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Immunofluorescence Reveals Ubiquitination of Retained Distal Cytoplasmic Droplets on Ejaculated Porcine Spermatozoa

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ABSTRACT: The purpose of the present study was twofold: 1) to determine if antibodies raised against ubiquitin would recognize antigens associated with the porcine cytoplasmic droplet (CD), and 2) to determine if the same antibody would identify ubiquitinated substrates on the surface of morphologically abnormal boar spermatozoa. Permeabilization with the detergent Triton X-100 (0.05%) showed virtually all CDs to be ubiquitin positive. Distal droplets (DDs) retained in situ on boar spermatozoa were readily labeled following Triton permeabilization, whereas DDs present on nonpermeabilized cells were not. Negative control preparations lacked the ubiquitin staining on the DD. The use of microtubes for fixation and incubation provided clearer images as well as better sperm cell distribution and density than an initial slide-mounted technique. Immunoblotting indicated that larger amounts of ubiquitinated proteins were present in extracts from sperm cells from an ejaculate with an abnormally high percentage of retained DDs (52% DDs) compared to a morphologically normal sample (6% DDs). The primary antibody recognized both mono-ubiquitin of bovine origin (8.5 kd) and human ubiquitin conjugate (35 kd), as demonstrated by Western blot. Preabsorption of the anti-ubiquitin antibody with purified bovine ubiquitin was successful in preventing diaminobenzidine staining of sperm extract from the high DD ejaculate. The presence of antigens recognized by anti-ubiquitin antibodies in the boar sperm CD, coupled with the possibility that superfluous ubiquitin species are detrimental to embryonic development by targeting critical paternally contributed zygotic organelles, raises concerns that retained DDs may be more detrimental to fertility than previously suspected.

Key words: Sperm, boar.
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The remnant of original spermatid cytoplasm remaining attached to the spermatozoon after spermiation is known as the cytoplasmic droplet (CD) (Fawcett and Ito, 1965). Ultrastructural analysis of the CD shows numerous internal vesicular elements surrounded by an intact plasma membrane (Kaplan et al, 1984). Early speculation that the CD contains remnants of the Golgi apparatus and endoplasmic reticulum (Bloom and Nicander, 1961) has been challenged by more recent structural and cytochemical evidence that suggests the presence of proteins native to the outer dense fibers, fibrous sheath, and perforatorium of spermatozoa (Hermo et al, 1988). Several glycolytic enzymes have been localized to the CD, which suggests a relationship to lysosomal activity (Dott and Dingle, 1968; Harrison and White, 1972). Antibodies to lipoxygenase, an enzyme capable of damaging cellular membranes through peroxidation, recognize antigens in the CD of sperm from the bull and boar (Fischer et al, 2002). The identification of P450 aromatase in the CD of several species has led to the implication of estrogens in the regulation of sperm maturation in the epididymis (Janulis et al, 1996).

The CD migrates from the proximal to the distal position during maturation in the distal caput and proximal corpus epididymis and is normally shed from boar spermatozoa during or shortly after ejaculation (Larsen et al, 1980; Kato et al, 1996). In contrast to rats, which dispose of the contents of the CD by epithelial phagocytosis following their loss in the distal region of the epididymis, as many as 70%–90% of cauda epididymal boar spermatozoa retain the distal droplet (DD) (Kaplan et al, 1984; Hermo et al, 1988; Kato et al, 1996). D-Fructose, a component of the seminal vesicular fluid, is thought to be the factor responsible for normal shedding of the DD from boar sperm (Harayama et al, 1996). In the bull and ram, phospholipid-binding protein, synthesized in the ampullary glands and seminal vesicles, is believed to induce the release of the CD (Matousek and Kysilka, 1984).

Proximal droplets (PDs) found on ejaculated spermatozoa are generally considered indicative of a defect of testicular origin and have been implicated in the depressed fertility of bulls and boars (Dausend, 1974;

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Amann et al, 2000; Thundathil et al, 2001). The effect of a retained DD on fertility is less well defined, although there is some evidence suggesting a negative impact for stored boar semen used in artificial insemination programs (Larsson et al, 1984; Zeuner, 1992; Waberski et al, 1994; Althouse, 1998). An elevated incidence of retained DDs has been described in conjunction with biochemically altered plasma membranes in heat-stressed boars (Althouse, 1992). Additionally, boar sperm with retained CDs have a reduced binding affinity for porcine oviductal epithelial explants in culture (Petrunkina et al, 2001).

