

UBIQUITIN AS AN OBJECTIVE MARKER OF SEMEN QUALITY AND FERTILITY IN BULLS

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Introduction

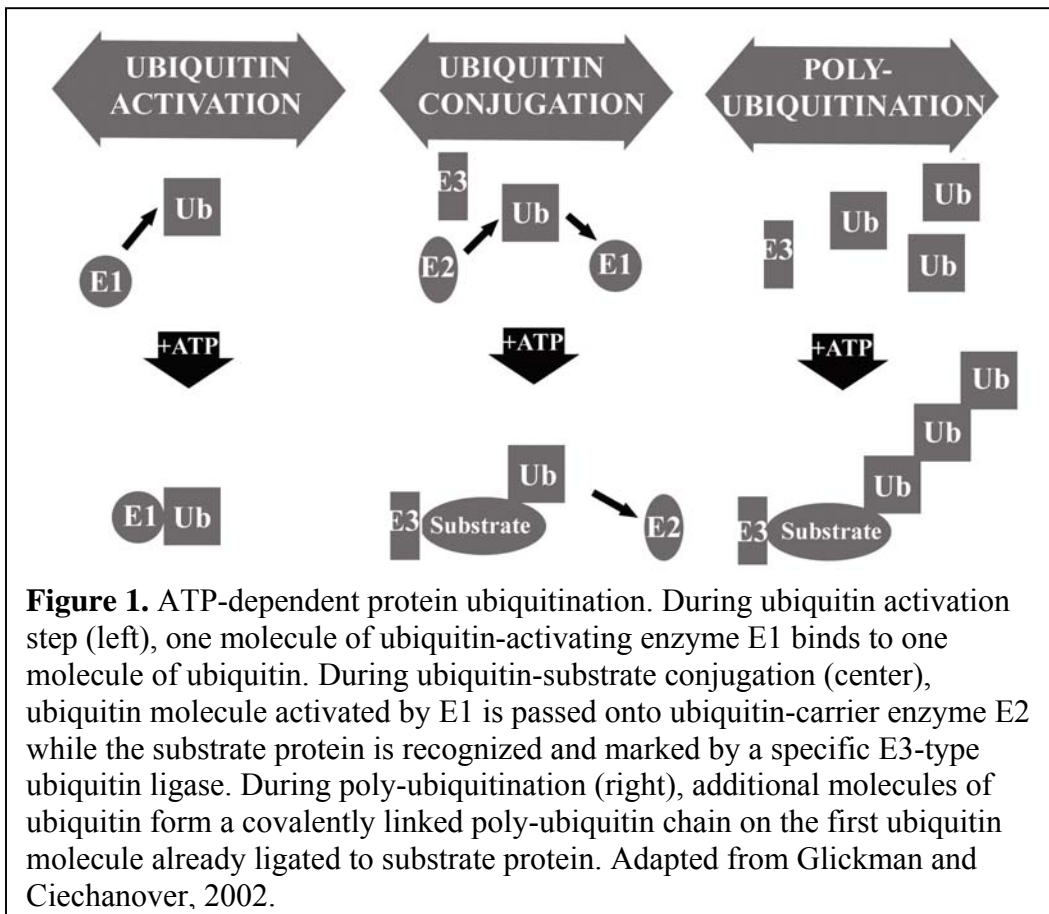
Andrology is one of few fields of medicine and biology in which biochemical and molecular tools are not routinely used for evaluation and diagnostics (discussed by Eliasson, 2003). Accurate evaluation of an infertile male is of paramount importance for proper diagnosis of couples in human infertility, but also for an accurate estimation of current fertility and prediction of future fertility in large farm animals including bulls, boars, and stallions. This is currently not possible due to the paucity of a universal, objective semen assay capable of detecting a wide array of both visible and cryptic sperm abnormalities (Amann, 1989; Nikoletos et al., 1999).

It is indisputable the currently available, subjective, and automated methods of semen evaluation provide important information about the quality of a semen sample. However, each method has major shortcomings that may result in a large portion of defective spermatozoa being missed by even a well-trained semen evaluator. These shortcomings are mostly due to the fact current semen evaluation techniques are either subjective and/or detect only certain types of sperm abnormalities found in fertile and infertile males. It is thus highly desirable to develop new, objective, unbiased semen quality assays that would reflect both current and future fertility of a male. Toward this goal, we have been working on the identification of “negative” sperm quality markers, i.e. proteins associated with morphologically and/or functionally defective spermatozoa in male farm animals and humans. The following presentation will discuss the identification of sperm protein ubiquitin as one such fertility marker.

