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Ubiquitin-Dependent Sperm Quality Control Mechanism Recognizes Spermatozoa With DNA Defects as Revealed by Dual Ubiquitin-TUNEL Assay

PETER SUTOVSKY,[1,3]* EVELYN NEUBER,[2] AND GERALD SCHATTEN[3]

[1]*Departments of Animal Sciences, and Obstetrics and Gynecology, University of Missouri-Columbia, Columbia, Missouri*
[2]*Department of Obstetrics and Gynecology, Yale University of Medicine, New Haven, Connecticut*
[3]*University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania*

ABSTRACT Defective mammalian spermatozoa become ubiquitinated during epididymal passage, a mechanism that may mark the abnormal spermatozoa for proteolytic destruction (Sutovsky et al., 2001a: J Cell Sci 114:1665–1675). It is not known how such spermatozoa are recognized by the epididymal ubiquitination pathway and whether there is a selection against certain types of sperm defects. We examined the relationship between sperm ubiqutination, lifelong sperm morphology and sperm DNA defects using a single chanel, ubiquitin-activated flow cytometric assay, and a dual, ubiquitin-TUNEL assay. Semen samples from nine service sires of good-to-average fertility were screened. A positive correlation was found between sperm ubiquitination and the average frequency of morphological semen abnormalities from field evaluations performed throughout the reproductive life of individual sires. Sample correlation coefficients were $r = 0.65$ for primary (head and tail) and $r = 0.60$ for total semen abnormalities in the single channel assay. In a dual assay, we found a high, positive correlation ($r = 0.93$) between the ubiquitin-positive sperm and the TUNEL positive sperm. Substantial correlations ($r = 0.47–0.64$) were observed when the measurements from these two respective assays were compared for individual sires. While anti-ubiquitin antibodies recognized most of the TUNEL-positive sperm cells, the TUNEL-positive spermatozoa represented only a subset (~20–40%) of all ubiquitin-positive cells. It appears that the ubiquitin-dependent sperm quality control, residing in the epididymal epithelium, has the ability to detect spermatozoa with apoptotic or necrotic DNA, while spermatozoa with defects other than DNA fragmentation are also recognized and ubiquitinated. *Mol. Reprod. Dev. 61: 406–413, 2002.*

Key Words: ubiquitin; epididymis; sperm morphology; DNA; TUNEL assay; apoptosis

INTRODUCTION

The selection and elimination of the defective spermatogenic cells occurs during spermatogenesis in the testis, presumably by the way of apoptotic pathway (reviewed by Sinha Hikim and Swerdloff, 1999). Our recent data (Sutovsky et al., 2001a) suggest the existence of an ubiquitin-dependent sperm quality control mechanism that resides in the epididymis. Ubiquitin, the universal proteolytic marker (reviewed by Hershko and Ciechanover, 1998), is a small peptide of 8.5-kDa that can ligate covalently to lysine residues of various proteins destined for degradation by the proteasomal or lysosomal pathway. Following the covalent ligation of one ubiquitin molecule to the substrate, additional ubiquitin molecules can bind to it, forming a variety of polyubiquitin chains. This accounts for the surprising diversity and substrate specificity of ubiquitination. Ubiquitin-dependent proteolysis occurs at a precisely defined point in the life cycle of the substrate protein, and at specific stages during the cell cycle (reviewed by Laney and Hochstrasser, 1999).

In the epididymis, ubiquitin appears to be secreted in apocrine fashion by the cells of the epididymal epithelium and binds preferentially to the surface of defective spermatozoa in bulls, men, rhesus monkeys, and mice (Sutovsky et al., 2001a,b). Some of such spermatozoa, along with the ubiquitinated cytoplasmic droplets, seem to be disposed of by the epididymal epithelium, explaining why there is a gradual decrease

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*Correspondence to: Peter Sutovsky, PhD, Assistant Professor Department of Animal Sciences, College of Agriculture, Food and Natural Resources, University of Missouri-Columbia, S141 Animal Sciences Research Center, 920 East Campus Drive, Columbia, MO 65211-5300. E-mail: sutovskyp@missouri.edu

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in the percentage of defective spermatozoa during epididymal passage (Roussel et al., 1967; Ramamohana Rao et al., 1980). Some ubiquitinated spermatozoa can be found in the ejaculate, though they are probably immotile and thus unable to compete for an oocyte.

It is not known what is the signal for the ubiquitination of abnormal spermatozoa. One possibility is that the apoptotic, or otherwise compromised spermatozoa with altered plasma membrane, permeabilized mitochondrial membranes and fragmented nuclear DNA are recognized by ubiquitin. We have developed the sperm-ubiquitin-tag immunoassay (SUTI), an objective semen quality assay encompassing the flow-cytometric measurement of fluorescence in semen samples processed with anti-ubiquitin antibodies and appropriate fluorescent conjugates (Sutovsky et al., 2001b). In the present study, SUTI is used in combination with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL assay) to probe bull spermatozoa. In addition to ubiquitin, this assay screens for DNA strand breaks in the sperm nuclei, indicative of sperm DNA fragmentation via the apoptotic or necrotic pathway. While only 20–40% of defective, ubiquitinated spermatozoa carry fragmented DNA, our results demonstrate a high degree of correlation between sperm ubiquitination and DNA defects. While almost all spermatozoa with defective nuclear DNA are subject to ubiquitination, many other ubiquitinated spermatozoa with visible defects do not carry fragmented DNA. These findings suggest that the spermatozoa with chromatin/DNA defects are recognized in the epididymis and marked for destruction by ubiquitin-dependent proteolytic pathway.

