean  $34.95 \pm 6.9$ ) and the ones in the normal group ranged between 20-63 yr (mean  $35.69 \pm 7.8$ ). No significant differences were found between the mean ages of the two groups. Sperm ubiquitination of semen samples were evaluated using appropriate fluorescent conjugates of polyclonal antibodies against human ubiquitin. Clinical profiles of the subjects and mean percentages of ubiquitinated sperm are summarized in Table 1. The most common pattern of ubiquitin localization included ubiquitination of whole sperm head and ubiquitination of the acrosomal region (Fig. 1). Most ubiquitinated spermatozoa showed relatively little intensity of ubiquitin signal on their tails. Along with spermatozoa, other contaminants of cellular origin such as leukocytes were ubiquitinated and thus were recognizable by anti-ubiquitin antibodies. Immunofluorescence analysis of permeabilized spermatozoa showed that nearly all spermatozoa were ubiquitinated. No fluorescent signal was detected in negative control slides (Fig. 1-C')

The mean percentage of ubiquitinated spermatozoa in the oligoasthenoteratozoospermic (OAT) group showed significant increase compared to the normozoospermic group. The average sperm ubiquitination in the normal and the OAT groups were  $1.91 \pm 2.37$  and  $7.68 \pm 6.6$  respectively (Table 1). The difference in the percentage of ubiquitinated spermatozoa between two groups was significant ( $P < 0.05$ ). Out of 58 men with normal semen parameters, seven had higher ubiquitin measurements, compared to the mean percentage of ubiquitinated sperm in the oligoasthenoteratozoospermic cases. Negative correlations were found between sperm ubiquitination and rapidly progressive motility (grade a) (Fig. 2-A), slowly progressive motility (grade b) (Fig. 2-B), normal morphology (Fig. 2-D) viability (Fig. 2-E), sperm concentration (Fig. 2-F) and sperm counts (Fig. 2-G).

Positive correlations were also determined between ubiquitinated sperm, the percentage of immotile sperm (grade d) (Fig. 2-C) and immature germ cell counts (Fig.2 H). The correlation coefficients and *P* for correlation analysis are shown in Table 2. The backward multiple linear regression analyses demonstrated that sperm ubiquitination is more

correlated with sperm morphology. It was shown that per unit increase in percentage of normal morphology would result in 0.187 reduce in percentage of ubiquitinated sperm ( $B = -0.187$ ,  $SD = 0.0032$ ,  $P < 0.001$ ). No significant correlation was found between the percentage of ubiquitinated sperm and the percentage of non-progressive motility.

**Table1:** Demographic (a), clinical semen quality (b), sperm ubiquitination (c) data of 147 male included in the present study