Ubiquitin-Proteasome Pathway

The ubiquitin system is unique due to the fact it is at the same time highly conserved and universal on one side, and highly selective and substrate specific on the other. Besides controlled protein degradation, ubiquitination helps regulate a diverse array of biological events including cell cycle progression, membrane receptor-endocytosis, antigen presentation in immune system, and even retroviral infection.

Ubiquitination is achieved through the covalent binding of 76-AA, 8.5 kDa ubiquitin to the ϵ -amino group on the substrate's Lys-residues via ubiquitin's C-terminal AA residuum (G76; **Figure 1**). Ubiquitination requires the hydrolysis of ATP and a set of ubiquitin-conjugating factors including ubiquitin activating (UBA) and conjugating (UBC) enzymes (review: Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002).



Common patterns of ubiquitination include mono-, di- and tetra-ubiquitination, each of them increasing the molecular weight of a substrate by an appropriate multiple of 8.5 kDa. Variations in the patterns of substrate ubiquitination appear as ladder bands, separated by 8.5 kDa spaces, in Western blotting analyses. Tetra- and poly-ubiquitination are consensus signals for the docking of ubiquitinated proteins to 26-S-proteasome, resulting in proteolytic degradation of the substrate and return of the intact ubiquitin molecules to the cytosolic pool (Pickart, 1998). Liberated poly-ubiquitin chains are disassembled by ubiquitin C-terminal hydrolases (e.g. PGP 9.5) to regenerate monoubiquitin, and the substrate is hydrolyzed into small peptides by specific endopeptidases (reviewed by Wilkinson and Hochstrasser, 1998). Poly-ubiquitin chain formation can occur on all seven internal Lys-residues (Pickart, 1998). Thus, an almost infinite number of ubiquitination patterns can be achieved through such covalent bonding. This, along with a variety of ubiquitin-conjugating enzymes and unique substrate protein motifs conducive to ubiquitination accounts for the unexpected substrate specificity of ubiquitin-protein conjugation (Laney and Hochstrasser, 1999; Varshavsky, 1997).

A chain of four or more ubiquitin molecules targets the ubiquitinated substrate to the 26S proteasome. The proteasome is a holoenzyme typically composed of a barrel-shaped 20S core capped with one or two 19S regulatory complexes. The 20S core of the proteasome is composed of four concentric rings containing seven proteasomal subunits of α -type (two outer rings) and seven subunits of β -type (two inner rings). While most α -type and β -type subunits are constitutively expressed in all tissue, three β -type subunits, including β 1, β 2, and β 5, can be replaced by their inducible analogues, β 1i /LMP2, β 5i /LMP7, and β 2i /LMP10. These “inducible” β -type subunits are present in eye lens and in immunoproteasomes responsible for

antigen presentation in lymphocytes (Glickman and Ciechanover, 2002), but they can also be induced in other tissues by heat shock or γ -interferon signal when there is an increased need for ubiquitin-dependent proteolysis.

Ubiquitin System in Spermatogenesis and Fertilization

Few reports exist on the respective roles of ubiquitin-proteasome pathway in gametogenesis and fertilization (reviews: Baarends et al., 1999b; 1999b; Bebington et al., 2001; Escalier, 2003; Sutovsky, 2003). Ubiquitination has been implicated in targeted proteolysis of histones and other proteins during spermatid elongation (Baarends et al., 1999; Wing et al., 1995; 1996; Chen et al., 1998), in the degradation of the sperm mitochondria after fertilization (Sutovsky et al., 1999; 2004), and in the sperm-zona penetration during fertilization (Sakai et al., 2003; Sutovsky et al., 2003). Proteasomes accumulate in sperm acrosome (Bialy et al., 2001; Wojcik et al., 2000; Sutovsky et al., 2004; Sakai et al., 2004), in spermatid manchette (Rivkin et al., 1997), in the centriolar vault of the sperm tail (Bialy et al., 2001; Mochida et al., 2000; Wojcik et al., 2000), and in nuclear vacuoles of defective spermatozoa (Bialy et al., 2001; Wojcik et al., 2000; Ziembra et al., 2002). There are at least two proteasomal subunits recognized by anti-sperm antibodies in patients diagnosed with autoimmune infertility (Bohring et al., 2001).

