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Memorandum

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From: Adele M. Childress, Ph.D., Program Official
Office of Extramural Programs, NIOSH, E-74

Subject: Final Report is declared 'Confidential' for Grant 5 R21 OH007324-02. Do not distribute until Nov 2007

To: William D. Bennett
Data Systems Team, Information Resources Branch, EID, NIOSH, P03/C18

The attached sheet lists the publications for final report that has been received from the principal investigator (PI) on the subject NIOSH grant. The PI requested that the final report remain confidential for a period of four years, as allowed, so the actual report is not being transmitted. If you receive inquiries about this final report, please refer the caller to us.

List of Publications

Sutovsky P: Ubiquitin-Dependent Proteolysis in Mammalian Spermatogenesis, Fertilization, and Sperm Quality Control: Killing Three Birds with One Stone. Micros Res Tech, in press, 2002

Rawe VY, Brugo Olmedo S, Benmusa A, Shiigi SM, Chemes HE, Sutovsky P: Sperm Ubiquitination in Patients with Dysplasia of the Fibrous Sheath. Hum Reprod, in press, 2002

Sutovsky P, Neuber E, Schatten G: Ubiquitin-Dependent, Sperm Quality Control Mechanism Recognizes Speratozoa with DNA Defects, as Revealed by Dual Ubiquitin-TUNNEL Assay. Mol Reprod Dev 61:406-413, 2002

Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Simerly C Schatten G: A Putative, Ubiquitin-Dependent Mechanism for the Recognition and Elimination of Defective Spermatozoa in Mammalian Epididymis. J Cell Sci 114:1665-1675, 2001

Sutovsky P, Terada Y, Schatten G: Ubiquitin-Based Sperm Assay for the Diagnosis of Male Factor Infertility. Human Repro 16:250-258, 2001

Cover Page.

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Ubiquitin-Based Semen Quality Assay in Toxicology

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Project Duration Dates:
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Principal Investigator (PI):
Peter Sutovsky, PhD
Assistant Professor
University of Missouri-Columbia
S141 ASRC
920 East Campus Drive
Columbia, MO 65211-5300
Telephone: (573) 882-3329
Fax: (573) 884-5540
http://www.asrc.agri.missouri.edu/faculty/Sutovsky.html

Project Sites:
University of Missouri Columbia, Columbia MO
(7-1-2001 to 9-29-2002)
Oregon Health & Science University, Portland, OR
(9-30-2001 to 6-30-2001)

1. TABLE OF CONTENTS.

2. LIST OF ABBREVIATIONS.

ART=assisted reproductive technologies
AsA=arylsulfatase A
Apo(a)=apolipoprotein(a)
CASA=computer assisted semen analysis
DBCP=1, 2-dibromo-chloro-propane
DFS=dysplasia of the fibrous sheath/stomp tail syndrome
EEC=epididymal epithelial cells
ICSI=intracytoplasmic sperm injection
SUTI=Sperm-ubiquitin-tag immunoassay
TCE=trichloroethylene
Ubi=ubiquitin

3. LIST OF FIGURES.

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5. ABSTRACT.

Ubiquitin is the omnipresent, house-keeping protein that binds to other proteins to mediate their degradation and recycling in both normal and diseased cells. PI's recent work implicated ubiquitin system in the control of human and animal spermatogenesis and fertilization. As the number of couples seeking treatment for male, female and unexplained infertility increases rapidly, so does the need for an objective, universal sperm quality assay. Sperm-Ubiquitin Tag Immunoassay (SUTI) has been proposed by PI as an objective diagnostic tool for male infertility diagnostics and toxicological/occupational evaluation of male reproductive health. This novel experimental approach based on the objective measurement of ubiquitin levels in sperm samples, however, needed to be validated by research deciphering the

various functions of ubiquitin system in male reproductive system. At the same time, it had to be determined whether the levels of sperm ubiquitin in laboratory animals commonly used in toxicological trials mirror those seen in subfertile human sperm samples. Therefore, Aims #1 and #2 of the present project sought to answer the following questions: Aim #1, Is the detection of sperm-surface ubiquitination a universal approach for assessing semen quality in men? Aim #2., Is the rhesus monkey the optimal model for assessing the effects of workplace chemicals on male fertility and semen quality? Studies performed during two years of this project conclude that there is a strong negative correlation between sperm ubiquitin levels and male fertility/semen quality parameters in both humans and animals. Male patients with self-reported histories of occupational exposure to toxic agents, as well as male rodents exposed to known workplace toxicants have semen samples with increased levels of sperm ubiquitin. Overall protein makeup and content of ubiquitinated proteins, as revealed by proteomic analysis of sperm samples, differs between infertile men and fertile donors. Several know sperm surface proteins appear to be ubiquitinated in the defective spermatozoa. Pending the final data analysis, it is conceivable that the reproductive system of male rhesus monkeys is more sensitive to solvents than that of rodents. The benefits of this research are numerous: Deciphering the ubiquitin-dependent events during spermatogenesis can improve reproductive health of the US population at many levels, including more accurate diagnosis and treatment of male and unexplained infertility, toxicological screening, amelioration of assisted reproductive technologies (ART) and possible prevention of developmental abnormalities after ART.