It has long been recognized that a selective mechanism may exist for the removal of abnormal spermatozoa in the efferent ductules and epididymis (Crabo et al, 1971). Rao (1980) recognized that the incidence of abnormal sperm heads decreased as spermatozoa passed along the length of the excurrent duct system in dairy bulls and that further reduction occurred in the epididymis of bulls with pathological semen. Although the process whereby abnormal sperm cells were recognized was not known, it was proposed that epithelial phagocytosis was the primary route of clearance (Rao, 1980). Recently, a ubiquitin-dependent sperm quality control mechanism functioning in the bovine epididymis was described, showing that ubiquitin is secreted by epithelial cells in the epididymis and is reported to bind to the surface of defective bull sperm (Sutovsky et al, 2001a). Most of the ubiquitinated, defective sperm are subsequently phagocytized by the epididymal epithelial cells, but some escape phagocytosis and can be identified in the ejaculate (Sutovsky et al, 2001a). In the stallion, sperm ubiquitination may be associated with seasonal changes in sperm numbers and semen quality (Sutovsky et al, 2003).

Ubiquitin, a small peptide of 8.5 kd, is a universal marker for proteolysis found in all tissues and organisms (Hershko, 1998). Since its initial discovery, ubiquitin has been assumed to operate exclusively in the intracellular space, where it marks proteins for recycling and identifies misfolded or damaged proteins for degradation (Hershko, 1998). Ubiquitin was recently identified at relatively high levels in an extracellular fluid—human seminal plasma (Lippert et al, 1993). Shortly thereafter, intense ubiquitin immunoreactivity was detected in the principal cells of the distal caput and in the clear cells of the cauda epididymidis of the rat (Martin et al, 1995). Epithelial clear cells of the rat epididymis actively dispose of the contents of CDs released from spermatozoa (Hermo et al, 1988).

The preferential binding of ubiquitin to the surface of defective spermatozoa has been reported in bulls, stallions, men, rhesus monkeys, and mice (Sutovsky et al, 2001a,b, 2003) but not in the boar. Although speculation abounds as to the significance of retained DDs to fertility and some work has been done to elucidate the factors involved in the normal release and abnormal retention of the CD, more can be learned about the CD at the molecular level. A greater understanding of the CD will help discern the potential impact it has on the sperm that carry it and on the oocytes those sperm encounter. The authors propose that a ubiquitin-dependent mechanism, similar to that identified in other species to remove morphologically abnormal sperm from the epididymis, functions in the boar by marking defective sperm for disposal. In addition, since retained CDs on spermatozoa represent the majority of abnormalities in boar ejaculates, we hypothesize that ubiquitination of the CD in particular should be expected to occur and will reinforce the need for studies addressing the interaction of retained CDs with in vivo fertility. Therefore, the purpose of the present study was twofold: 1) to determine if antibodies raised against ubiquitin would recognize antigens associated with the porcine CD, and 2) to determine if the same antibody would identify ubiquitinated substrates on the surface of morphologically abnormal boar spermatozoa.

Materials and Methods

Porcine Semen

Modified sperm-rich ejaculates were collected by the gloved-hand technique, diluted 1:4–1:25 with commercial porcine semen extender (Androhep; Minitube of America, Verona, Wis; or X-CELL; IMV Int, Minneapolis, Minn), cooled to 15°C–19°C, and transported at that temperature to the laboratory. Samples were obtained from a total of 12 adult crossbred boar studs housed at 3 separate locations. On arrival, sperm morphology was determined by mixing 500 μL of the extended semen with an equal volume of 0.2% buffered glutaraldehyde, placing 10 μL of the fixed sperm solution on a clean glass slide covered with a coverslip, and counting 200 sperm per sample using differential interference contrast at 400× magnification with an Olympus BH-2 microscope (Barth and Oko, 1989). Sperm were classified into one of the following categories: normal, abnormal heads (including acrosomes), abnormal midpiece, abnormal tails (including distal midpiece reflex), proximal CDs, distal CDs, coiled tails, or separated heads. The percentage of sperm in each morphology category was determined by dividing the number of cells in each classification by the total number of cells counted. Sperm cell concentration was determined using the HTR-IVOS computer-assisted semen analysis system (Software version 12.1a; Hamilton Thorne Biosciences, Beverly, Mass).

Reagents

Anti-ubiquitin (whole antiserum) developed in rabbits using bovine red blood cell ubiquitin conjugated to keyhole limpet hemocyanin as the immunogen was purchased from Sigma Chemical Co (St Louis, Mo). Secondary antibodies were obtained from Zymed Laboratories, Inc (South San Francisco, Calif). Normal goat serum (NGS) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif). Vectashield Mounting Medium with propidium iodide (PI) was purchased from Vector Labora-

tories (Burlingame, Calif). Beltsville Thaw Solution (BTS) was supplied by IMV (Minneapolis Minn). Buffered formalin was purchased from Fisher Scientific (Pittsburgh, Pa), and glutaraldehyde was obtained from Electron Microscopy Sciences (Fort Washington, Pa). Unless otherwise noted, all other reagents were purchased from Sigma-Aldrich (St Louis, Mo).