MATERIALS AND METHODS

Sperm and Fertility/Morphology Data

Semen samples from nine Holstein bulls of varied, but at least average fertility, were purchased from the keeper of sires, ABS Global, Inc. (De Forest, WI). Due to limited availability, samples from Bull #5 were not analyzed by dual, TUNEL/ubiquitin assay. Sperm were cryopreserved in egg yolk extender and thawed prior to analyses. Neither the dilution in extender nor the freezing and thawing, seemed to affect the labeling generated by ubiquitin and TUNEL assays. Sperm were washed by the centrifugation in Sperm TL medium (Parish et al., 1986) for 5 min at 500*g*.

Relative rates of primary (% of sperm with head and/or tail defects) and total (% of all defects including semen contaminants other than defective sperm and broken heads/tails) were assessed by semen sellers for each the nine bulls during their reproductive life. The age of sires at the day of collection, information on collection frequency, records of repeated or discarded collections, and the dates of collections were not available. Semen quality in sires varies substantially during first 2 years after birth (Johnson et al., 1998), therefore, only the collections/evaluations performed after 2 years of age (16–348 collections in individual sires) were included in the statistical analysis.

Single Channel Flow Cytometry, Ubiquitin Assay

Sperm suspensions were pelleted by a 5 min centrifugation at 500*g* and fixed for 40 min in 2% formaldehyde in phosphate buffered saline (PBS, Sigma St. Louis, MO). No permeabilization of the sperm membranes was performed. Samples were blocked in 5% normal goat serum (NGS) in PBS for 25 min and incubated with the mouse monoclonal antibody MK-12-3 against bovine erythrocyte ubiquitin (MBL, Nagoya, Japan; dil. 1/100), washed by resuspension in 10 ml of PBS with 1% of NGS, then incubated with FITC-conjugated goat anti-mouse IgG (Zymed Laboratories, South San Francisco, CA; dil. 1/80), washed again, and resuspended in 500 μl of pure PBS without serum. Blank, negative control samples were prepared for each experimental sample by omitting the primary antibody.

Ubiquitin median values (channel number at which half the cells are dimmer and half the cells are brighter; increases with the increasing number of brighter cells), and the histograms of relative fluorescence, were generated by FACS Calibur Analyzer (Becton Dickinson, San Diego, CA) at 488 nm wavelength. Relative levels of fluorescence in 5,000 individual cells/sample were recorded. A sample of PBS buffer was used to eliminate nonspecific fluorescence and to calibrate the cytometer. A blank sample from the corresponding sire, labeled with secondary antibody alone, was measured before each anti-ubiquitin-labeled sample. Analysis thresholds were set at channel zero. The shape of the fluorescence histogram, the scatter plot of visible light reflecting the overall cell shape, the shift of the curve towards high relative fluorescence, and the shift of the peak of relative fluorescence curve on the X-axis, were also noted.

Dual Channel Flow Cytometry, Ubiquitin-TUNEL Assay

Sperm were collected and fixed as described for single channel flow cytometry assay, and permeabilized for 10 min with 0.1% Triton-X-100 (Sigma). Permeabilization of bull sperm does not substantially affect the reading of ubiquitin fluorescence, since it does not unmask the intrinsically ubiquitinated substrates of sperm mitochondria, as seen in human sperm (Sutovsky et al., 1999, 2000). Samples were stained for 1 hr at 37°C using In Situ Death Detection Kit AP (TUNEL kit; Roche Diagnostic Corp., Indianapolis, IN), according to the manufacturer protocol. After a cycle of resuspension/centrifugation in PBS, the samples were processed with anti-ubiquitin antibody MK-12-3, as described above, except that the Cy-5 (far red-spectrum: >650 nm) conjugated goat anti-mouse IgG was used to avoid the overlap of ubiquitin signal with the green-fluorescent TUNEL labeling. Blank, double-negative control samples were generated by the omission of substrate from TUNEL labeling kit and by

the omission of anti-ubiquitin antibody from the immunolabeling protocol.

Ubiquitin/TUNEL double labeling was measured sequentially in each of the 5,000 cells screened in each sample by two-channel flow cytometry using FACS Calibur Analyzer. Instead of medians and two-dimension (fluorescence x/number of cells y) histograms, three dimension (fluorescence x, fluorescence y, number of cells xy) scatter diagrams were obtained. Fluorescence thresholds were set subjectively for both TUNEL and ubiquitin-generated signals, based on the scatter diagram of Bull #1, which had the lowest number of ubiquitinated sperm and was taken as a standard. In such scatter diagrams (see Fig. 5A), the lower left portion (LL) represented cells with low levels of both TUNEL- and ubiquitin-activated fluorescence, (i.e., the presumed normal spermatozoa), the upper left (UL) portion contained cells with high TUNEL and low ubiquitin fluorescence; upper right portion (UR) had cells with high TUNEL and high ubiquitin fluorescence, and lower right (LR) portion was occupied by cells with low TUNEL, but high ubiquitin fluorescence.