6. SIGNIFICANT FINDINGS.

The present project resulted in the following discoveries:

1. Ubiquitin, the house-keeping gene product marking other proteins for degradation serves as a signal for the selective recognition, and possibly degradation of defective spermatozoa in humans, primates, rodents and other mammals.

2. Defective spermatozoa become ubiquitinated during epididymal passage, and some of them remain in semen after epididymal passage, thus qualifying ubiquitin as an objective marker of sperm/semen abnormalities in men, primates and non-primate mammals.

3. Relative levels of ubiquitin in the human spermatozoa correlate negatively with semen parameters (sperm count, motility, normal sperm morphology) and parameters of early embryonic development after IVF and ICSI (% normal pronuclear development, % of cleaved embryos, % of embryos selected from embryo transfer).

4. Patients with self-reported histories of occupational exposure to toxic agents, as well as male rodents exposed to known workplace toxicants have semen samples with increased levels of sperm ubiquitin.

5. Overall protein makeup and content of ubiquitinated proteins, as revealed by proteomic analysis of sperm samples, differs between infertile men and fertile donors.

6. Several known sperm-surface antigens, including aryslulfatase A, apolipoprotein(a) and platelet-activating-factor-receptor show diminished immunoreactivity to specific antibodies in the defective, ubiquitinated spermatozoa.

7. USEFULLNESS OF FINDINGS.

This work established a novel, previously unrecognized mechanism contributing to the normal occurrence of spermatogenesis and epididymal sperm maturation, and opened a new area of infertility research. Of particular importance are the findings of increased sperm ubiquitin being linked to human male infertility in patients with various ethiologies including, but not limited to occupational exposure. While the data from this project are highly indicative of ubiquitin's role in human infertility, further research on both the human male gametes and on the animal gametes, tissues and embryos will be necessary to fully decipher pertinent pathways. Ubiquitin can be used as an objective marker for the diagnosis of male human infertility. Sperm Ubiquitin Tag Immunoassay (SUTI) has been developed and patented. If the assay is successfully disseminated, it can enhance the reproductive health of US population at many levels, including more accurate diagnostics and treatment of male infertility due to heritable and epigenetic factors (including toxic workplace exposure), improvement of ICSI and related assisted fertilization technologies and possible prevention of developmental abnormalities caused by poor development after ART. Identification of the ubiquitinated substrates on the sperm surface will provide additional markers for objective semen analysis and infertility diagnostics. Identification and characterization of the respective roles of the ubiquitin-proteasome pathway components in human spermatozoon may also provide new contraceptive targets and diagnostic tools. Finally, the comparison of various animal models may help selecting the best ones for the testing of the effects of solvents and pharmaceuticals on male reproductive system.

8. SCIENTIFIC REPORT.

This scientific report briefly recapitulates data already published and provides a more detailed, technical description of data in preparation for publications, some of which still await final analysis. Complete analysis of data obtained in the course of this project is described in the enclosed reprints and manuscripts **(Appendix 1-5). Please note that some of the data presented here contain data from manuscripts and other restricted information (e.g. data from collaborative projects) not suitable for distribution to the public prior to publication.**

Besides extending the research on the molecular and cellular mechanisms of epididymal sperm ubiquitination, the present project was aimed at gathering sufficient data to establish the ubiquitin based assays as an objective, rapid, non-invasive and cost-effective tool for wide-range screening of semen from men exposed to hazardous workplace chemicals. Consequently, the following specific aims were formulated:

Aim #1. Is the detection of sperm-surface ubiquitination a universal approach for assessing semen quality in men? Aim #1 sought evidence in the support of the hypothesis that ubiquitin-based assays are more sensitive than conventional sperm morphology assays of semen samples from infertile men and workers exposed to hazardous workplace chemicals. This hypothesis was tested by evaluating semen samples from male patients with various ethiologies of male factor and unexplained infertility, including but not limited to men exposed to workplace chemicals, to determine if an increased level of sperm-surface ubiquitin is present in semen samples of those men, as compared to semen samples from fertile donors. Further exploratory research involving a variety of animal models (mouse, rhesus monkey, rat, bull, boar) was proposed in order to simplify and further improve the ubiquitin based sperm assays.