Immunofluorescence

Ten samples of fresh extended porcine semen were prepared for the ubiquitin immunofluorescence assay (U-IFA) by an initial slide mounting technique similar to that of Sutovsky (2001a), while 2 others were prepared by conducting the fixation, incubation, and washing steps in microtubes rather than on slides. For the slide-mounted technique, extended semen was initially separated on an isotonic discontinuous Percoll gradient (72%/90%) to concentrate the sperm cells, remove the extender, and isolate sperm cells from bacteria, free CDs, and other debris (Parrish et al, 1986; Grant et al, 1994). Following Percoll separation, sperm cells were washed once in Tyrode albumin lactate pyruvate (Bavister et al, 1983); then, 10 μL of concentrated cell suspension was added to a 250-μL drop of warm (37°C) BTS (Johnson et al, 1988) on a charged slide (Superfrost/Plus, Fisher) and incubated at that temperature for 5 minutes. After attachment, excess fluid was shaken off, and the sperm were fixed by first covering the attachment area with 200 μL of phosphate-buffered saline (PBS) and then slowly adding 50 μL of 10% neutral buffered formalin (NBF). After 40 minutes of fixation, the samples were blocked and permeabilized for 30 minutes using 5% NGS in PBS containing 0.1% Triton X-100 (TX-100) and then incubated for 40 minutes in a 1:100 dilution of anti-ubiquitin in PBS. Following primary antibody incubation, the samples were rinsed 3 times in PBS and incubated for 40 minutes with the secondary antibody, fluorescein isothiocyante (FITC)-conjugated goat anti-rabbit diluted 1:100 in PBS. After 3 rinses in PBS, a coverslip was mounted using Vectashield with PI for the DNA counterstain. Fluorescence was evaluated with an Olympus IX70 microscope (NIB filter), and images were captured with a Spot RT digital camera (Diagnostic Instruments Inc, Sterling Heights, Mich).

For the microtube technique, a volume of extended semen containing 3.5–5.0 × 10^6 sperm cells was added to a 1.5-mL microtube and centrifuged at 200 × *g* for 3 minutes (all subsequent centrifugations were at 200 × *g* for 4 minutes). The supernatant was removed, and the pellet was resuspended with 500 μL of 0.2% glutaraldehyde in PBS. After 45 minutes of fixation, the supernatant was removed by centrifugation, and the sperm cells were resuspended in a blocking solution containing 10% NGS and 0.05% TX-100 in PBS for 45 minutes. Blocking/permeabilization fluid was removed by centrifugation, and the sperm cells were incubated for 1 hour in 100 μL of anti-ubiquitin antibody diluted 1:100 in PBS. The samples were then centrifuged, the supernatant was discarded, and the sperm cells were washed once in 500 μL of PBS prior to a 45-minute incubation with 100 μL of FITC-conjugated goat anti-rabbit secondary antibody diluted 1:100 in PBS. The samples were then washed once with 500 μL of PBS and resuspended in 100 μL of fresh PBS. After gentle mixing, a 10-μL drop of the sperm suspension was placed on a Superfrost/Plus Slide, and the edge of a coverslip was used to make a smear that was allowed to air dry. A coverslip (22 × 50 mm) was mounted over the area of the smear using Vectashield medium with PI as described above.

Rat cauda epididymidis served as the positive controls. Negative controls included the following: 1) reagent control—exclusion of the primary antibody (pure PBS), 2) normal rabbit serum (NRS) control—incubation with NRS (1:100 dilution in PBS) in place of the primary antibody, and 3) peptide competition—preabsorption of the primary antibody with excess ubiquitin prepared from bovine erythrocytes.

Western Blot

To reduce contamination with free CDs and remove excess seminal plasma and extender components, diluted boar semen was first separated through an isotonic Percoll gradient (72%/90%). After washing once with PBS, the sperm pellet was resuspended in precooled (4°C) PBS containing 1% TX-100 and selected protease inhibitors (5 mM benzamidine HCl, 5 μg/mL leupeptin, 1 μg/mL pepstatin A, and 25 mg/mL phenylmethylsulfonyl fluoride); it was then incubated on ice for 30 minutes with light vortexing at 5-minute intervals. Following detergent extraction, the sample was clarified by centrifugation at 10 000 × *g* for 5 minutes, and the supernatant was drawn off and stored at −20°C. Protein concentration was determined using the BCA Protein Assay Reagent Kit from Pierce Biotechnology (Rockford, Ill). Supernatant fluid containing 65 μg of sperm proteins was mixed 5:1 with 6× sample buffer and boiled for 3 minutes prior to 1-dimensional discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% stacking gel and a 15% resolving gel (Laemmli, 1970). Proteins were transferred to 0.2-μm-thick nitrocellulose membranes, with the transfer efficiency evaluated using Ponceau S prior to immunostaining (Towbin et al, 1979; Salinovich and Montelaro, 1986). Membranes were blocked in Tris saline (TS 20) containing 5% nonfat dried milk for 1 hour, incubated for 90 minutes with anti-ubiquitin antibody (1:100 in TS 20 with 1% dried milk) at room temperature, washed 3 times in TS 20, and incubated for 45 minutes with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG) (1:100). After 3 washes, blots were developed using the ImmunoPure Metal Enhanced DAB (diaminobenzidine) Substrate Kit (Pierce). Specificity of labeling was evaluated by including a negative control that was incubated with primary antibody preabsorbed to excess bovine ubiquitin and 2 positive controls: 1 μg of bovine red blood cell ubiquitin (Sigma) and 1 μg of Ub (FL-76), a 35-kd tagged fusion protein produced in *Escherichia coli* representing full-length ubiquitin of human origin (Santa Cruz Biotechnology).