Epifluorescence Microscopy

Spermatozoa were attached to poly-L-lysine-coated microscopy coverslips (Sutovsky et al., 2000) and fixed in 2% formaldehyde as described for flow cytometry. Samples were blocked in 5% normal goat serum (NGS) in PBS and sequentially incubated with the TUNEL kit, antibody MK-12-3 and TRITC-conjugated goat anti-mouse IgM (Zymed Inc., South San Francisco, CA; dil. 1/80). DNA was counter-stained with blue-fluorescent DAPI (Molecular Probes, Eugene, OR). Samples were washed and mounted in an anti-fade medium (Vectashield; Vector Labs, Burlinghame, CA). Conventional epifluorescence microscopy was performed using a Zeiss Axiophot microscope. Images were captured using a Princeton RTE/CCD 1217 digital camera (Princeton Instruments, Inc., Trenton, NJ) and MetaMorph software (Universal Imaging Corp., West Chester, PA), edited using Adobe Photoshop 4.0 (Adobe Systems Inc., Mountain View, CA) and printed on a SONY UP-D 8800 dye sublimation printer (Sony Corp., NY).

Statistical Analysis

Histograms and scatter diagrams of relative fluorescence were evaluated, and the predicted linear regression lines were plotted based on sample regression coefficients (b_{yx}; Snedecor and Cochran, 1973; Sokal and Rohlf, 1981), calculated using average flow cytometry medians from three separate repeats of single-channel, ubiquitin-only flow cytometry and available morphology data (rates of primary and total semen abnormalities, as determined by ABS Global). Data on the lifelong histories of semen evaluation, including the percentages of primary and secondary abnormalities (Table 1), were received from semen distributors after all tests were performed. All data were entered into Microsoft Excel spreadsheets and correlation (r) and regression (b) coefficients were calculated using Microsoft Excel Statistical Tool Package (MS Excel 98). The same data sets were used to calculate the percentages of cells with four different types of combined TUNEL/ubiquitin signals and to evaluate the correlation between such groups of cells (Fig. 4B).

RESULTS

To establish the relationship between ubiquitin and sperm morphology, semen samples from nine bulls of good/average fertility were assayed for ubiquitin by fluorescence-activated flow cytometry and the acquired data were compared with the records of semen quality provided by the distributors of semen. Median intervals of fluorescence (ubiquitin medians) were obtained for each sample, reflecting the value of relative fluorescence from which half the cells are on the left side and half on the right side in the histogram of fluorescent cell distribution (Fig. 1). Average ubiquitin medians from three repeats are presented in Table 1. The lowest ubiquitin medians were obtained from sires #1, 5, 7, and 8. These bulls also displayed the best (i.e., lowest) average frequencies of primary and total semen abnormalities (Table 1). The sires with elevated ubiquitin

TABLE 1. Frequency of Primary and Secondary Sperm Defects, and Ubiquitinated Spermatozoa in Nine Bulls of Varied Fertility*

Bull #	n-value	Primary abnormalities	SD	SE	Total abnormalities	SD	SE	Median ubiquitin
1	19	6.3	1.8	0.41	12.8	3.4	0.78	2.08
2	109	7.7	4	0.38	14.2	6.3	0.61	12.71
3	206	12.6	5.8	0.4	17.1	5.8	0.4	10.42
4	348	10.5	3.9	0.21	16.5	4.4	0.24	14.08
5	47	6.1	2.5	0.36	10.7	4	0.58	4.44
6	129	7.6	2.6	0.23	12.5	3.6	0.32	9.86
7	16	7	1.9	0.48	9.8	2.5	0.63	7.54
8	61	4.8	2	0.26	7.9	2.9	0.37	5.47
9	236	8	2.9	0.19	12.2	4.1	0.27	12.06
Avgerage		7.844			12.633			l8.74

*n-value represents assessments performed during the reproductive lifetime of the sire, excluding the first 2 years. Ubiquitin median is a relative value with no dimension. SD, standard deviation; SE, standard error.