Aim #2. Is the rhesus monkey the optimal model for assessing the effects of workplace chemicals on male fertility and semen quality? The hypothesis was that semen samples from rhesus males have relative levels of surface ubiquitin similar to those seen in men, and that the

subfertile rhesus males, similar to subfertile men, have elevated levels of surface ubiquitin. This was to be compared with sperm ubiquitin data in male rodents, i.e. mice and rats commonly used in reproductive toxicology studies. Confirming this hypothesis would provide the grounds for using rhesus monkey as a preferred model for assessing toxic risks of workplace chemicals, effectively replacing the commonly used rodent models, which may not be sufficiently sensitive to such external factors. This aim also included further exploratory research using gametes and reproductive tissues from rodents, ruminants and primates, as well as the epididymal cell cultures.

8.1. Aim #1. Mechanisms of sperm ubiquitination.

After leaving the testis via testicular rete, spermatozoa undergo final maturation in the epididymis, composed of three distinct compartments: caput, corpus and cauda. Numerous proteins, secreted in apocrine fashion by the epididymal epithelium, are implicated in sperm immobilization, stabilization of sperm perinuclear structure by disulphide bond-formation and acquisition of fertilizing potential (reviewed in Sutovsky, 2003; **Appendix 1**). Accumulation of ubiquitin is seen in the stereocillia, apical blebs and vesicles detaching from the apical epithelial surface in the epididymis of men, rhesus monkeys, mice, bulls, boars and stallions (Fraile et al., 1996; 1993; Hermo and Jacks, 2002; Santamaria et al., 1993; Sutovsky et al., 2001a, 2002, 2003). In vitro culture system was established to investigate the capability of epididymal epithelial cells (EEC) to secrete ubiquitin and remove defective spermatozoa from the epididymal lumen (Sutovsky, 2003; Sutovsky et al., 2001a). In this culture system, ubiquitin was pulse-chased in the cultured EEC and immuno-precipitated from the EEC-conditioned medium and is present in epididymosomes, the secretory vesicles isolated from bovine epididymal fluid (Sutovsky et al., 2001a; **Appendix 2**). Components of the ubiquitin-proteasome pathways, including monoubiquitin, polyubiquitin chains, ubiquitin-conjugating enzyme E2, ubiquitin C-terminal hydrolase PGP 9.5 and various proteasomal subunits (e.g. MECL-1, LMP-1, LMP-2; see **Fig. 1**) were detected in the clear and principal cell of epididymal epithelium in humans, primates, rodents and large farm animals (see also Aim 2 for results and references). As a result of sperm surface ubiquitination during epididymal passage, the expression of certain proteins on the sperm surface is either reduced or they are not expressed at all. Immunodetection and MALDI-TOF sequencing of proteins separated by 2-D SDS-PAGE indicates that the ubiquitinated substrates in the defective spermatozoa include platelet-activating factor-receptor, apoliprotein (a) and arylsulfatase A (**Fig. 2**).

Fig.1. Detection of polyubiquitin chains (red) and proteasomal subunit α/β (green) in the clear cells of the rat epididymal epithelium after exposure to theophilline

Fig.2. Absence of arylsufatase A immunoreactivity (red) from a defective, ubiquitinated spermatozoon (green).

8.2. Aim #1. SUTI analysis of human sperm samples.

Immunocytochemical analysis (**Fig. 3**) demonstrated that the increased amounts of ubiquitin immuno-reactive proteins are present on the surface of spermatozoa in the semen samples of men from couples seeking infertility treatment. To date, five quantitative studies of human sperm ubiquitin by SUTI have been completed with support from

Fig. 3. Immunodetection of ubiquitin (red) in semen samples from a fertile donor (left) and an infertility patient (right).

this award. The *First Study,* published in 2001 (**Appendix 3;** Sutovsky et al., 2001b), evaluated sperm samples of men from 17 couples seeking infertility treatment. Six couples (35%) were previously diagnosed with unexplained,
idiopathic infertility. Using SUTI assay, men from 5 of those 6 couples (83%) showed an increased amount of sperm ubiquitin. None of the six couples (0%) achieved pregnancy by IVF. In addition to detecting male infertility in those idiopathic cases, SUTI assay established a negative correlation between Ubi-median and % embryonic cleavage after IVF (r=-0.43; Pearson's correlation).