Ubiquitin System of the Epididymis

Pertinent to the search for new semen quality/fertility markers, ubiquitin is one of the secretory proteins in the epididymis. Accumulation of ubiquitin is seen in the stereocillia, apical blebs, and epididymosomes in humans and other mammals studied to date (Fraile et al., 1996; Hermo and Jacks, 2002; Santamaria et al., 1993; Sutovsky et al., 2001; 2003). Unconjugated mono-ubiquitin can be pulse-chased in the cultured epididymal epithelial cells (EEC) and immuno-precipitated from the EEC-conditioned medium (Sutovsky et al., 2001a). Ubiquitin from epididymal fluid may be regenerated by the ubiquitin C-terminal hydrolase, PGP 9.5, abundantly expressed in the epididymal tissue (Fraile et al., 1996; Santamaria et al., 1993). Prostate-like secretory particles, epididymosomes from bull epididymal fluid, were shown to transfer epididymis-secreted proteins to the bull sperm plasma membrane (Frenette and Sullivan, 2001). Such ubiquitin-containing epididymosomes arise from the release of the apical blebs from the epididymal epithelial lining (Andonian et al., 2002; Hermo and Jacks, 2002; Sutovsky et al., 2001a).

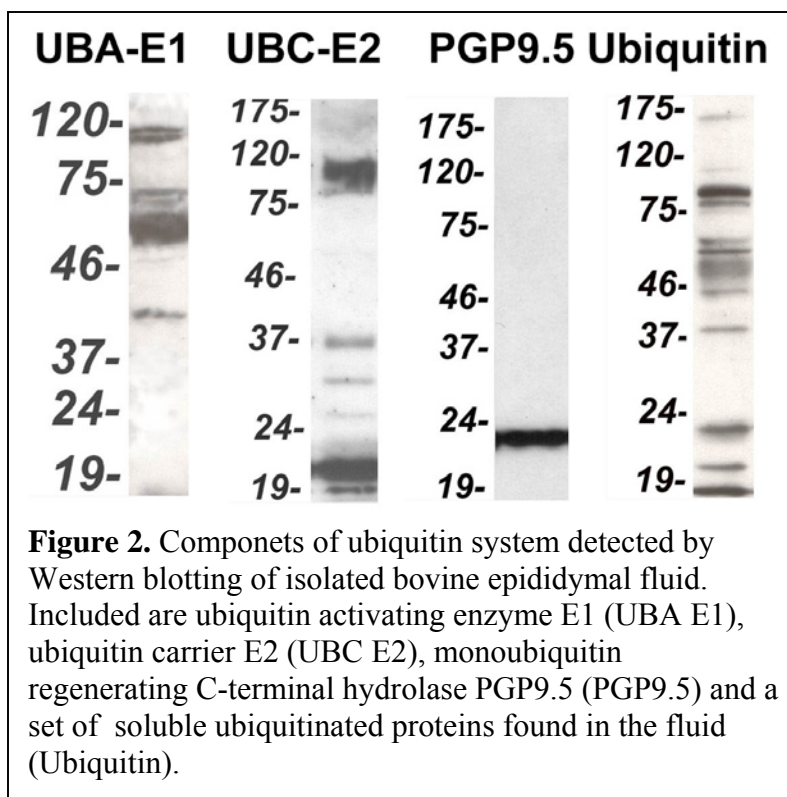
We have shown recently that the defective bull (Sutovsky et al., 2001a; 2002), stallion (Sutovsky et al., 2003), rat (Tengowski et al., 2004), and human (Sutovsky et al., 2001b; 2004; Rawe et al., 2002) spermatozoa express ubiquitin on their surface and this defective sperm ubiquitination occurs during epididymal passage (reviewed by Sutovsky, 2003). These observations led us to propose the existence of an epididymal ubiquitin-based sperm quality control mechanism (Sutovsky et al., 2001), a hypothesis recently confirmed by others in the rat (Hermo and Jacks, 2002).

In our studies, we also observed some of the defective bull spermatozoa are removed during epididymal passage while others can be found in the ejaculated semen and identified by specific anti-ubiquitin antibodies (Sutovsky et al., 2001 a). Such an ability of epididymal environment to selectively recognize and remove defective spermatozoa and sperm cytoplasmic droplet has been promoted by some scientists (see articles by Axner et al., 1999; 2002; Hermo et al., 1988;

Ramamohana Rao et al., 1980) but rejected by others. This ongoing dispute underlines the importance of studying epididymal proteolysis. The loss of defective spermatozoa during epididymal passage has been extensively documented (e.g. Axner et al., 1999, 2002; Chenoweth et al., 2000; Ramamohana Rao et al., 1980). Whole sperm phagocytosis in the epididymis has been disputed, yet occasionally documented in the epididymis of fertile animals (e.g. Goyal, 1982; Lopez Alvarez and Bustos Obregon, 1995) and in cultured epididymal epithelial cells (Sutovsky et al., 2001a; Sutovsky, 2003). Sperm liquefaction and intraluminal phagocytosis, a process that is most evident in vasectomized epididymis, could be an alternative pathway for removing defective spermatozoa during epididymal passage (Barrat and Cohen, 1987; Flickinger, 1982). Endocytotic, clear cells of the epididymal epithelium display high accumulation of both polyubiquitinated proteins and proteasomes (Tengowski et al., 2004). Proteasomes are also present in fully differentiated spermatozoa and could be involved in the degradation of sperm remnants within epididymal lumen. Our new data show that ubiquitin cofactors such as ubiquitin-activating (UBA), conjugating (UBC) and regenerating (ubiquitin-C-terminal hydrolases) enzymes are present in epididymal fluid.