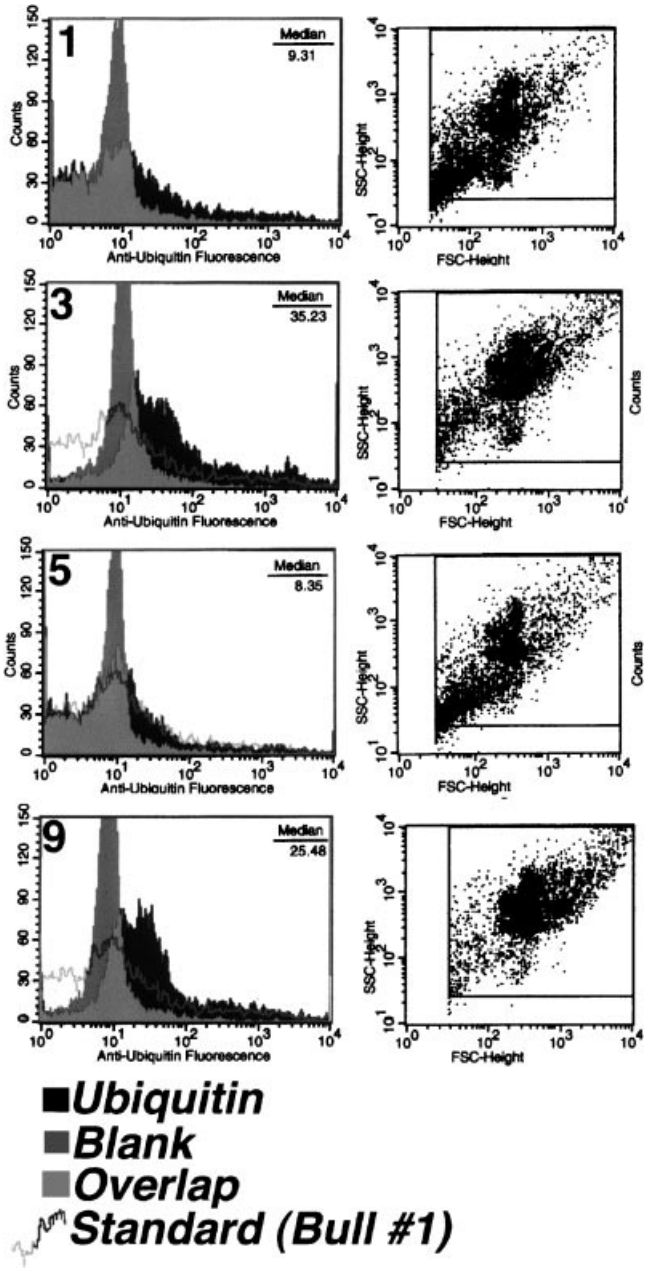


Fig. 1. Flow cytometry histograms rendered by the fluorescence of sperm samples processed with anti-ubiquitin antibodies and appropriate fluorescent conjugates (left: X = anti-ubiquitin fluorescence, Y = counts). Corresponding, visible light scatter plots are shown on right (FCS/SSC height). Representative semen samples originate from four bulls (numbering corresponds to Table 1) with varied, but good fertility. Median values of ubiquitin fluorescence are shown in upper right corner of each histogram. Empty curve in **panels 3**, **5**, and **9** outlines the histogram of a standard sample (Bull #**1**) from a bull with lowest occurrence of ubiquitinated spermatozoa. Note higher median values, reflecting the increase in the number of ubiquitinated, defective sperm, in Bull # 3 and 9. Results from the same run are shown for all bulls.

medians (Bull #3, 4, 6, and 9; Table 1) also showed a higher percentage of primary and total semen abnormalities. In flow cytometry, the histograms of such bulls were typically shifted to the right, reflecting an increase in the number of highly-fluorescent cells (Fig. 1).

A significant positive correlation ($P < 0.05$) was found between ubiquitin medians and the lifelong, average frequencies of primary (Fig. 2A; $r = 0.65$) and total (Fig. 2B; $r = 0.60$) semen abnormalities. Eight of the nine sires were subsequently screened by a dual, TUNEL/ubiquitin assay (Figs. 3 and 4). Fluorescence microscopy of TUNEL- and ubiquitin-positive cells revealed DNA fragmentation in the ubiquitin-positive sperm displaying a variety of morphological defects (Fig. 3). These defects included coiled tails ("Dag defect"; Blom, 1966), twin tails, twin heads, abnormal sperm tail midpieces, deformed sperm heads and detached tails as well as occasional cellular debris and immature spermatogenic cells. In some cases, the