Table A	Value	Ubiquitin intensity	
		Correlation	P
Count	74.3 ± 10.0	-0.55	0.004*
% Normal Morphology	11.6 ± 0.7	-0.58	0.002*
% Motility	44.0 ± 4.2	-0.02	0.93
VAP (IVOS)	50.9 ± 1.7	0.12	0.61
VSL (IVOS)	43.2 ± 1.6	0.08	0.74
VCL (IVOS)	74.0 ± 2.9	0.10	0.67
ALH (IVOS)	3.8 ± 0.2	-0.06	0.81
BCF (IVOS)	19.8 ± 0.5	0.25	0.29
LIN (IVOS)	58.8 ± 1.2	0.02	0.92

Double labeling experiments were performed on globozoospermic samples (Escalier, 1990) with anti-Ubi probes and antibodies against sperm head capsule, perinuclear theca (Oko et al., 2001). The preliminary results **(Fig. 4,** left panel) document the presence of ubiquitin in the round-headed cells with aberrant PT. Similarly, Ubi was detected at high levels in teratospermic human spermatozoa with fragmented DNA, revealed by dual, ubiquitin-TUNEL staining (**Fig. 4**, right panel).

In the *Second Study* (**Appendix 4**; Rawe et al., 2002), five men with the dysplasia of fibrous sheath (DFS or stomp tail syndrome; Chemes, 2000) were screened by SUTI along with 8 fertile donors, showing significantly higher ubiquitin levels in the former group. DFS is believed to be a heritable syndrome, often manifested in brothers of the patients (Chemes, 2000; and H. Chemes, personal communication). These data demonstrate that the increased sperm ubiquitin correlates not only with male infertility of unknown etiology, but also with the well defined, heritable sperm defects.

Fig. 4. Dual assays for ubiquitin and other markers of sperm abnormalities. Left panel: High ubiquitin (green) and deviant perinuclear theca (red) assembly in a round headed, globozoospermic sperm sample. Right: Dual labeling of ubiquitin (red) and apoptotic/necrotic, single stranded DNA (green, TUNEL assay) in a teratospermic sample with high degree of nucleomalasia .

In the *Third Study* (**Appendix 5,** Sutovsky et al., 2003), 28 subjects seeking infertility treatment were screened by SUTI along with 15 fertile donors. Table 2B in **Appendix 5** shows strong negative correlation (Pearson's correlation coefficient) between relative ubiquitin levels (Ubi-median) and clinical semen parameters, including sperm count and motility. Intriguingly, there was a positive correlation of sperm ubiquitin medians with % of abnormal spermatozoa by WHO criteria, yet the WHO-based evaluation did not show as strong a correlation with sperm count as ubiquitin did. This reaffirms PI's belief that SUTI assay, or similar ubiquitin-based screens are superior of conventional semen analysis (see also results of *Fifth Study).* Table 1 in **Appendix 5** shows that among all screened men, six men with self-reported workplace exposures had the highest relative levels of ubiquitin, followed by current smokers and former smokers. Intriguingly, current smokers had very good motility and relatively low % of abnormalities, but high ubiquitin levels. While it was

not know whether this was strictly because of paternal/male factors, it is safe to assume that many subjects within the "infertile" group were indeed subfertile.

Further confirmation of PI's data on the efficiency of SUTI assay for the measurement of human sperm ubiquitin was provided by the *Fourth Study* conducted by a collaborating team of an established andrologist, Dr. S. E. Lewis (Queen's University of Belfast UK). Armed with PI's input and probes, yet without direct participation of PI himself, and using a different flow cytometry system, these collaborators applied SUTI to samples from 35 infertility patients (**Table A**), and found the correlation coefficients between Ubi and sperm count (r=-0.55) and Ubi and % of normal morphology (r=-0.58) to be almost identical to those obtained by PI in his studies. As expected, the parameters of automated semen motility analysis (CASA/IVOS) did not correlate with ubiquitin values (**Table A**) or with clinical semen parameters.

Table B	*Ubi Med Mn 1 (F)*	*Ubi Med MN2 (G)*	*Ubi Med Mn3 (H)*	*Ubi Med Md 1 (F)*	*Ubi Med Md 2 (G)*	*Ubi Med Md 3 (H)*	*% abnorm Kruger*
Vol.	**-0.37**	-0.14	0.09	**-0.37**	-0.11	0.07	-0.07
Count	-0.27	0.20	0.24	-0.19	0.28	**0.39**	-0.20
Motil	**-0.27**	-0.18	-0.01	-0.25	-0.16	-0.06	0.09
Kruger Abn	-0.21	**-0.24**	**-0.24**	-0.22	-0.21	-0.20	1
Age	-01	-0.23	-0.24	-0.01	-0.20	-0.25	**0.48**
%2PN	-0.36	-0.56	-0.54	-0.40	-0.55	**-0.58**	0.14
% 0 PN	0.40	0.58	0.54	**0.60**	0.54	0.52	0.016
%Multi PN	-0.36	-0.40	**-0.42**	-0.40	-0.39	-0.35	-0.12
%cleaved	-0.47	-0.62	-0.65	-0.49	-0.61	**-0.63.**	0.2
%transferred embryos	**-0.61**	-0.44	-0.41	**-0.61**	-0.38	-0.33	0.06

