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Apical blebs on sperm storage tubule epithelial cell microvilli: Their release and interaction with resident sperm in the turkey hen oviduct

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ABSTRACT

Located at the anterior end of the turkey hen's vagina are numerous discrete tubular invaginations of the surface epithelium, collectively referred to as the sperm storage tubules (SSTs). After mating or artificial insemination, sperm ascend the vagina, enter the SSTs, and over the ensuing days and weeks, gradually exit the SSTs and are transported to the anterior end of the oviduct to fertilize a daily succession of ova. Little is known regarding the cellular and molecular mechanisms responsible for sperm subsistence in the lumen of the SST. In this study, the origin of microvillus blebs (MvBs) on the apical tips of SST epithelial cells was examined, and their possible role in sperm survival was discussed. Regardless, if sperm are present or not, transmission electron microscopy revealed two types of microvilli differentiated by the presence or absence of pleomorphic unilaminar MvBs localized to their apical tips. Although some MvBs appeared to be discharging their contents into the SST lumen, others appeared to have pinched off the microvillus stem. When SSTs contained clusters of densely packed sperm, the sperm heads of those sperm adjacent to the SST epithelial cell surface were surrounded by the microvilli. Associated with the plasmalemma of sperm throughout the SST lumina were membrane fragments and small vesicles (30–130 nm in diameter), some of which appeared to have fused with sperm. It is concluded that the MvBs are a form of shedding vesicle released from the SST epithelial cell microvilli by apocrine secretion. On the basis of observations described herein and those of other authors, it is suggested that the MvBs contribute to sustained sperm storage in the SSTs by (1) supplying metabolic substrates used by resident sperm, (2) serving as fusogenic vehicles providing exogenous macromolecules that reversibly suppress sperm functions associated with fertilization (decapacitation?) and stabilize the sperm plasmalemma, and (3) acting as transport vesicles actively transporting fluid from the SST epithelial cells to the SST lumen.

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1. Introduction

Sperm transferred into the vagina by copulation or artificial insemination has the capacity to maintain their fertilizing capacity for days to months depending on the avian species [1]. This prolonged fertile period is the result of sperm residing in discrete tubular invaginations of the

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surface epithelium lining the lumen of the uterovaginal junction (UVJ). There are about 30,000 such tubular invaginations, referred to as the sperm storage tubules (SSTs), in the UVJ mucosa of the turkey. (See Bakst [2] and Froman et al. [3] for comprehensive reviews of oviductal sperm storage in poultry.) While the female is in egg production, sperm are gradually released from the SST, ascend to the infundibulum where sperm interact with the inner perivitelline layer (IPL) enveloping the ovulated ovum, hydrolyze the IPL (polyspermy is normal in birds), and fertilize the ovum.

Unlike mammals, ejaculated chicken sperm do not require a period of capacitation to fertilize an ovum *in vitro* [4]. Furthermore, Howarth [5] reported that chicken sperm removed from the testes, epididymides, or ductus deferens and surgically transferred into the magnum portion of the oviduct resulted in fertilized eggs. However, there were no fertilized eggs if the same sperm were vaginally inseminated into the hen. More recently, Nixon et al. [6] concluded that capacitation is not a prerequisite for Japanese quail sperm to hydrolyze IPL fragments *in vitro*. These authors also noted that posttesticular maturation of Japanese quail sperm was limited to an increase in sperm motility after passage through the epididymis.

Considering the relatively short period of sperm viability after semen collection and storage *in vitro* [7], and the long duration of sperm storage in the UVJ, it has been suggested that sperm residing in the SSTs undergo a reversible suppression of metabolic activity, motility, and acrosomal enzyme activity [2,8]. In addition, the functional integrity of the sperm plasmalemma is maintained by suppression of the formation of reactive oxygen species [8], which appear to be the basis for decreased sperm viability after *in vitro* semen storage for longer than 6 hours [7]. Given the above, Bakst and Akuffo [9] alluded to the possibility that sperm within the SST lumen are in a decapacitated-like state and reach a capacitated state when released from the SSTs and enter the uterus [8].

Sperm within the SST subsist on exogenous fatty acids [3,10] and possibly other lipids derived from the SST epithelial cells [2,8]. Possible mechanisms of lipid transfer from the SST epithelial cells or its luminal contents to resident sperm were reviewed by Bakst et al. [8]. One such mechanism included the dilation of the apical tips of the SST microvilli and the shedding of microvillus vesicles into the SST lumen to interact with sperm. Several other investigators, including Burke [11], Tingari and Lake [12], and Schuppin et al. [13], observed dilation of the SST epithelial cell microvilli and similar sized vesicles in the SST lumen but did not elaborate further on possible roles of these blebs in SST function. However, Schuppin et al. [13] and Bakst et al. [8] did allude to the possibility that such blebbing was an artifact of fixation.