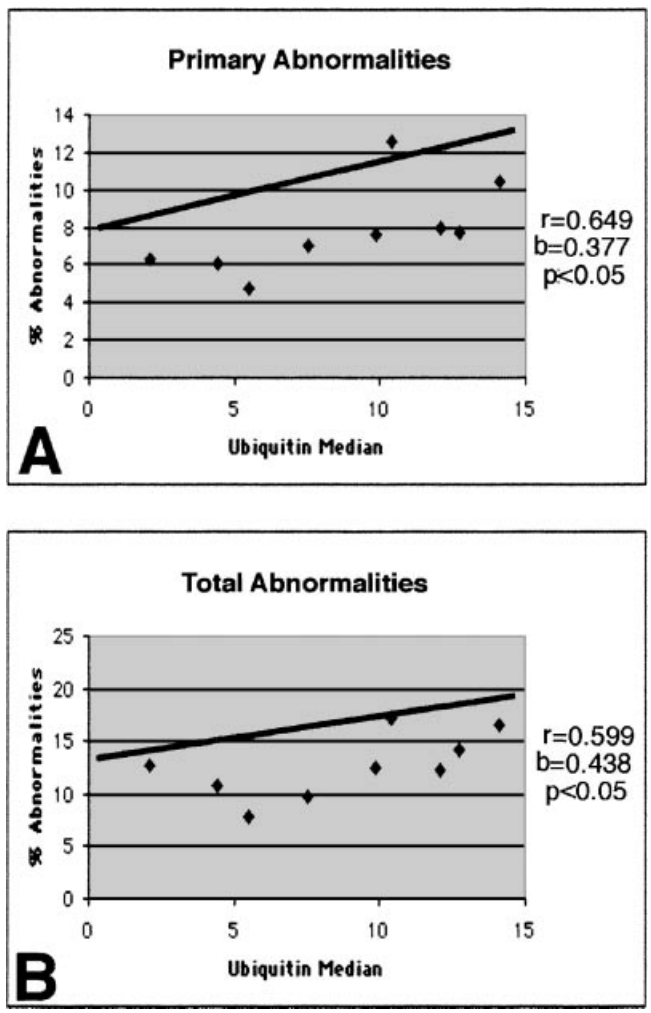


Fig. 2. Correlation between the median fluorescence of ubiquitin (median) in semen samples of the screened bulls, and the lifelong averages of sperm morphology parameters provided by semen distributors. Primary, sperm abnormalities (**A**: $r = 0.649$) and total abnormalities (**B**: $r = 0.599$) show significant positive correlation with ubiquitin medians. Ubiquitin medians shown are an average from three runs; r is a coefficient of correlation; b is a coefficient of regression used to plot the regression lines shown in respective diagrams.