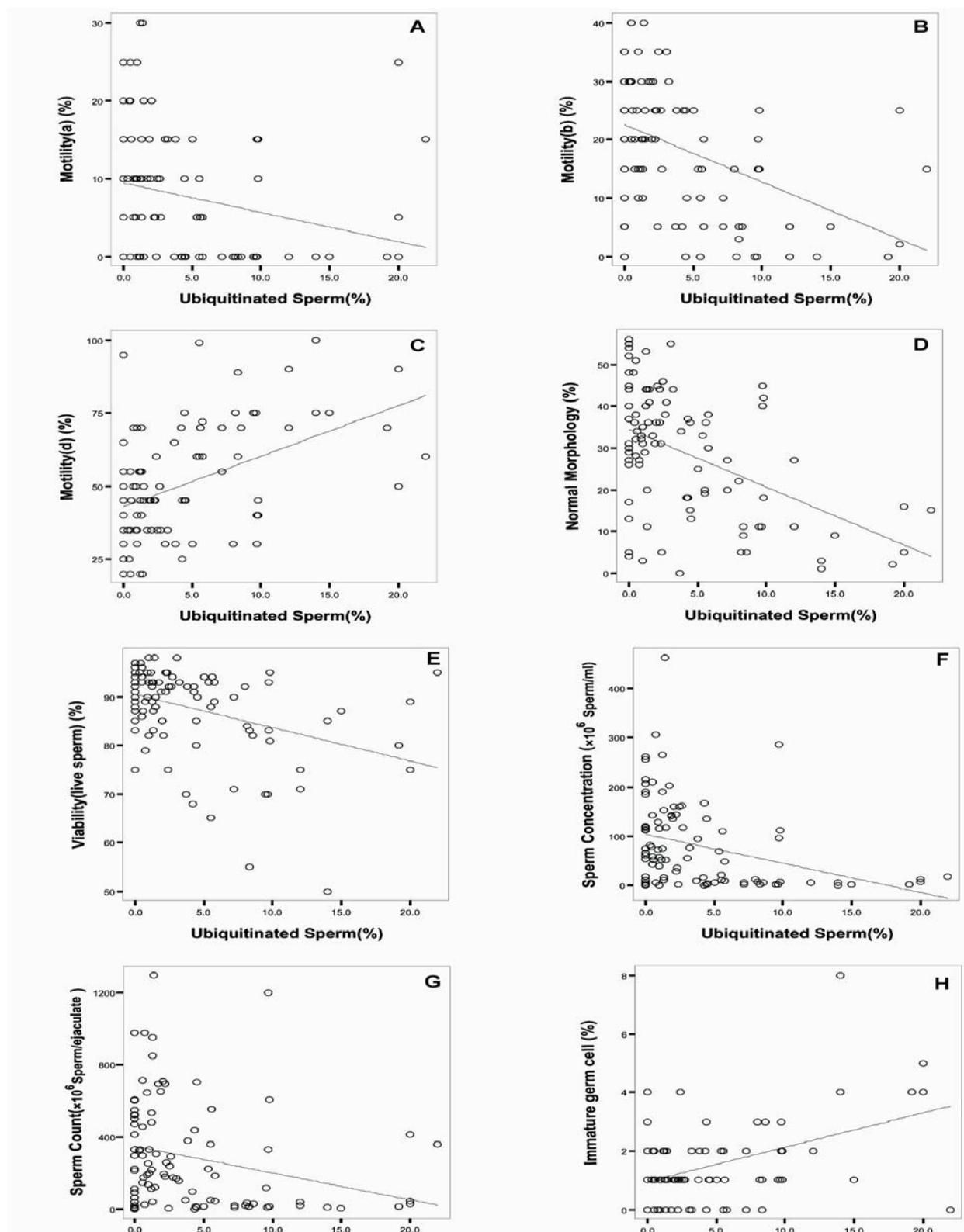
Subject parameters	Oligoasthenoeratozoospermia	normozoospermia
<b>(a)Demographic</b>		
Number of cases	42	58
Age <sup>a</sup>	34.95± 6.9	35.69 ± 7.8
<b>(b)semen quality</b>		
Sperm count(10 <sup>6</sup> sperm) <sup>a</sup>	80.5± 159.8	430.7 ±277
Concentration (10 <sup>6</sup> sperm/ml) <sup>a</sup>	7.2 ± 5.57	130.2 ± 81
Viability(live) % <sup>a</sup>	80 ± 10.3	92.28±3.6
Normal morphology % <sup>a</sup>	13.86 ± 8.41	39.8±11.4
Motility		
Rapidly progressive motility % <sup>a</sup>	2.7 ± 5.6	11.47± 8.1
Slowly progressive motility % <sup>a</sup>	8.14 ± 7.9	25.69± 6.6
Non progressive motility% <sup>a</sup>	25.36± 13.64	22.8± 12.4
Immotile % <sup>a</sup>	65.07± 18.8	39.8± 11.4
<b>(c)sperm ubiquitination</b>		
Ubiquitinated sperm % <sup>a</sup>	7.68± 6.67	1.91±2.37

<sup>a</sup> These parameters are an average ± SD.