Active Ubiquitin System is Present in the Epididymal Fluid

All crucial components of the ubiquitination machinery, including UBA-E1, UBC-E2, ubiquitin-C-terminal hydrolase PGP9.5, and mono-, di- and poly-ubiquitin were detected in bovine epididymal fluid (**Figure 2**). The expected, multi-band patterns of E1, E2, and ubiquitin Western blots arise from the presence of intermediates of ubiquitin conjugation such as E1-ubiquitin and E2-ubiquitin thiol-esters in the epididymal fluid. We performed Western blotting on bovine epididymal fluid isolated under stringent conditions to avoid contamination from red blood cells, epididymal spermatozoa and cellular debris, and epididymal epithelial cells. Those materials, along with purified erythrocyte ubiquitin were probed by affinity purified, precisely characterized antibodies. Negative controls were performed with appropriate non-immune sera. Apart from the single PGP9.5 band, expected in all samples except purified ubiquitin, none of the band patterns seen in the isolated epididymal fluid matched the multi-band patterns seen in erythrocytes, purified ubiquitin, spermatozoa, or epithelial cells.



Surface of Defective Bull Spermatozoa is Altered by Ubiquitination

We have discovered aryl-sulfatase A (AsA) is one of the sperm surface proteins ubiquitinated in the defective spermatozoa. AsA is an enzyme implicated in the sperm-zona interactions (Carmona et al., 2002; Tantibhedhyangkul et al., 2002). AsA is already present in fully differentiated testicular spermatozoa, though a small portion of sperm surface AsA could be contributed by epididymal secretions (Tantibhedhyangkul et al., 2003). AsA was identified in our laboratory by MALDI-TOF sequencing of protein spots identified by antisera raised against a putative, ubiquitin-immunoreactive protein of bull spermatozoa. Immunofluorescence with anti-AsA serum showed that AsA immunoreactivity is reduced in the ubiquitinated spermatozoa, probably by allosteric masking of AsA epitopes by polyubiquitin chains (**Figure 3**). Presence of high MW AsA- and ubiquitin-immuno-reactive bands, indicative of AsA ubiquitination, was seen in Western blots. We further determined ubiquitinated spermatozoa that lack AsA glycoprotein immunoreactivity also show an increased affinity to LCA-lectin. While normal spermatozoa only bind LCA to their acrosome, the ubiquitinated ones show high affinity for LCA binding on the whole surface of their sperm heads and tails. Other lectins known to bind to mammalian spermatozoa, including PNA, WGA and UEA-1, display neither high preference for mannose residues nor a distinct pattern of co-localization with ubiquitin immunoreactive proteins in defective spermatozoa (data not shown).

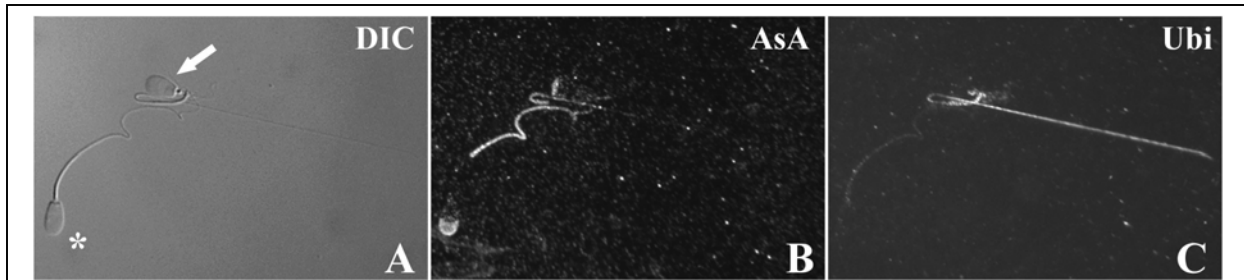


Figure 3. Ubiquitin (Ubi) marks defective spermatozoa deficient in proteins associated with fertility such as the arylsulfatase A (AsA). Normal spermatozoon (A; asterisk), shows the presence of AsA (B) and lack of ubiquitin (C), while the defective spermatozoon with coiled tail (A; arrow) displays high level of ubiquitin (C) in absence of AsA (B). Spermatozoa were fixed in formaldehyde and labeled with anti-ubiquitin mouse IgG and anti-AsA rabbit IgG antibodies detected by red fluorescent anti-rabbit IgG (AsA) and green fluorescent anti-mouse IgG, respectively.