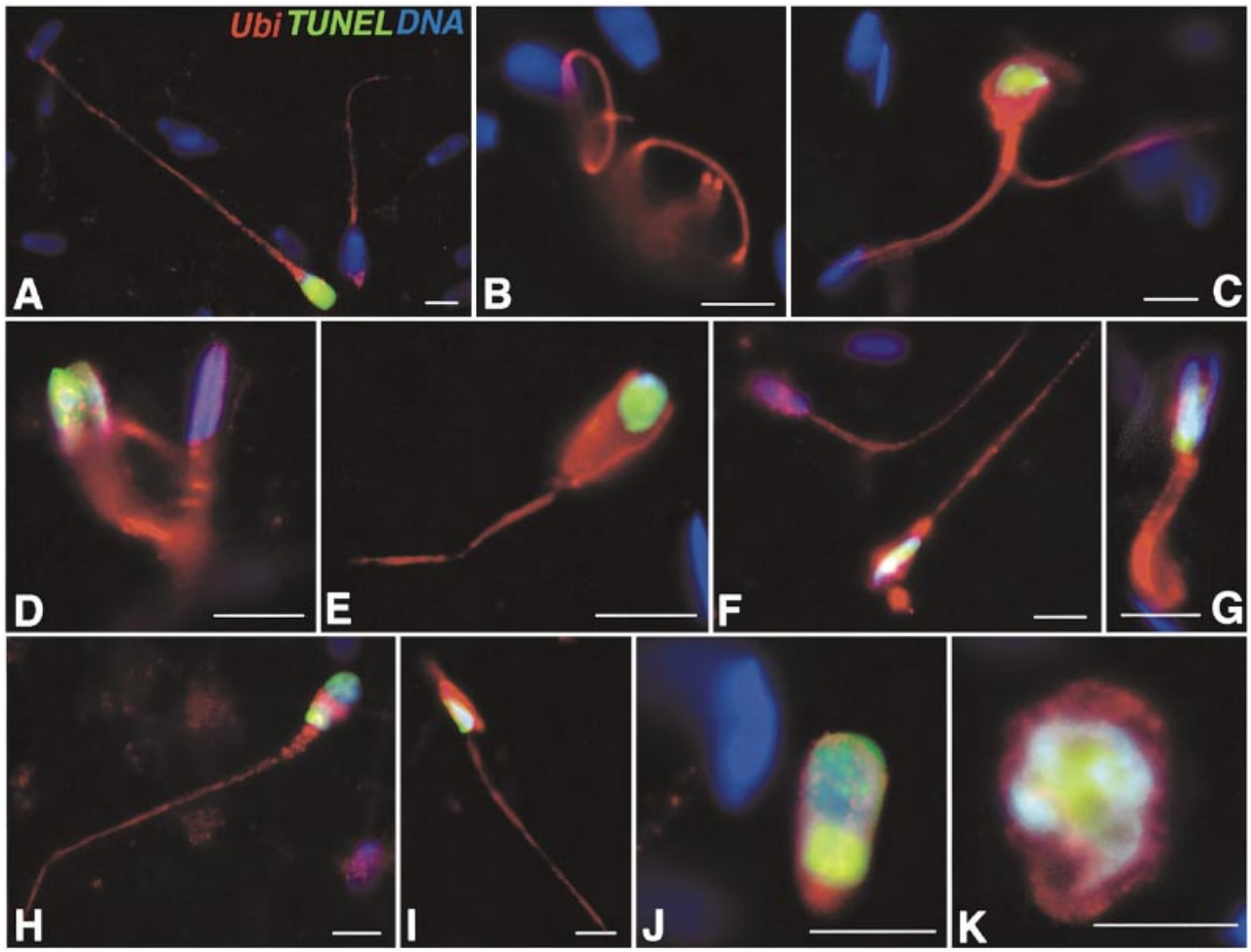


Fig. 3. Fluorescent detection of fragmented DNA by TUNEL assay (green), and ubiquitin (red) in bull spermatozoa. DNA in both normal and defective spermatozoa is counter-stained with DAPI (blue). **A**: Two morphologically normal sperm, in which hidden defects were revealed by ubiquitin (both sperm) and TUNEL (left sperm only) assays. **B**: An ubiquitinated spermatozoon with coiled tail (Dag defect) shows no signs of DNA damage detectable by TUNEL. DNA damage was detected by TUNEL in the sperm with twin tails **(C)**, twin heads (**D**; note an ubiquitin-positive spermatozoon with normal DNA on the right), abnormal midpieces **(E–H)**, deformed sperm heads **(I)**, and detached tails **(J)**, as well as in the cellular debris and immature spermatogenic cells **(K)**, occasionally found in the semen samples. Scale bars in all panels are 10 μm.

seemingly normal sperm displayed both ubiquitin and TUNEL labeling (Fig. 3A). In other cases, the defective, ubiquitin-positive sperm displayed no TUNEL-generated fluorescence (e.g., Fig. 3A, right spermatozoon; and Fig. 3B). Very few TUNEL-positive sperm without ubiquitin labeling were recorded.

The dual detection of TUNEL- and ubiquitin-generated fluorescence in flow cytometer (Fig. 4) revealed low levels of ubiquitinated sperm cells in sires #1 and 8 (Fig. 5; semen of Bull #5 was not available for this double assay). The double-labeling also indicated low fluorescence in Bull #9, that scored a high median in single channel assay. With the exception of Bull #9, all sires that had high medians in the first assay (Bull #2, 3, 4, and 6) also had high levels of positive cells in the combined, TUNEL/ubiquitin assays (Fig. 5A,B). TUNEL-positive and ubiquitin-positive cells were highly correlated (Fig. 5B; $r = 0.93$), indicating almost complete overlap. When all TUNEL positive cells (UL + UR) were pooled and compared with the ubiquitin medians generated by the first set of experiments (ubiquitin-only assay), the correlation ($r = 0.64$; Fig. 5B) was closer than when this was done for all ubiquitin positive cells (UR + LR; Fig. 5B; $r = 0.55$). However, a comparable correlation coefficient ($r = 0.63$) was found in the ubiquitin-positive cells that did not display TUNEL-fluorescence (LR; see legend in Fig. 5A).

DISCUSSION

The present data show that anti-ubiquitin antibodies recognize the TUNEL-positive sperm with DNA strand breaks resulting from apoptosis or necrosis, and a wide range of other abnormalities that are not accompanied by DNA damage. These include the coiled