Results

As reported in the Table, virtually all CDs were identified as ubiquitin positive (96% of CDs and 97% of DDs) when TX-100 was included in the blocking solution for permeabilization. Two additional ejaculates were analyzed using the microtube technique in which the sperm cells were not mounted on glass slides until all incubations and washes had been completed; 0.2% glutaraldehyde was

Percentage of ubiquitin-positive cytoplasmic droplets by U-IFA*†

Ejaculate	Source Stud	% CD in Ejaculate	% CD U-IFA+ (N)‡	% DD in Ejaculate	% DD U-IFA+ (N)
1	A	45	94 (63/67)	22	95 (52/55)
2	B	51	82 (55/67)	39	85 (44/52)
3	B	44	100 (15/15)	40	100 (15/15)
4	B	49	95 (90/95)	23	98 (54/55)
5	B	50	95 (19/20)	36	93 (14/15)
6	C	50	100 (107/107)	42	100 (90/90)
7	C	68	100 (148/148)	17	100 (105/105)
8	C	22	100 (35/35)	15	100 (21/21)
9	C	60	100 (44/44)	22	100 (30/30)
10	C	83	97 (142/147)	35	98 (51/52)
	Mean (SD)	52 (16%)	96 (6%)	29 (10%)	97 (5%)

* Cytoplasmic droplets % (CD) includes proximal and distal cytoplasmic droplets as well as cytoplamic droplets trapped within a distal midpiece reflex.

† DD, distal droplets; U-IFA, ubiquitin immunofluorescence assay.

‡ N = number of sperm with a retained CD that were U-IFA +/total number of sperm identified with a retained CD.

used in place of 2% NBF for fixation, and 0.05% TX-100 was used for permeabilization. Figure 1 illustrates the presence of ubiquitin within the DD using this method. DDs retained in situ on boar spermatozoa are readily labeled following permeabilization, whereas DDs present on nonpermeabilized cells are not. Negative control preparations consisted of the following: 1) reagent control with PBS replacing the primary antibody, 2) peptide competition control with preabsorption of the primary antibody with purified bovine ubiquitin, and 3) NRS substituting for anti-ubiquitin antiserum; all lacked the ubiquitin staining of the DD. Western blot (Figure 2) demonstrated affinity of the primary antibody for both mono-ubiquitin of bovine origin (8.5 kd) and human ubiquitin conjugate (35 kd). A clear difference can be seen in the relative amounts of ubiquitinated proteins present in extracts from sperm cells from an ejaculate with an abnormally high percentage of retained DDs (52% DDs) compared to a morphologically normal sample (6% DDs). Preabsorption of the anti-ubiquitin antibody with purified bovine ubiquitin was successful in preventing DAB staining of sperm extract from the high-DD ejaculate.

Discussion

The initial slide-mounted U-IFA technique that was used in the present study is similar to the protocol described for bull sperm (Sutovsky, 2001a), with laboratory-specific procedural modifications including the antibody source and the use of charged slides rather than poly-L-lysine–coated coverslips for sperm cell attachment. Although the permeabilization of boar spermatozoa with TX-100 in the initial protocol showed intense fluorescence labeling of the CD, exposure to this detergent also caused a high proportion of DDs to be lost from the sperm cells, effectively reducing the population of sperm with retained DDs and complicating the interpretation of the U-IFA. Improvements made in the sperm fixation and mounting techniques are described elsewhere (Kuster and Althouse, 2003). The potential value of these modifications in studies using other species' spermatozoa warrants consideration.