The *Fifth Study*, currently at the stage of data evaluation, provided further insight into how SUTI or a related ubiquitin assay could be useful even in case with obvious male factor. Nineteen couples undergoing ICSI were examined. Three different arbitrary thresholds were set for "positive and negative" cells in flow cytometer and two related ubiquitin median values (Mn 1-3 and Md 1-3) were obtained for each threshold. Intriguingly, various Ubi-median values showed strong correlation with several parameters of early embryonic development **(Table B).** This included a negative correlation between ubiquitin, and % of two pronuclear zygotes (%2 PN), % of the cleaved embryos and % of embryos suitable for transfer/transferred to recipient mothers. While some indication of the predictive value of SUTI for embryonic development after assisted fertilization already arose from the *First Study*, the *Fifth Study* provided a more conclusive and highly encouraging data to this regard. Altogether the above *Five Studies* provide a strong incentive for further developing SUTI assay and for testing it on a large pool of semen samples.

Fig.5. Whole protein SELDI profile ("gel" view) of isolated sperm samples from infertility patients (teratospermia; samples 1-12) and fertile donors (13-21). A major band of 12,200 Da is present in all fertile men, but absent from all patients' samples. Insert shows a diagram view of this band/peak in donor #21. Protein loading was standardized.

Fig. 6. Matching whole protein SELDI profile ("gel" view) of isolated seminal plasma complementing sperm samples shown in fig. 6 (1-12=patients, 13-21= fertile donors). Several, but not all patients lack a major band of 50 kDa, and show some unique, higher MW bands.

8.3. Aim #1-Exploratory Studies.

The goal of these studies was to develop more time and cost effective, highly accurate variants of SUTI. For this purpose, the surface-enhanced laser ionization-desorption (SELDI) was adapted to determine the overall sperm and seminal plasma protein makeup and to identify the ubiquitin-immunoreactive sperm proteins in semen samples of fertile donors and infertility patients. While PI and his collaborators have yet to establish optimal conditions for SELDI, first trials show major differences of overall sperm protein profile between patients and fertile donors (**Fig. 5**). The absence of several major bands consistent with increased ubiquitination (each ubiquitin molecule increases the mass of a substrate by 8.5 kDa and increased ubiquitination has been detected by flow cytometric SUTI in the same set of samples), yet it is not proven that all of those proteins are indeed ubiquitinated. Particularly striking is the absence of a 12,200 Da protein from virtually all patient samples (**Fig. 5,** samples 1-12) and the fact that this band, along with several others is present in all samples from fertile donors (**Fig. 5**, samples 13-21). In the preliminary MADI-TOF trials, this band was identified as apoliprotein(a), a known sperm surface protein. Similar to whole spermatozoa, the matching seminal plasma from these fertile and infertile samples isolated in the process of sample preparation for SUTI or SELDI was informative of differential ubiquitination or other protein modification between patients (**Fig. 6**, #1-12) and fertile donors (**Fig. 6**; #13-21), especially if one considers that varied levels of ubiquitin were previously reported in seminal plasma of humans (Lippert et al., 1993). Using dedicated SELDI biomarker software by Cyphergen, both isolated sperm and seminal plasma can then be searched for markers of normal sperm profile, i.e. bands that are specific for only the normal, or only the defective spermatozoa. Insert in **Fig. 5** shows the profiles of the above described 12,200 Da band, which was identified using this software. In other preliminary trials with varied protein extraction and chip coating protocols, the whole protein profiles generated by SELDI showed this and other missing and extra protein bands in some patients (not shown). The antibody KM691 against recombinant human ubiquitin, a tool used for flow cytometric SUTI, binds ubiquitinated sperm proteins to antibody-coated chip (**Fig. 7**). Further supporting such effort, the western blotting of human (see **Fig. 8**) sperm samples shows that semen samples from infertile men display differential ubiquitination profiles of the sperm substrates. The SELDI assay, coupled to proteomic analysis/micro sequencing by MALDI-TOF is being used for identification and characterization of substrates that are differentially ubiquitinated in the defective spermatozoa.