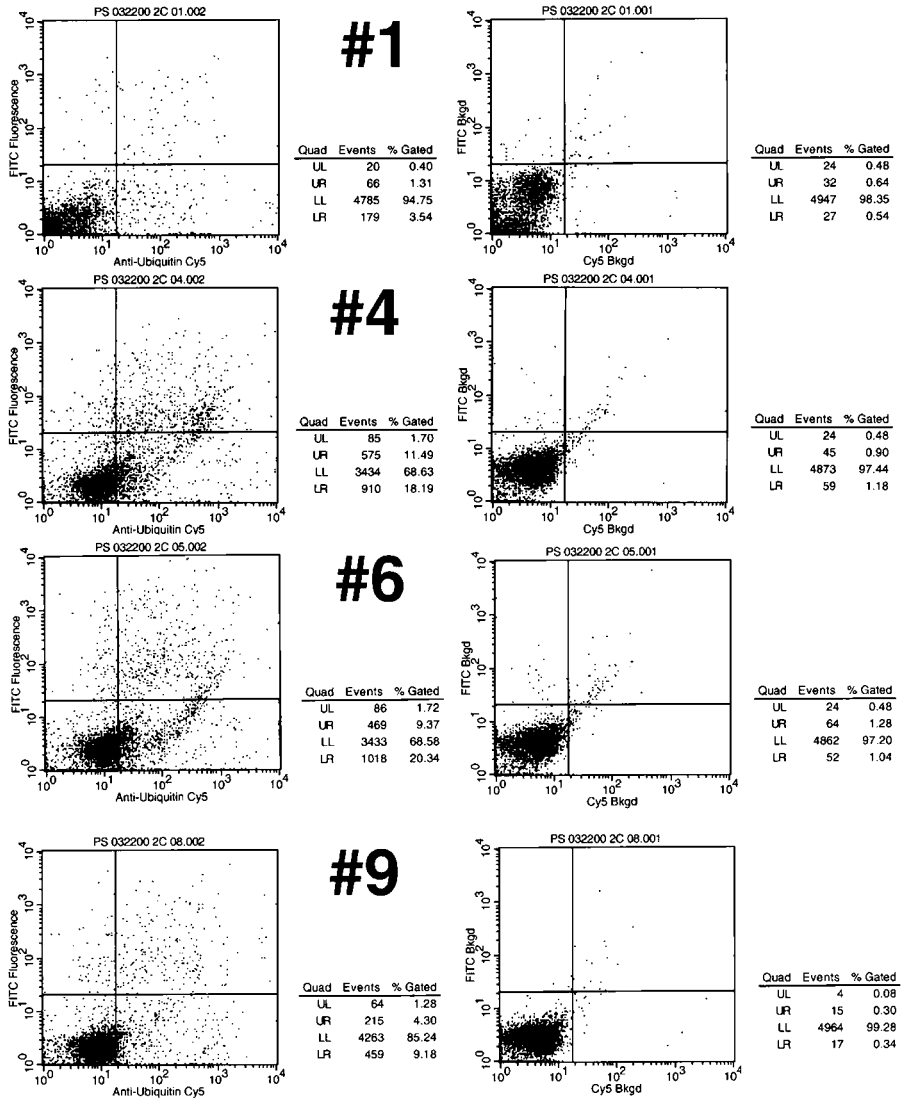
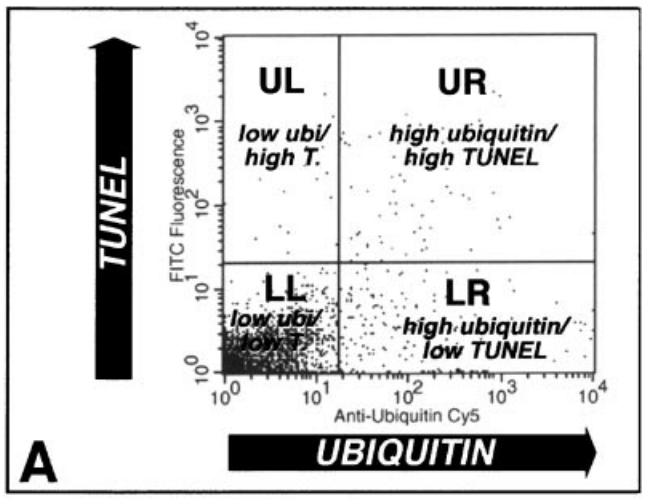
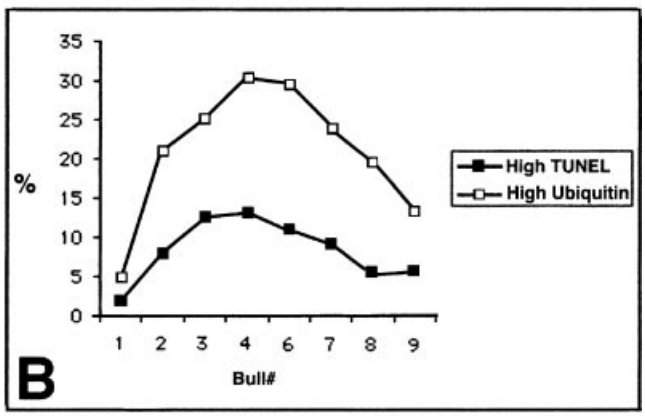


Fig. 4. Dual flow cytometric detection of ubiquitin and TUNEL fluorescence in semen samples of four sires from the analyzed group. Histograms of fluorescent cell distribution from a representative run are shown. Cut off lines were set arbitrarily, based on the distribution of high/low fluorescent cells in Bull #1, which had the lowest rate of ubiquitin-positive cells in all assays performed for this study. Axis X = relative ubiquitin fluorescence, axis Y = relative TUNEL fluorescence (no dimension for either).

tails ("Dag defect"; Blom, 1966), abnormal mitochondrial sheaths, split tails, twin tails, twin heads, and leukocytes, spermatids and cellular debris present in ejaculate (Sutovsky et al., 2001a,b; this study). Our recent research shows that the ubiquitination of the surface of defective spermatozoa occurs during epididymal passage (Sutovsky et al., 2001a). Similar to some other epididymal proteins (e.g., Agrawal and Vanha-Perttula, 1988; Cooper et al., 1988; Coulter, 1992; Hermo et al., 1992; Kirchhoff, 1998), ubiquitin appears to be secreted in apocrine fashion by the epididymal epithelial cells (Sutovsky et al., 2001a). The tagging of defective spermatozoa with ubiquitin may be responsible for the removal of a portion of defective sperm during epididymal passage (Roussel et al., 1967; Ramamohana Rao et al., 1980). It is interesting to note that the defective spermatozoa are often partially or completely demembranated (Barth and Oko, 1989; Sutovsky et al., 2001b) and that the levels of Annexin V, a marker of apoptotic plasma membrane rearrangement, are elevated in the sperm of infertile men (Oosterhuis et al., 2000). Other early-apoptotic markers, including activated caspase 3 (Weil et al., 1998) and Fas-ligand (Sakkas et al., 1999), have been found in defective spermatozoa of mice and men, respectively. The modifications of plasma membrane in defective spermatozoa (Oosterhuis et al., 2000) may account for their high, nonspecific uptake of the histological stain, trypan blue-Giemsa (Nagy et al., 1999). Perhaps the epididymal ubiquitinationand partial removal of the defective spermatozoa is the common endpoint at which different mechanisms of testicular and epididymal