Surface ubiquitination was not apparent on nonpermeabilized morphologically abnormal porcine spermatozoa. Although this finding could be because of species differences in epididymal ubiquitination, it is more likely that the probe used in the present study was limited in its affinity for certain types of polyubiquitinated chains (Hershko and Ciechanover, 1998). The primary antibody (U5379) was generated against mono-ubiquitin purified from bovine erythrocytes, whereas ubiquitin potentially located on the surface of spermatozoa would be covalently bound to sperm surface proteins in the form of polyubiquitinated chains (Sutovsky et al, 2001a). That positive IFA results were obtained following permeabilization of the CD supports the belief that the antibody was effective at detecting ubiquitinated sperm proteins; however, further investigation may be elected to determine if using a broader range of epitope-specific ubiquitin probes and recombinant isopeptides will definitively verify the presence of ubiquitin-protein conjugates on the surface of the boar spermatozoa. The ubiquitinated proteins revealed within the boar CD after permeabilization suggest that the antibody used in the present study detects mainly intracellular ubiquitin that would most likely have originated from ubiquitin-dependent proteolysis during spermatid elongation (Sutovsky, 2003). As a remnant of the spermatid cytoplasm, the CD contains numerous vesicular elements and other small particles that may have originated from the Golgi apparatus, sperm centrosome, and sperm histones (Bloom and Nicander, 1961; Moreno et al, 2000; Sutovsky, 2003). The detection of intracellular ubiquitin