Inducible Proteasomal Subunits Are Expressed in the Epididymis

While proteasomes are universally present within all tissues and cell types, their subunit composition may be altered under certain conditions. Specifically, the inducible 20 S core subunits LMP2, LMP7 and LMP10 are expressed and replace constitutive subunits $\beta 1$, $\beta 2$ and $\beta 5$ in T-lymphocytes during antigen presentation and in some other cell types after interferone stimulation or heat shock (Glickman and Ciechanover, 2000). Thus, the expression of these three core subunits indicates special requirements for proteasomal activity. We have detected all three inducible subunits in the mammalian epididymis, indicating that increased protein turnover is required there, consistent with ubiquitin secretion in the epididymal fluid.

The Use of Sperm-Ubiquitin Tag Immunoassay (SUTI) for Bull Fertility Evaluation

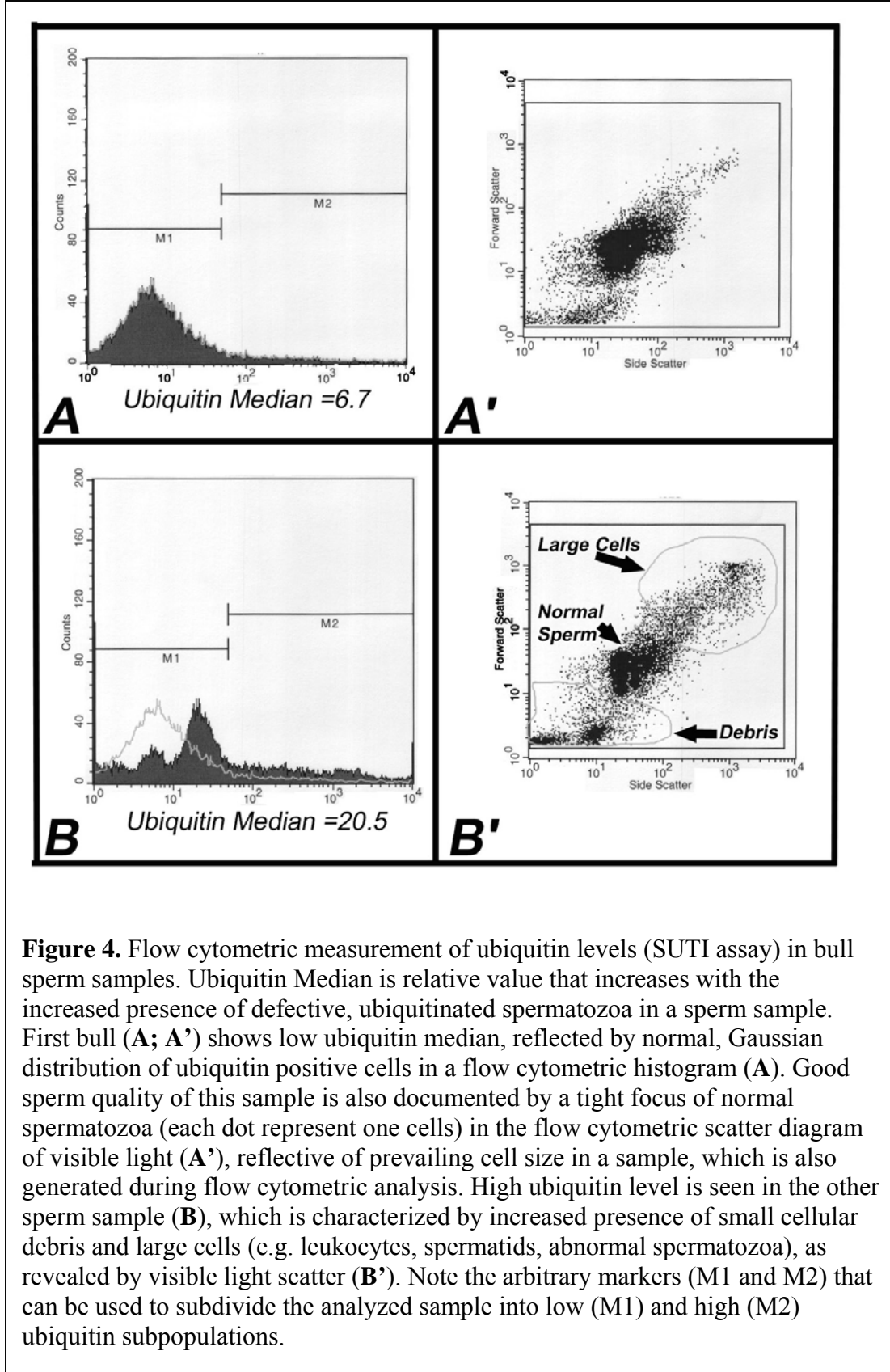
Our initial observations of defective sperm ubiquitination in bulls were made by immunocytochemical analysis of sperm samples processed for immunofluorescence microscopy. While this technique allowed us to describe various patterns of bull sperm defects, it was not suitable for large scale screening of multiple sperm samples. For these reasons, we adapted our SUTI assay for flow cytometry, allowing a high throughput (10,000 cells measured in 5-10 seconds), objective, automated measurement of sperm ubiquitin levels in multiple samples (typically 25-100 samples in one hour) in a single trial. Details of this technique are described in Sutovsky et al., (2002). Briefly, sperm samples are washed in SpermTL Medium, and the sperm pellet is fixed in 2% formaldehyde, washed, and stored for up to 8 weeks in phosphate-buffered saline at +4 °C. Samples are then blocked and sequentially incubated with anti-ubiquitin mouse IgG and fluorescently labeled goat-anti-mouse IgG-FITC, resulting in a green-fluorescent labeling of surface-ubiquitinated, defective spermatozoa. The levels of fluorescence, reflective of the abundance of ubiquitinated, defective spermatozoa, are then measured by flow cytometer in 10,000-20,000 cells per semen sample.