8.4. Aim #1: Conclusions.

Sperm samples from 125 patients and fertile donors were screened by flow cytometric SUTI assay in the course of this project. Additional samples were screened using biochemical, immunocytochemical and proteomic techniques. Altogether, the above data strongly indicate the value of SUTI for diagnostics of male infertility, and even for the prediction of treatment outcome in obvious teratospermic cases. Furthermore, even the limited number of subjects with self-reported workplace exposure provides an indication that the

Fig. 7. Gel view of SELDI on a chip coated with anti-ubiquitin KM 691. Samples from patients (1-12) and donors (131-21) are identical to those in fig. 6 and 7. Note the absence of several major low MW bands in all patients.

Fig.8. Detection of ubiquitinated substrates by Western blotting of sperm extracts from infertility patients (lanes 2-7) and fertile donors (lanes 8-10).

ubiquitin-based assay will be a powerful tool for reproductive toxicology. The exploratory research provides a basis for the development of sophisticated, proteomic approaches to the evaluation of male infertility and detection of even the most subtle toxic effects on male reproductive system.

8.5. Aim#2. Studies in rhesus monkey.

Controlled trial of exposure to a known insecticide/pesticide compound 1,2-dibromo-chlor-phosphate (DBCP) was designed in accordance with the previously studies of rhesus monkeys exposed to DBCP, as published by Overstreet et al. (1988). Briefly, two healthy, fertile rhesus males of reproductive age were exposed for 4 weeks to descending dose of DBCP gradually reduced from 100 mg/kg body weight at day 1 to 0 mg at day 28. Semen samples from two exposed males and two control males were collected weekly in a time period from 30 days prior to day 1 of exposure to 30 days after the end of exposure (including the period of exposure). Sperm motility, count, volume and % abnormalities were evaluated at collection. Samples were cryopreserved for the evaluation by light microscopic and flow cytometric SUTI assays. DBCP exposure induced decline in sperm motility and semen volume in both exposed males, accompanied by an increased % of sperm abnormalities (**Fig. 9**). Final evaluation by flow cytometric SUTI will determine if this decline in sperm quality was paralleled by increased sperm ubiquitination. For this to occur, a number of different anti-ubiquitin probes were tested to determine which ones recognize the ubiquitin-protein conjugates and specific types of poly-ubiquitin chains present on the surface of defective rhesus spermatozoa. From among 10 probes tested, antibodies Ab1690 (rabbit IgG from Chemicon Inc., Temecula, CA) and antibody KM 691, which we also use for human sperm evaluation (mouse IgM from Kamiya Biomedical Co., Seattle, WA) yielded best results in immunofluorescence and flow cytometry (**Fig. 10**). Similar to human and non-primate mammalian tissues, anti-ubiquitin antibodies detected ubiquitin accumulation in the secretory cells of the rhesus epididymal epithelium. Therefore, for the final cytometric analysis, scheduled for March-April 2003, the combination of these two antibodies will be used and antibody binding will be detected by a combination of green-fluorescent anti-mouse IgG-FITC and far-red-fluorescent anti-rabbit IgG-Cy5.

Fig.9. Decline in sperm motility and volume, paralleled by an increase in sperm abnormalities in two male rhesus monkeys exposed to DBCP for 4 weeks.

Fig.11. Ubiquitin profiles (flow cytometric histograms) of representative semen samples of control mice (left column) and mice exposed to TCE (right column) for 1-4 weeks (W1-W4). Relative levels of sperm levels are shown on x-axis and are increased in the TCE-exposed males at weeks 1 and 2, but not at weeks 3 and 4.

8.6. Aim#2: Studies in rodents.

Studies of sperm ubiquitin levels in male mice exposed to a common workplace solvent, trichloroethylene (TCE) were conducted in collaboration with Dr. Poh-Gek Forkert (Queen's University, Kingston, ON), whose group previously detected TCE metabolites in the seminal plasma of exposed workers (Forkert et al., 2003). Mice were exposed to TCE (1000 ppm) for 6 h/day, 5 days/week for 1 to 4 weeks. Urinary excretion of TCE metabolites, TCA and TCOH was evaluated following inhalation

Fig.10. Immunofluorescence (A-C) and flow cytometric detection (D) of ubiquitin in the rhesus monkey spermatozoa.

exposure to TCE. Urine was collected over a period of 24 h prior to the end of TCE exposure for that week (including the 6-h exposure). Data were expressed as mean ± S.E.M. ($n = 6$), and were analyzed by two-way ANOVA and the Bonferroni test. Cauda-epididymal spermatozoa were collected at week 1, 2, 3, and 4 from control and exposed mice (3-6 males/group/week) and evaluated by flow cytometric SUTI assay. Spermatozoa collected at these respective time points were used for in vitro fertilization of mouse oocytes obtained by superovulation of female mice. Relative levels of sperm ubiquitin peaked at week 2 a dropped below the control level by week 4 in the exposed males (**Fig. 11**). Surprisingly, the peak of sperm ubiquitin levels coincided with a transient loss of fertility, evidenced by low rates of in vitro fertilization in the males exposed for 2 weeks, but not in those exposed for 1, 3, and 4 weeks (Forkert, Tanpaichitr and Sutovsky, In preparation). This re-acquisition of fertility and sperm quality was not paralleled by a reduction of TCE metabolites in urine (**Table C**), suggesting that this reacquisition of fertility was due to reproductive system adaptation rather than accelerated metabolization of TCE.