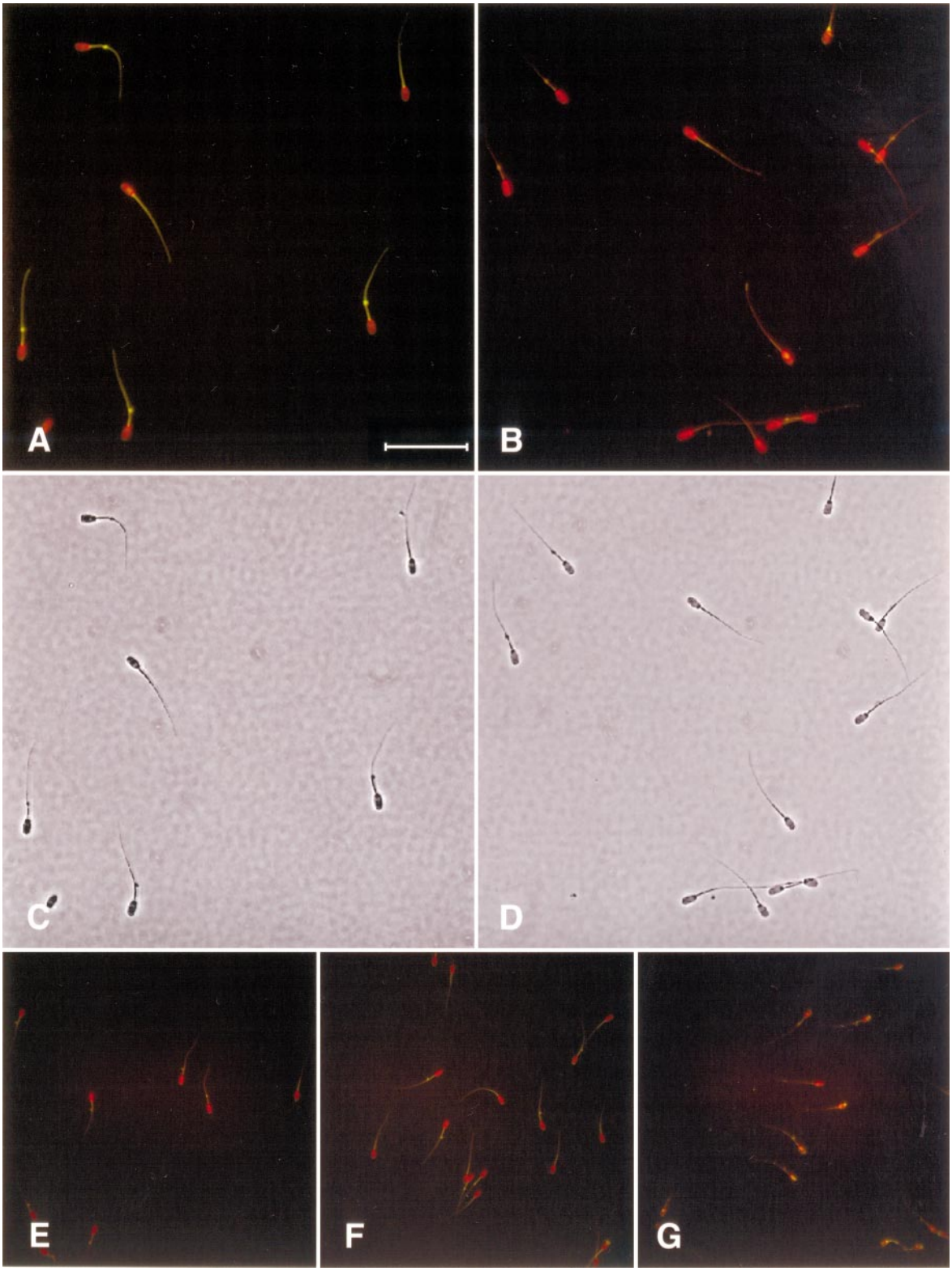

Figure 1. Immunofluorescence **(A, C, E–G)** of ejaculated boar spermatozoa labeled using anti-ubiquitin antibodies conjugated with fluorescein isothiocyante (FITC) with propidium iodide (PI) for DNA counterstaining. Note the fluorescence of the distal droplet (DD) for permeabilized, glutaraldehyde-fixed sperm in **(A)** but the lack of staining for nonpermeabilized cells in **(B)**. Phase-contrast images of the same fields show cell morphology in **(C)** and **(D)**. Negative control samples: **(E)** reagent control (no primary antibody), **(F)** peptide competition (primary antibody preincubated with purified bovine ubiquitin), and **(G)** normal rabbit serum substituted for anti-ubiquitin antiserum all lacked ubiquitin staining of the DD. Scale bar **(A)** = 0.05 mm.

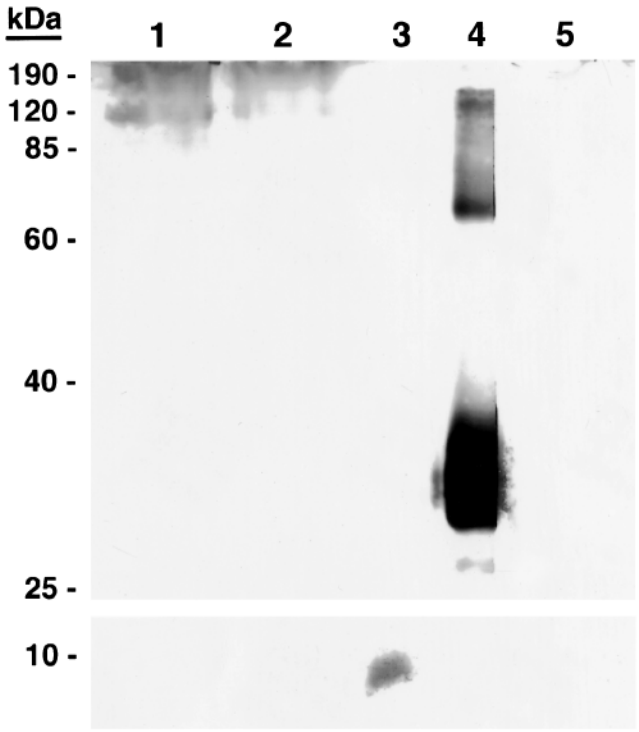

Figure 2. Western blot of extracted sperm proteins probed with anti-ubiquitin antiserum U5379. Staining intensity indicates that more ubiquitin-conjugated proteins are present in the high distal droplet (DD) ejaculate (52% retained DDs) in lane 1 than in the normal ejaculate (6% DDs) in lane 2. Purified bovine mono-ubiquitin (8.5 kd) and human ubiquitin conjugate (35 kd) are shown in lanes 3 and 4. Preabsorbing the primary antibody with bovine ubiquitin succeeded in preventing the high DD ejaculate from being stained in lane 5 (peptide competition). Relative molecular weights are indicated by the numbers to the left of the blot.

does not rule out the possibility that the surface of the CD will also become ubiquitinated at a later stage, such as during epididymal passage and/or during postejaculatory sperm modifications.

Ubiquitination of the retained CDs has important implications when coupled with the knowledge that PDs and DDs have been associated with depressed fertility in vitro and in vivo. In mammals, some of the paternally derived organelles, such as mitochondria, are degraded in the lysosomes of the oocyte after fertilization, while others, such as the centrosome and male pronucleus, become vital zygotic components (Yanagimachi, 1994; Sutovsky et al, 1999). It has been theorized that following natural fertilization, the ubiquitin present on the surface of spermatozoa from subfertile ejaculates are carried over to the oocyte cytoplasm, where it could potentially target vital paternal organelles for destruction by the proteolytic machinery of the oocyte, effectively preventing further embryonic development (Sutovsky et al, 2001b). In support of this theory, a relatively high correlation coefficient ($r = -0.432$) was obtained by comparing Sperm Ubiquitin Tag Immunoassay (SUTI) to cleavage rate after in vitro fertilization (IVF) in human infertility patients, even though fertilization rates were poorly correlated ($r = 0.046$) (Sutovsky et al, 2001b).

Although ubiquitin was not simultaneously investigated, another study found that semen from yearling beef bulls containing more than 30% spermatozoa with PDs had severely compromised IVF fertility, which subsequently improved as the bulls matured and the incidence of PDs decreased (Amann et al, 2000). It has been reported that bull sperm with PDs do not bind to the zona pellucida at the same rate as morphologically normal sperm (Thundathil et al, 2001), and boar sperm with retained CDs have a reduced binding affinity to porcine oviductal epithelial explants in culture (Petrunkina et al, 2001); however, the ability of porcine sperm with retained DDs to bind to the zona and fuse to the oolema has not been ruled out.