The median values of ubiquitin-induced fluorescence (Ubi-medians) and fluorescence means are recorded and averaged for each semen sample. Histograms of relative fluorescence (**Figure 4**) provide additional useful information, typically showing the normal, Gaussian distribution in

highly fertile bulls with excellent semen quality, and a shifted, flat, or multi-peaked distribution in subfertile bulls with poor semen quality. Arbitrary, quartile thresholds can be set on the histograms and cell subpopulations within those thresholds will be analyzed by measuring their Ubi-medians and determining what percentage of all measured cells they represent.

The diagrams of the visible light scatter are also generated by flow cytometric SUTI and used to analyze the relative size of cells within the screened samples. Visible light-scatter diagrams in animals with low ubiquitin medians and good field semen parameters invariably show a tight focus of presumably normal-sized and shaped spermatozoa in the center of the diagram. Samples of subfertile bulls frequently show cells that are larger or smaller than typical spermatozoa, resulting in the reduction of the focus of cells in the center of the diagram.

To assure unbiased analysis of experimental data, corresponding field semen analysis data and fertility records including but not limited to sperm count, motility, and no-return rates after AI are provided by the staff of collaborating institutions, only after all SUTI analyses are completed and sent to the said collaborators. The data are analyzed statistically (ANOVA; MS-Excel Stat. Tools) and correlated with semen parameters and fertility data.



Increased Sperm Ubiquitin Levels Coincide with Reduced Fertility in Breeding Bulls

We have previously determined the increased ubiquitin levels coincided with both the increased levels of sperm DNA damage and with the reduced fertility in a small group of breeding bulls (Sutovsky et al., 2002). We are currently conducting trials involving several hundred bulls in collaboration with USDA and major bull semen distributors. A study of 261 bulls from USDA Experimental Station, Fort Keogh, MT, revealed a negative correlation between sperm ubiquitin and field semen parameters (sperm count, swirl, % normal morphology) in this group of bulls. We also performed two studies of 8 and 10 sires, respectively, paired in a two-sire pasture system in which the pairs of sires competed to mate with multiple cows. Intriguingly, there was a strong negative correlation ($r=-0.5$ to -0.7) between sperm ubiquitin levels and the percentage of calves sired in both trials (**Figure 5**). This provides indication that sperm ubiquitin evaluation has significant value for assessing and possibly even predicting bull fertility. Increased ubiquitin levels in bull sperm are predictive of both poor semen quality and fertility in bulls.