r-value	UL	UR	LL	LR	UL+UR	UR+LR
UBIMED	0.630	0.625	-0.556	0.469	0.635	0.551
UL+LL						*0.931

Fig. 5. A: Schematic rendering of the arbitrary cutoffs used to evaluate flow cytometry histograms generated by dual TUNEL/ubiquitin assay. UL, upper left (cells with high TUNEL and low ubiquitin fluorescence); LL, lower left (cells with low TUNEL and low ubiquitin signals); UR, upper right (cells with high TUNEL and high ubiquitin); LR, lower right (cells with low TUNEL and high ubiquitin fluorescence). **B**: Statistical analysis of the relationship between the relative rates of TUNEL- and ubiquitin-positive cells in dual assay, and ubiquitin medians (UBIMED) obtained by single channel flow cytometry measurements (see also Table 1). Note high correlation between the TUNEL- and ubiquitin-positive cells (UL + UR vs. UR + LR; $r = 0.93$). Substantial levels of correlation ($r = 0.47–0.64$) are seen between the results from single channel assay and dual assay (ubiquitin medians compared to percent of positive cells).

sperm quality control converge. The remaining ubiquitinated sperm, found in the ejaculated semen, may therefore be a reliable marker of overall sperm quality.

Flow cytometric double-detection of TUNEL and ubiquitin fluorescence in semen samples of eight bulls from the analyzed group revealed very close correlation between the sums of TUNEL and ubiquitin positive cells ($r = 0.93$). Most TUNEL-positive cells were thus recognized by anti-ubiquitin antibodies. Only 12–15% of TUNEL-positive cells were not recognized by anti-ubiquitin antibodies in bulls with elevated ubiquitin levels. It is common in both bulls (this study) and humans (Sutovsky et al., 2001a) that defective spermatozoa are ubiquitinated mainly on the sperm tail, rather than on the sperm head (e.g., Fig. 3A,B), and the heads and tails of defective spermatozoa can separate inside the epididymis or during semen handling. High TUNEL-readings in this fraction, which are not accompanied by high levels of ubiquitin, likely account for the TUNEL-positive sperm heads detached from the ubiquitinated tails.

Two different approaches can be used to analyze sperm ubiqutination. In the ubiquitin-only assays, the relative levels of ubiquitin signal were displayed in the form of two-dimensional histograms. These histograms show the ubiquitin-generated fluorescence on X-axis and the numbers of cells corresponding to each value of relative fluorescence on the Y-axis. The advantage of this approach is that it does not require any arbitrary (i.e., subjective) setting of threshold levels and provides a single numeric value of median fluorescence for each sample. This is useful for the screening of large number of samples. Scatter diagrams used for the combined TUNEL/ubiquitin assay add the third-dimension, i.e., the relative levels of TUNEL-generated fluorescence, to the ubiquitin-generated fluorescence readings and sums of cells in each interval of fluorescence intensity. The TUNEL-readings seem to correlate well with ubiquitin readings. General DNA stains such as DAPI, propidium iodide or Hoechst 33342/35258 could also be added to antibody solutions to add yet another level of detection.

Objective, flow cytometric assays are a superior tool for semen analysis. Membrane permeant nuclear stains such as propidium iodide (Garner et al., 1986) and vital mitochondrial dyes (Evenson et al., 1982; Garner and Thomas, 1999) are used to discriminate between live a dead spermatozoa. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), ELISA or Comet assays are suggestive of the apoptotic or necrotic process in spermatozoa (Baccetti et al., 1996; Sun et al., 1997; Hughes et al., 1999; van der Schans et al., 2000) and can be used to screen for DNA strand breaks in bull sperm nuclei. DNA-specific dyes were also applied to intact (Ferrari et al., 1998) or partially denatured bull sperm (Ballachey et al., 1987) to extrapolate sperm head morphology. Such tests show high correlation with fertility of screened animals (Ballachey et al., 1987), while they may not correlate well with the results of microscopic semen analysis (Evenson et al., 1999). The DNA-based assays provide useful information about sperm quality, though they may not cover the whole spectrum of sperm head and tail abnormalities found in both fertile and subfertile semen samples. Sperm-ubiquitin-tag immunoassay (SUTI), as used in this study, is a candidate method for automated semen evaluation in service sires. Average percentages of primary abnormalities and total abnormalities, assessed by the sellers of semen throughout the reproductive life span of individual sires, show positive correlation ($r = 0.65$ and 0.60, respectively) with ubiquitin-medians, suggesting that ubiquitin is a reliable

marker of semen abnormalities in cattle. A similar tendency has been previously reported in humans (Sutovsky et al., 2001a). It is yet to be investigated whether this assay could be used to predict future fertility in young sires.

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