A possible explanation for this reduced binding capacity of sperm with a CD relates to the maturity level achieved during spermiogenesis. In humans, the presence of a chaperone protein, HspA2, throughout the tail and on the surface of the tail plasma membrane has been associated with the level of sperm cellular maturity, function, and fertility (Huszar et al, 2000; Ergur et al, 2002). Expression of HspA2 in human sperm occurs during remodeling of the plasma membrane when zona pellucida–binding sites are formed (Huszar et al, 1994, 1997). Human sperm with a diminished HspA2 expression retain

cytoplasm, are considered immature, and fail to bind to the zona pellucida (Ergur et al, 2002).

The boar sperm CD contains numerous membranous structures (Kaplan et al, 1984). One comparison finds the area of these internal CD membranes equivalent to 54% of the total surface area of the external sperm plasma membrane (Kaplan et al, 1984). The demonstrated presence of antigens recognized by anti-ubiquitin antibodies in the boar sperm CD, coupled with the possibility that superfluous ubiquitin species are detrimental to embryonic development through targeted destruction of critical paternally contributed zygotic organelles, raises concerns that retained DDs may be more detrimental to fertility than previously suspected. Further studies are warranted to investigate the binding, penetrating, and fertilizing ability of boar sperm with retained distal droplets and the fate of the CD and its contents during and after fertilization, as well as the developmental competence of any embryos produced from sperm with a CD.

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References

Althouse GC. *Biochemical Composition of the Spermatozoal Plasma Membrane in Normal and Heat Stressed Boars* [thesis]. Ames: Iowa State University; 1992.

Althouse GC. Cytoplasmic droplets on boar sperm cells. *Swine Health Prod.* 1998;6:128.

Amann RP, Seidel GE, Mortimer RG. Fertilizing potential in vitro of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. *Theriogenology.* 2000;54:1499–1515.

Barth AD, Oko RJ. *Abnormal Morphology of Bovine Spermatozoa.* Ames: Iowa State University Press; 1989.

Bavister DB, Leibfried ML, Lieberman G. Development of preimplantation embryos of the golden hamster in defined culture medium. *Biol Reprod.* 1983;28:235–247.

Bloom G, Nicander L. On the ultrastructure and development of the protoplasmic droplet of spermatozoa. *Zeit Zellfrosch.* 1961;55:833–844.

Crabo B, Gustafsson B, Nicander L, Rao AR. Subnormal testicular function in a bull concealed by phagocytosis of abnormal spermatozoa in the efferent ductules. *J Reprod Fertil.* 1971;26:393–396.

Dausend P. *Vorkommen und fertilitatsdiagnostische bedeutung von morphologisch abwechenden samenzellen im ebersperma unter besonderer berucksichtigung der sogenannten anhangenden protoplamatropfen* [med vet thesis]. Hannove Universitat; 1974.

Dott HM, Dingle JT. Distribution of lysosomal enzymes in the spermatozoa and cytoplasmic droplets of bull and ram. *Exp Cell Res.* 1968; 52:523–540.

Ergur AR, Dokras A, Giraldo JL, Habana A, Kaovanci E, Huszar G. Sperm maturity and treatment choice of in vitro fertilization (IVF) of intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure. *Fertil Steril.* 2002;77:910–932.

Fawcett DW, Ito S. The fine structure of bat spermatozoa. *Am J Anat.* 1965;116:567–610.

Fischer KA, Safranski TJ, Van Leyen K, Rucker EB, Sutovsky M, Sutovsky P. Components of lipoxygenase and ubiquitin-dependent pathways for organelle degradation co-localize in the cytoplasmic droplet of mammalian spermatozoa. *Biol Reprod.* 2002;66(suppl 1):114.

Grant SA, Long SE, Parkinson TJ. Fertilizability and structural properties of boar spermatozoa prepared by Percoll gradient centrifugation. *J Reprod Fertil.* 1994;100:477–483.

Harayama H, Shibukawa T, Miyake M, Kannan Y, Kato S. Fructose stimulates shedding of cytoplasmic droplets from epididymal boar spermatozoa. *Reprod Fertil Dev.* 1996;8:1039–1043.

Harrison RAP, White IG. Glycolytic enzymes in the spermatozoa and cytoplasmic droplets of bull, boar and ram and their leakage after shock. *J Reprod Fertil.* 1972;30:105–115.

Hermo L, Dworkin J, Oko R. Role of epithelial clear cells of the rat epididymis in the disposal of the contents of cytoplasmic droplets detached from spermatozoa. *Am J Anat.* 1988;183:107–124.

Hershko A. The ubiquitin system: past, present and future perspectives. In: Peters JM, Harris JR, Finley D, eds. *Ubiquitin and the Biology of the Cell.* New York, NY: Plenum Press; 1998: 1–17.

Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* 1998;67:425–479.

Huszar G, Sbracia M, Vigue L, Miller D, Shur B. Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane β-1,4-glactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. *Biol Reprod.* 1997;56:1020–1024.