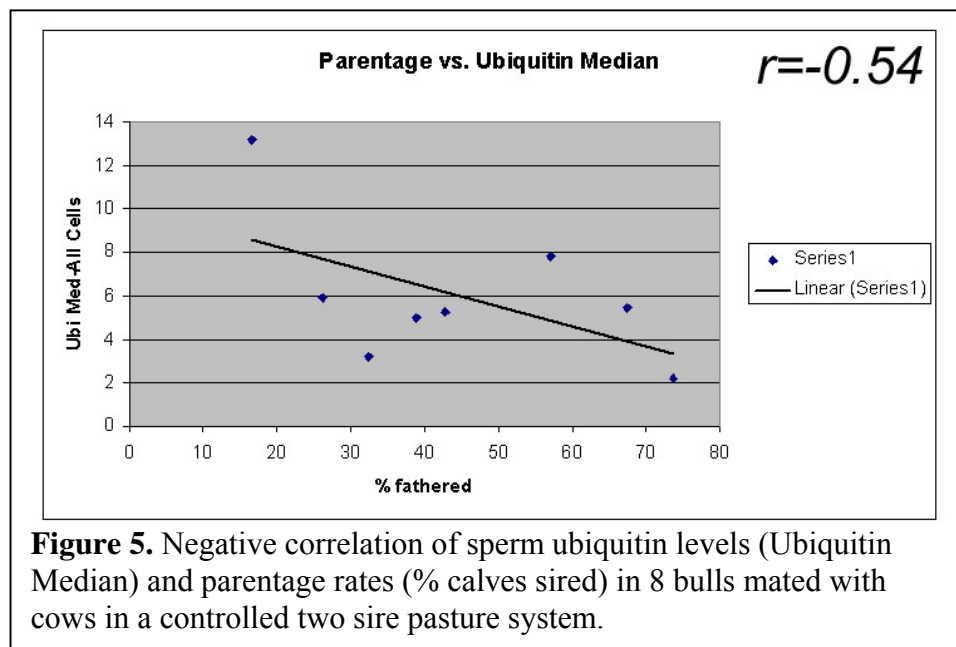


Figure 5. Negative correlation of sperm ubiquitin levels (Ubiquitin Median) and parentage rates (% calves sired) in 8 bulls mated with cows in a controlled two sire pasture system.

Advantages of Ubiquitin-Based Assays over Other Semen Evaluation Techniques

It is undisputable the currently available, subjective, and automated methods of semen evaluation provide important information about the quality of a semen sample, and there is no intention to dismiss the value of each of these techniques, as listed below. However, each method has major shortcomings that may result in a large portion of undiagnosed male factor cases because such methods are subjective and/or detect only certain types of sperm abnormalities found in an infertile male. The advantages of ubiquitin-based assays over these other methods can be summarized as follows:

- 1.** *Subjective light microscopic evaluation of sperm morphology and motility* by arbitrary standards such as WHO or Kruger's strict criteria used for human semen evaluation (Kruger et al., 1987; WHO, 1987, 1992, 1999). The value of sperm morphology