The marking and elimination via ubiquitin-proteasome pathway of defective spermatozoa were investigated in the epididymis of an infertile *azh* mutant mouse, producing spermatozoa with gross sperm head malformations (Moreno and Sutovsky 2003, submitted). Increased levels of sperm surface ubiquitination were found in the epididymal *azh/azh* spermatozoa, as compared with epididymal *azh/+* and wild type (wt; +/+) spermatozoa, and with the testicular *azh/azh, azh/+* and +/+ spermatozoa. Immunofluorescence analysis detected such surface ubiquitination in 100% of azh/azh spermatozoa, but only in 10.7% and 4.5% in the azh/+ and wt spermatozoa, respectively. Two major, unique, anti-ubiquitin-immuno-reactive bands were detected in the extracts of epididymal *azh/azh* and *azh/+* spermatozoa by Western blotting (**Fig. 12**). Together, these data show that the defective and the morphologically normal mouse spermatozoa are

Fig. 12. Sperm-ubiquitin immunoreactivity of wild type (wt) and azh mutant mice. Note that banding patterns are identical in the testis, yet differ between wt and *azh* sperm isolated from the epididymis.

ubiquitinated differentially during epididymal passage in rodents.

A study in rat, conducted in collaboration with Dr. Mark Tengowski (Pfizer Inc., Groton, CT) investigated testicular and epididymal sperm changes induced by the non-specific phosphodiesterase inhibitor, theophylline. Male Sprague-Dawley rats housed singly were fed either a control diet or a theophylline (1,3-dimethylxanthine, 8000 ppm) diet. Experimental groups were on diet for 8, 16, 24, or 32 days. Body weights and food consumption over the 32 d study were recorded. *In vivo* MRM imaging was performed under isofluorane anesthesia. Increased signal observed in the theophylline-treated rats suggests that a breakdown of the blood-testis barrier was occurring. Histology and immunohistochemistry were performed on the tissues. *In vivo* MRM results indicate that day 16 testis displayed an increased T1-weighted water signal in the area of the seminiferous tubule that decreased by day 32. Pathology commonly observed in this model includes dilatation of the seminiferous tubules, vacuoles and multinucleate giant cell formation within the tubules, congestion of sperm in the rete testis, and impaired spermatogenesis. Histology confirmed these findings and validated the *in vivo* MRM to predict its sensitivity to recognizing changes in testis and epididymal tissues. The participation of the ubiquitin system in clearing damaged cells was investigated, using probes for various markers of the ubiquitin-proteasome pathway. Changes in the up-regulation/mobilization of ubiquitin-proteasome pathway may be one of the mechanisms used in theophylline-treated epididymis to remove damaged cells before storage in the cauda epididymis

(see **Fig. 2**). The combined use of *in vivo* MRM and subsequent tissue or seminal analysis for the presence of ubiquitin in longitudinal studies may become an important biomarker for assessing testis toxicities in drug and solvent studies.

TABLE C. Urinary excretion of TCE and TCOH following inhalation exposure to TCE.

TCE Exposure (weeks)	TCE Metabolites (μmole/24 h)	
	TCA	TCOH
1	12.7 ± 1.1	79.0 ± 4.8^{a}
2	22.3 ± 2.6^{b}	99.4 ± 6.4^{a}
3	21.1 ± 2.3^{b}	$104.9 \pm 6.7^{a,c}$
4	23.0 ± 2.4^{b}	$106.1 \pm 4.3^{a,c}$

[a]Significantly different from levels of TCA ($p < 0.001$).
[b]Significantly different from TCA levels detected at 1week ($p < 0.05$).
[c]Significantly different from TCOH levels detected at 3 and 4 weeks ($p < 0.05$).

8.7. Aim#2: Conclusions.

Ubiquitin appears to be a superior indicator of male infertility and reduced sperm/semen quality in both non-human primates and rodents. However, while the effects of toxic exposure on sperm ubiquitin may last beyond the period of exposure in primates, the TCE-exposed rodents showed a surprising adaptability, wherein both the loss of fertility and increased sperm ubiquitination were only transient and did not last until the end of exposure trial. This, if supported by further research and final analysis of primate data, suggest that non-human primates could indeed be a superior model for human male reproductive toxicology. This benefit however, could be outweighed by the ethical and financial concerns associated with the maintenance of primate colonies and exposure of non-human primates to reproductive toxicants.