The contribution of shedding vesicles derived from the mammalian epididymis (epididymosomes) and prostate gland (prostasomes) in posttesticular sperm maturation has been addressed by numerous authors and reviewed by Sullivan and Saez [14]. Proteins associated with the epididymosomes and prostasomes, secreted by apocrine secretion and exocytosis, respectively, have been shown to alter sperm motility patterns, render protection to sperm against oxidative stress, modulate capacitation or decapacitation, function in ovum–sperm interaction, and modify the cholesterol:phospholipid ratio in the sperm plasmalemma. Prostasomes in particular, with their high cholesterol content, have been proposed to fuse with sperm and inhibit capacitation and the acrosome reaction [15].

Resident sperm no doubt interact with secretions, including macromolecules, from the SST epithelium, and such interactions contribute to prolonged sperm storage in the hen. Considering the multiple roles of shedding vesicles secreted by the mammalian epididymis and prostate gland in posttesticular sperm maturation, the following study examines the origin and secretion of SST epithelial cell microvillus blebs (MvBs) and their possible role in prolonged sperm survival in the hen's oviduct.

2. Materials and methods

The care, use, and procedures involving animals in this study were approved by the Beltsville Area Animal Care and Use Committee. A total of six Large White commercial turkey hens in egg production were used in this study. All hens were kept in single cages in an environmentally controlled house, exposed to a photoperiod of 14 hours of light and 10 hours of dark, and provided water *ad libitum*. Standard corn-based commercial breeder diets were provided to the hens (17% protein) and toms (14% protein). Hens were inseminated weekly with 300 million sperm for 7 or more weeks before tissue collection. At 42, 46, and 51 weeks of age, two hens were euthanized by cervical dislocation 24 hours after their last insemination, and the uterus and vagina were removed as one piece. Removal of the connective tissue enveloping the uterus and the coiled vagina exposed the UVJ, the site of the SSTs. The contiguous segments were then straightened and cut from the vagina through the uterus to expose the longitudinal mucosal folds of the vagina and UVJ [16]. To isolate UVJ folds (about 1.5–2 mm in both height and width) containing SSTs, an individual fold was grasped with fine forceps and then cut longitudinally through the lamina propria. Isolated folds about 10 mm long were then transferred to a Petri dish containing fixative and the SSTs were localized by stereomicroscopy (Nikon SMZ1500). Once localized, individual folds with SSTs were trimmed to about 2 to 3 mm in length, transferred to fresh fixative, and used for transmission electron microscopy (TEM). After samples for TEM were kept in fixative, additional unfixed UVJ folds containing SSTs were isolated as described previously, and a squash preparation was prepared and immediately examined by differential interference contrast microscopy (Zeiss Axioskop with the QICam digital camera using the NIS-Elements Software package).

Samples were fixed overnight at room temperature in either 2% glutaraldehyde in 0.1-M cacodylate buffer or 3.0% glutaraldehyde and 2.0% paraformaldehyde in 0.1-M cacodylate buffer. After fixation, the mucosal folds were washed in buffer and further trimmed to 3- to 4-mm pieces containing SSTs. All trimmed specimens were postfixed in 2% osmium tetroxide buffered in 0.1-M cacodylate. Samples were dehydrated in ethanol and embedded in Spurr Low-Viscosity Embedding Media (Polyscience). Thin sections

were cut on a Reichert A/O Ultracut microtome using a diamond knife (Diatome) and mounted on 400 mesh Ni grids. Sections were stained with 4% aqueous uranyl acetate (wt/vol) for 15 minutes followed by 3% aqueous lead citrate (wt/vol) for 5 minutes. Specimens were viewed with a Hitachi HT7700 TEM (Hitachi High Technologies America), and the images were captured with an AMT XR-41C 4 megapixel camera.