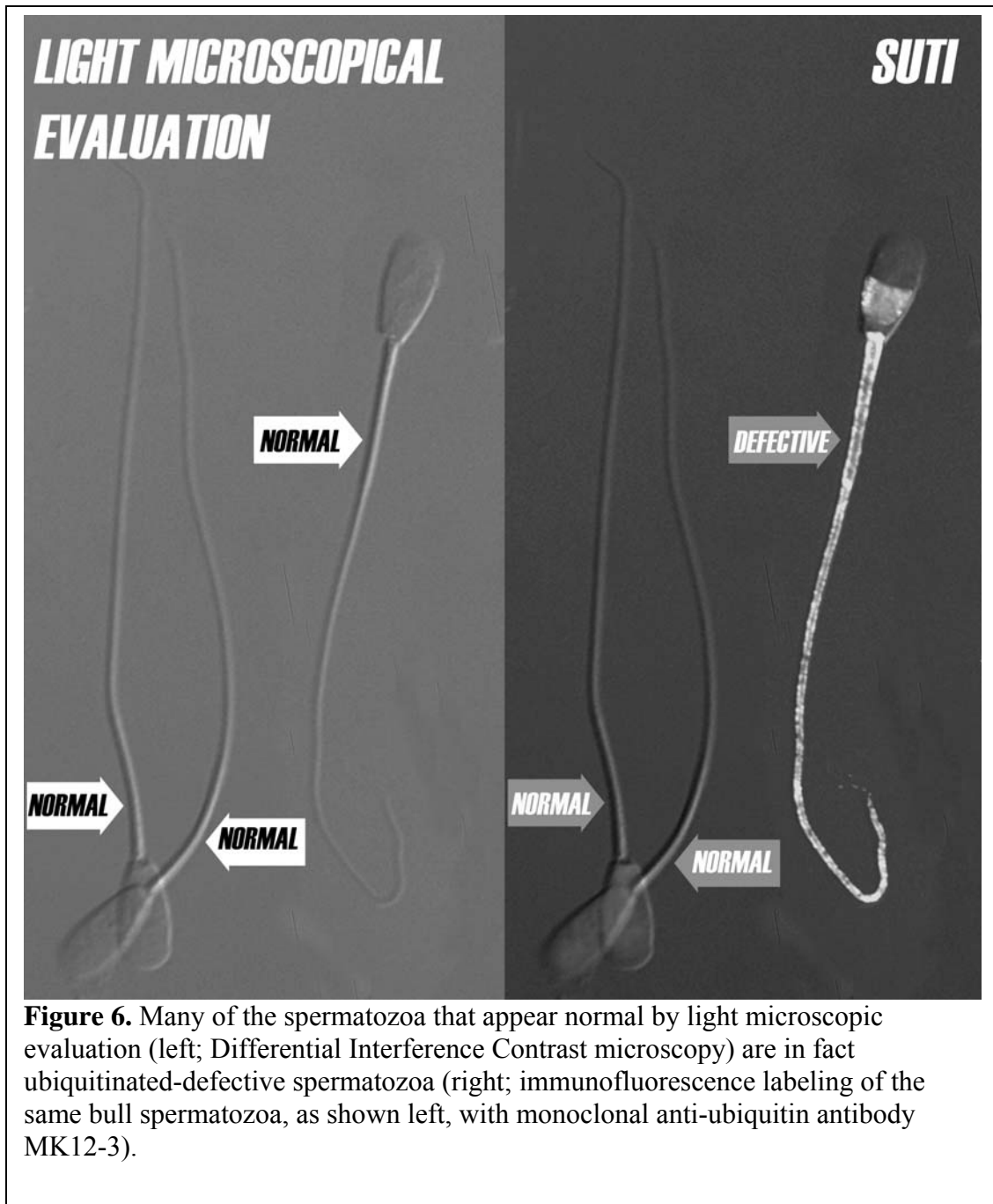
evaluation has been debated fiercely for the last two decades and there is still no clear distinction between fertile and infertile sperm sample (see Eliasson, 2003). One major reason is the fact many intrinsic sperm defects may go unnoticed even to the trained eye of an experienced evaluator. Ubiquitin-based assays recognize defective spermatozoa that appear to be morphologically normal by light microscopic evaluation (**Figure 6**; next page).

- 2.** *Automated morphometry and motility evaluation* (CASA and IVOS systems) (Douglas-Hamilton, 1995; Krause, 1995). Motility is a variable sperm characteristic that declines rapidly after sample donation and depends largely on the length of time between collection and evaluation (typically up to 2 h post collection in humans; Drobnis, 1992, Eliason, 1981, Jorgensen et al., 1997). While there is a significant overlap between semen parameters of fertile and infertile men, sperm morphology is thought to be a stronger infertility predictor than sperm motility (Guzick et al., 2001). Ubiquitin-based assays are not affected by sample handling or storage as the polyubiquitin chains are stably, covalently linked to substrates on defective sperm surface.
- 3.** *Flow cytometric evaluation of sperm chromatin integrity* (SCSA; Ballachey et al., 1987; Evenson et al., 1982; Garner and Thomas, 1999) or sperm DNA fragmentation caused by apoptotic process (TUNEL and COMET assays; e.g. Baccetti et al., 1996 Sun et al., 1997). Ubiquitin-based assays recognize spermatozoa with DNA damage (see Sutovsky et al., 2002), but also spermatozoa with intact chromatin that have defects other than DNA damage.
- 4.** *Vital stain-based assays* (e. g. Garner and Thomas, 1999) measure the uptake of vital nuclear or mitochondrial dyes by live, presumably fertile spermatozoa. Ubiquitin-based assays are not biased towards live or dead spermatozoa, and normal spermatozoa damaged during sample isolation and handling will not be falsely identified as defective.

While ubiquitin based assays show distinct advantages over each of the above methods, it is possible a combination of ubiquitin evaluation with one or more of these methods would provide the most comprehensive testing of bull semen.

Conclusions

Our and other research indicates ubiquitin protein is secreted by the epididymal epithelium into epididymal fluid and covalently linked to the surface of defective mammalian spermatozoa. Since many of such spermatozoa can be found in the semen, ubiquitin is a suitable marker of sperm abnormalities. Ubiquitin-based sperm quality assays are relatively easy and not costly to perform and have a number of advantages over other methods of semen evaluation including objectivity, independence from semen handling and storage methods, and universality with regard to recognizing multiple types of obvious and cryptic sperm defects. Further effort will be focused on validation of such assays and their dissemination of artificial insemination and natural breeding programs.



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