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11. PUBLICATIONS.

11.1. Present Publications.

*Sutovsky P. (2003). Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization, and sperm quality control: killing three birds with one stone. *Micros. Res. Tech.,* In press. (Invited article)

Thompson WE, Ramalho-Santos J, Sutovsky, P. (2003) Ubiquitination of prohibitin in mammalian sperm mitochondria: Possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biol. Reprod* 68: In press.

Sutovsky P, Turner RM, Hameed S, Sutovsky M (2003) Differential ubiquitination of stallion sperm proteins: Possible implications for infertility and reproductive seasonality. *Biol. Reprod.,* 68, 688-698

*Rawe, V.Y., Brugo Olmedo, S., Benmusa, A., Shiigi, S. M., Chemes, H.E., and Sutovsky, P. (2002). Sperm ubiquitination in patients with dysplasia of the fibrous sheath. *Human Reprod.,* 17:2119-2127.

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*Sutovsky, P., Moreno, R., Ramalho-Santos, J., Dominko, T., Thompson, W. E., 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. *Human Reprod.* , 16, 250-258

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11.2. Submitted Publications.

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Dalzell LH, Sutovsky P, McClure N, Lewis SEM. (2003) Sperm ubiquitin and its relationship with sperm count, motility and morphology. *Human Reproduction*, Submitted 2-17-03.

Moreno R, Sutovsky P (2003) High ubiquitin levels in the defective spermatozoa of infertile *azh/azh* mutant mice. *Molecular Reproduction & Development*. Submitted 1-30-03

M.W. Tengowski, P. Sutovsky, L.W. Hedlund, D.J. Guyot, J.E.Burkhardt, W. E. Thompson, M. Sutovsky, and G.A. Johnson (2003) Reproductive cytotoxicity is predicted by magnetic resonance microscopy and confirmed by ubiquitin-proteasome immunohistochemistry in a theophylline-induced model of rat testicular and epididymal toxicity. *Toxicologic Pathology*. Submitted 2-10-03.

11.3. Anticipated Future Publications.

Sutovsky, P, Rawe V, Powell, M., Rivnay B, Sutovsky M. (2003). Sperm ubiquitin levels measured by SUTI assay are predictive of developmental potential of human embryso conceived by ICSI with teratospermic spermatozoa. In preparation.

Powell M, Sutovsky M, Rawe, V, Sutovsky P. Proteomic analysis of sperm of teratospermic infertility patients and identification of apoliporotein (a) as a potential substrate of ubiquitin in the defective spermatozoa. (2003). In preparation.

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Forkert PG, Sutovsky P (2003). Transient increase in sperm ubiquitin levels parallels a transient loss of fertility in male mice exposed to a toxic workplace chemical, trichloroethylene (TCE). (In preparation)

Baska K, Thompson WE, Sutovsky M, Sutovsky.P. (2003) Identification of arylsulfatase A as a ubiquitinated substrate in the defective mammalian spermatozoa. In preparation.

Fischer KA, Safranski TJ, Van Leyen K, Rucker EB 3rd, Sutovsky M, Sutovsky P. (2003). Components of lipoxygenase and ubiquitin-dependent pathways for organelle degradation are expressd in the epididymis, testis and in the cytoplasmic droplets of mammalian spermatozoa. In preparation.

11.4. Patents.

Ubiquitin-Based Sperm Quality Assay. P. Sutovsky, Inventor. Submitted to U.S. Patent Office 12/99, i.e. before the starting date of this project. International patents and updates submitted 5/00-9/02, updates included data from this project. Claims were accepted by US patent office 1/03. SUTI assay is being developed into a commercial male infertility test by Repromedix Inc., Woburn, MA.

11.5. Media Coverage (Partial List).

Rawe et al., 2002, *Human Reprod* 17:2119-2127. (Partial List) MedStar TV News (Distributed Nationwide via ABC, CBS and NBC); MSNBC; HealthScout.com; BabyCenter.com; Penthouse magazine; Men's Health Magazine; also covered by press in Argentina

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BBC Radio, London, UK, Interview, January 21, 2002, documentary series *"Beyond the Genome."*

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Sutovsky et al., 2001 *Human Reproduction* 16, 250-258

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12. APPENDICES.

Appendix 1. Sutovsky, 2003.
Appendix 2. Sutovsky et al., 2001a
Appendix 3. Sutovsky et al., 2001b
Appendix 4. Rawe et al., 2002
Appendix 5. Sutovsky, Hauser et al., 2003