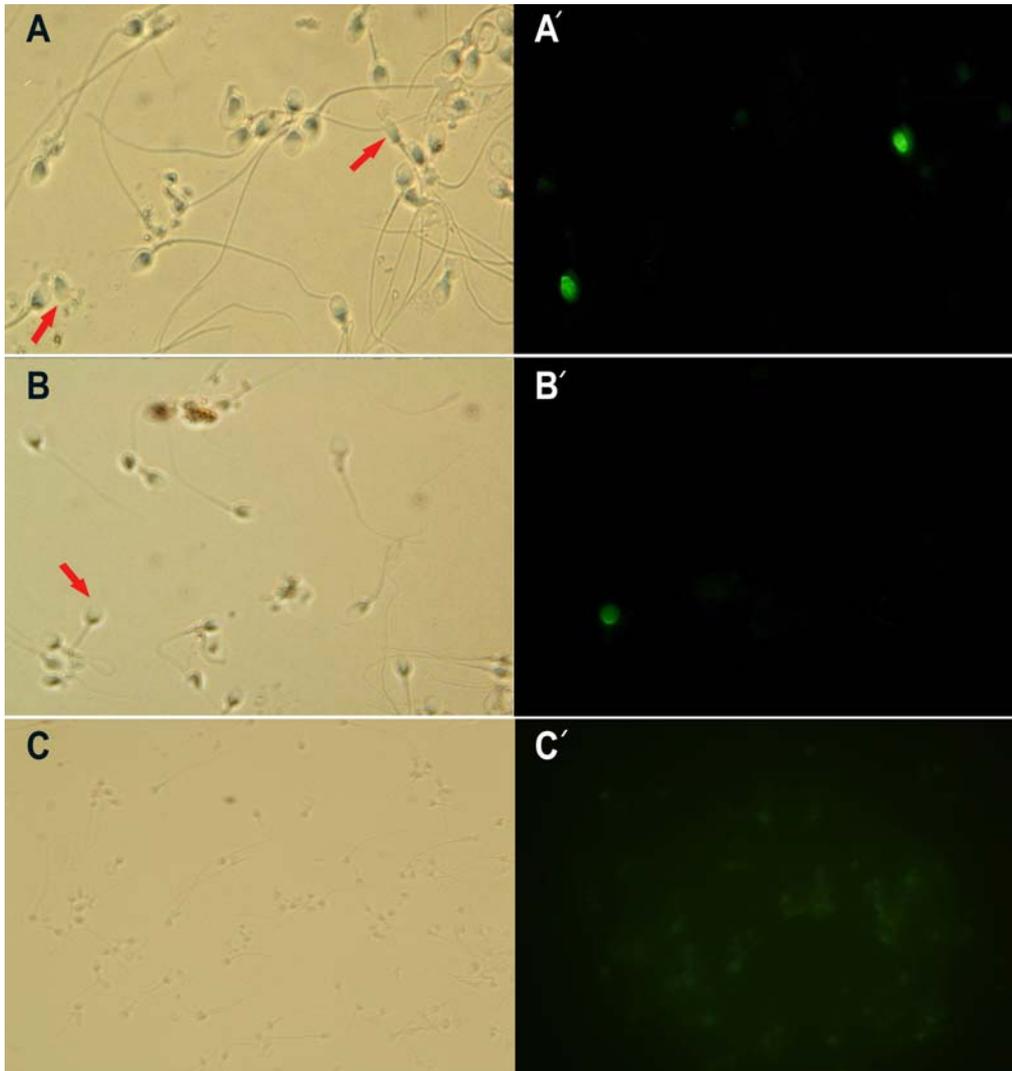
**Table 2:** Correlation analysis of the relationship between the percentages of ubiquitinated sperm and standard semen parameter

Variables	r	P
Sperm count (sperm/ejaculate)	- 0.278	0.005
Sperm concentration (sperm/ml)	-0.37	0.000
Normal morphology (%)	-0.509	0.000
Rapidly progressive motility (%)	- 0.246	0.014
Slowly progressive motility (%)	-0.474	0.000
Non progressive motility (%)	-0.073	0.471
Immotile sperm (%)	0.486	0.000
Immature germ cell (%)	0.469	0.000
Viability (%)	-0.407	0.000

r. Pearson's correlation coefficient.



**Fig. 1:** Sperm ubiquitination as shown by light (A, B) and immunofluorescence microscopy (A' B'). Negative control using FITC-IgG(C, C')



**Fig. 2:** Scatter plots between percentage of ubiquitinated sperm of 100 semen samples, and percentage of rapidly progressive motility (A), Slowly progressive motility (B), Immotile sperm (C), Normal morphology (D), Viability (E), Sperm concentration ( $\times 10^6$  sperm/ml) (F), Sperm count ( $\times 10^6$  sperm/ejaculate) (G) and Immature germ cell (H) . Pearson's correlation coefficients are reported in Table 2

## Discussion

In the present study, the percentage of ubiquitinated sperm were evaluated in 58 normozoospermic and 42 oligoasthenoteratozoospermic cases, using a simplified immunofluorescence analysis technique. The study focused on the possible differences in sperm ubiquitination between two groups and relationship of sperm ubiquitination with semen parameters. The significant increase found in the average percentage of ubiquitinated sperm of oligoas-

thenoteratozoospermic group was similar to the previous findings (17, 22), demonstrated increased semen ubiquitination in patients with dysplasia of fibrous sheath and porcine distal cytoplasmic droplets. This result also confirms studies which have claimed ubiquitin-cross-reactive sperm surface proteins to be apparently a marker for semen abnormalities (16). Increased sperm ubiquitination were also found in seven normozoospermic subjects. This may probably be because semen analyses have

limited values in revealing hidden sperm abnormalities. In other words, anti-ubiquitin antibodies could bind to many sperm with intrinsic, hidden defects that would not be detected by light microscopic evaluations. Hence, further sperm analytical tests could be performed to determine ultra-structural sperm abnormalities (mitochondrial damage, DNA damage, etc) and the exact correlations between sperm ubiquitination and sperm functional parameters. This issue will be considered in our future study.