Huszar G, Stone K, Dix D, Vigue L. Putative creatine kinase M-isoform in human sperm is identified as the 70-kilodalton heat shock protein HspA2. *Biol Reprod.* 2000;63:925–932.

Huszar G, Vigue L, Oehninger S. Creatine kinase (CK) immunocytochemistry of human hemizona–sperm complexes: selective binding of sperm with mature CK-staining pattern. *Fertil Steril.* 1994;61:136–142.

Janulis L, Hess RA, Bunick D, Nitta H, Janssen S, Asawa Y, Bahr JM. Mouse epididymal sperm contain active P450 aromatase which decreases as sperm traverse the epididymis. *J Androl.* 1996;17:111–116.

Johnson LA, Albers JG, Grooten HJG. Artificial insemination of swine: fecundity of boar semen stored in Beltsville TS (BTS), Modified Modena (MM), or MR-A and inseminated on one, three, and four days after collection. *Zuchthygiene (Berl).* 1988;23:49–55.

Kaplan M, Russell LD, Peterson RN, Martan J. Boar sperm cytoplasmic droplets: their ultrastructure, their numbers in the epididymis and at ejaculation and their removal during isolation of sperm plasma membranes. *Tissue Cell.* 1984;16:455–468.

Kato S, Shibukawa T, Harayma H, Kannan Y. Timing of shedding and disintegration of cytoplasmic droplets from boar and goat spermatozoa. *J Reprod Fertil.* 1996;42:237–241.

Kuster CE, Althouse GC. A technique for preserving retained distal cytoplasmic droplets in situ for immunofluorescence evaluation of ejaculated porcine spermatozoa. *Prep Biochem Biotechnol.* 2003;33:301–310.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680–685.

Larsen RE, Crabo B, Leman AD. Physical and chemical influences on loss of the cytoplasmic droplet (CD) from porcine spermatozoa during ejaculation. In: *Proceedings of the 9th International Congress on Animal Reproduction and Artificial Insemination.* Madrid, Spain: Ministerio Agricultura. 1980;3:45–48.

Larsson K, Darentius K, Johansson K. Sperm morphology and in vitro

viability in diluted semen in relation to fertility of AI boars. *Nord Vet Med.* 1984;32:533–542.

Lippert T, Seeger H, Schieferstein G, Wolfgang V. Immunoreactive ubiquitin in human seminal plasma. *J Androl.* 1993;14:130–131.

Martin R, Santamaria L, Fraile B, Paniagua R, Polak JM. Ultrastructural localization of PGP 9.5 and ubiquitin immunoreactivities in rat ductus epididymidis epithelium. *Histochem J.* 1995;27:431–439.

Matousek J, Kysilka C. The phospholipid-binding protein of the reproductive tract of the bull—effect on the removal of spermatozoal cytoplasm droplets in the other species and influence of antibodies on its reactivity. *Anim Reprod Sci.* 1984;7:433–440.

Moreno RD, Ramalho-Santos J, Chan EK, Wessel GM, Schatten G. The Golgi apparatus segregates from the lysosomal/acrosomal vesicle during rhesus spermiogenesis: structural alterations. *Dev Biol.* 2000;219: 334–349.

Parrish JJ, Susko-Parrish JL, Leibfreid-Rutledge ML, Crister ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology.* 1986;25:591–600.

Petrunkina AM, Gehlhaar R, Drommer W, Waberski D, Topfer-Peterson E. Selective sperm binding to pig oviductal epithelium in vitro. *Reproduction.* 2001;121:889–896.

Rao AR, Bane A, Gustafsson BK. Changes in the morphology of spermatozoa during their passage through the genital tract in dairy bulls with normal and impaired spermatogenesis. *Theriogenology.* 1980;14: 1–9.

Salinovich O, Montelaro RC. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Anal Biochem.* 1986;156:341–347.

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

Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. Ubiquitin tag for sperm mitochondria. *Nature.* 1999;402:371–372.

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

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

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

Thundathil J, Palasz AT, Barth AD, Mapletoft RJ. The use of in vitro fertilization techniques to investigate the fertilizing ability of bovine sperm with proximal cytoplasmic droplets. *Anim Reprod Sci.* 2001; 65:181–192.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A.* 1979;76:4350–4354.

Waberski D, Meding S, Dirkson G, Weitz KF, Leiding C, Hahn R. Fertility of long-term-stored boar semen: influence of extender (Androhep and Kiev), storage time, and plasma droplets in the semen. *Anim Reprod Sci.* 1994;36:145–151.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* New York, NY: Raven Press Ltd; 1994: 189–317.

Zeuner A. On the relations between sperm-morphology and the fertility of boar semen. In: *Proceedings of the 12th International Congress on Animal Reproduction and Artificial Insemination.* The Hague, Netherlands, 1992:1617–1619.