By using the present subjective immunofluorescence technique, we were able to exclude other ubiquitinated semen components such as debris, immature germ cells and leukocytes. The present method for the detection of ubiquitinated spermatozoa is the modified protocol, previously described for bull sperm (12). Modifications include antibody source and the use of cytospin centrifugation, rather than poly L lysine-coated slides for sperm cell attachments. The source of anti-ubiquitin antibodies used in the present study was polyclonal antibodies generated against human polymerized ubiquitin. Compared to the epitope specific monoclonal antibodies, which are limited in their affinity ubiquitinated protein epitopes, the applied antibodies in this study may recognize broader ranges of sperm ubiquitinated proteins. However, further investigation may be needed to determine if using different epitope-specific ubiquitin probes will verify the presence of ubiquitin-protein conjugates on the surface of spermatozoa. To make the procedure simpler for clinical settings, the applied immunofluorescence assay has been streamlined by using anti-ubiquitin antibody; conjugated with Fluorescein Isothiocyanate (FITC). Since many intrinsic substrates such as mitochondrial membrane proteins (23, 24) and nuclear histones (25, 26) become ubiquitinated during spermatogenesis, no permeabilization occurs during immunofluorescence assay procedures. Therefore, it is presumed that the observed staining was due to anti-ubiq-

uitin immunoreactive proteins on the sperm surface. In addition, the increased immunofluorescence signals, detected following detergent-permeabilization of spermatozoa of the same samples, further confirms the fact that the immunofluorescence labeling of spermatozoa, in the main procedure, were obtained in the absence of permeabilization.

When the percentage of ubiquitinated sperm in 100 men with different infertility etiologies were evaluated, a negative correlation was found with good quality of sperm parameters. The resulted negative correlation, confirms similar studies performed by Ozanon (27) using immunofluorescence based assay. This negative correlation also implies that the biological sperm ubiquitination system in epididymis will specifically tag defective spermatozoa. This result also suggests that by excluding ubiquitinated cell debris and other ubiquitinated contaminants of seminal plasma, sperm ubiquitination positively relate to poor quality of semen parameters.

Our analysis was not in agreement with the results of Muratori (19) which found positive correlation between sperm ubiquitination and good quality of semen parameters through flow cytometric assays (excluding some apoptotic ubiquitinated bodies). They found a positive correlation between sperm ubiquitination and good quality of semen parameters, which indicates that ubiquitination is a normal process in spermatozoa maturation. However, their result was inconsistent with the previously studied role of ubiquitin in fertilization and gametogenesis (10, 28) and the global role of ubiquitin in proteolytic degradation of misfolded and damaged proteins.

Although it is likely that defective sperm are ubiquitinated, it is not clear how such sperm are recognized by ubiquitination machinery. The analyzed correlations between sperm Ubiquitination and semen parameters may help us to realize the mechanism of this machinery. According to the backward multiple linear regressions, among standard semen parameters,

the variable mainly influencing sperm ubiquitination is morphology. This analysis may imply that morphology has a critical role in targeting abnormal spermatozoa. The data is also in agreement with Sutovsky studies reported that ubiquitin immunoreactivity measured by SUTI assay correlated closely with sperm count and percentage of abnormal morphology (16).

Sperm ubiquitination is a smart system at molecular levels in recognizing abnormal spermatozoa and marking them for elimination during epididymal transit. Using such biological systems as predictive sperm functional tests, have the benefit of more accurately detecting intrinsic sperm abnormalities. The present subjective immunofluorescence assay can be completed by flow cytometry trials, providing the means for objective quantification of ubiquitin measurements in sperm samples. Such flow cytometric analyses could be performed by double staining of spermatozoa with FITC labeled anti-ubiquitin and specific antisperm antibodies, in order to reduce false positive results, which are attributed to ubiquitinated contaminants.

In summary, the present subjective immunoassay of sperm-surface ubiquitination provides a promising approach to semen evaluations in clinical settings. In contrast to the existing subjective assays, this technique may provide a reliable and measurable parameter of suboptimal semen quality, acquired prior to sperm discharge from urogenital tract.

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The authors declare that they have no Conflict of Interests.

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