3. Results

3.1. General observations

The microvillus border of the SST epithelium was observed when unfixed squash preparations of UVJ mucosa containing SSTs were examined by differential interference contrast (Fig. 1A). Sperm in compact bundles were longitudinally aligned with their heads oriented toward the base of the SST and in close apposition to the SST microvilli. The 51-week-old hens had fewer SSTs containing sperm bundles compared to the SSTs from the 37- and 46-weeks-old hens but still contained sperm. When examined by TEM, no discernible differences were observed in the fine structure of the SST epithelia with respect to the type of fixative or the hen's age. Briefly, the turkey's SST epithelium was columnar (18–20 μm from the basement membrane to the apical tip of the microvilli), nonciliated, and generally lacked an accumulation of secretory granules observed in the pseudostratified columnar, ciliated, secretory epithelial cells lining the UVJ lumen (Fig. 1A, B). Complex lateral interdigitations between the adjacent cells were observed either tightly apposed or dilated revealing lateral cytoplasmic extensions in the intercellular spaces (Fig. 1B). Dilated intercellular spaces appeared both above and below desmosomes but not in a 2- to 3-μm apical zone that contained the tight junctions (Fig. 1B, C). Tight junctions were prominent and extended 1 to 2 μm deep into lateral borders of the cells. Desmosomes (Fig. 1C) were prevalent, and less frequently, hemidesmosome-like complexes were observed connecting adjacent SST epithelial cells.

Oval shaped nuclei dominated the basal half to third of the SST epithelial cells. Also, seen in this region of some cells were clusters of mitochondria and a few lipid droplets (Fig. 1A, B). Observed in the supranuclear cytoplasm were partially extracted lipid droplets, numerous elongated mitochondria with longitudinally oriented cristae, lysosomes, multivesicular bodies, and scattered rough endoplasmic reticula (Fig. 1C). Golgi and glycogen granules were rarely observed.

3.2. Apical zone of the SST epithelial cell and the SST lumen

For descriptive purposes, the apical zone consisted of the 2- to 3-μm zone of cytoplasm and the apical plasmalemma that formed the microvilli (Figs. 1A–C and 2A–C). Other than the junctional complexes and strands of rough endoplasmic reticula, the apical zone generally had few of the organelles described in the previous section. Variable numbers of both electron lucent and dense vesicles from 20 to 280 nm in diameter populated the apical zone (Fig. 2A–C).

The apical surface of the SST epithelial cells formed a dense array of microvilli that varied in height (0.8–2.0 μm) and width (0.120–0.160 μm). The core of the microvilli possessed scattered longitudinally oriented core filaments (Fig. 2A–D) that were associated or not with tufts of fine filaments immediately subjacent to the apical plasmalemma. Transversely oriented filaments typical of a terminal web were rarely observed. In addition to the core filaments, particles similar to that were observed in the apical cytoplasm, and 20 to 40 nm diameter vesicles were discernible in the core of the microvilli (Fig. 2A, B).

Whether sperm were present or not in the SST lumen, two types of microvilli were observed. One "inactive" type was relatively straight and capped with an electron dense material (Fig. 2D). The other "active" type of microvilli formed conspicuous MvBs of various sizes and shapes at their tips. This dense cap may or may not be observed on the surface of the MvB (Fig. 2D). Also observed with the active microvilli were subtle transverse constrictions along their length giving the microvillus a slight undulating appearance (Fig. 2A). Occasionally, the apical cytoplasm with associated microvilli jutted into the SST lumen (Fig. 2A).

The unilaminar MvBs appear to have began as dilations of the apical tip of the microvillus. One type of the MvB maintained a somewhat spherical-shaped appearance and enveloped a granular or flocculent material and possibly one or two larger vesicles (70–100 nm in diameter) or multiple smaller vesicles (20–40 nm in diameter) (Fig. 2A–D). The other type of MvB, which may be derived from the spherical MvB, appeared as a roughly oblong, irregular-shaped vesicle containing a fine flocculent material or a more granular ground substance (Fig. 2A–D). The central regions of the oblong MvBs often were partially empty with whatever contents remaining being relegated to the inside perimeter of the MvB (Fig. 2A–D).

Secretion of the spherical MvBs or their contents resulted either from a pinching-off of the MvB at a constriction on the microvillus immediately beneath the MvB or a rupture of the plasmalemma enveloping the MvB (Figs. 2A–D and 3A). On the basis of their appearance in the SST lumen, the oblong MvBs also appeared to pinch off the microvilli or they rupture. The luminal contents of the SSTs, presumably derived from the MvBs, varied considerably in appearance. Unilaminar spherical vesicles ranging in diameter from 100 to 250 nm (Figs. 3A, B) containing a fine granular or flocculent material may be derived from ruptured MvBs or from constrictions and release off the tip of the microvilli. Larger unilaminar spherical to elongated oblong vesicles (500 nm to 1 μm in length) that may or may not possess a flocculent material or a fine granular material were less frequently observed. Membrane fragments were also present in the SST lumen (Figs. 2A, B and 3A).

3.3. Sperm:MvB interaction

The heads and midpieces of sperm in the periphery of the sperm bundles observed in the SST lumen were often between and closely associated with the microvilli (Fig. 3A). While sperm were observed between microvilli, none had penetrated into or between the SST epithelial cells. The

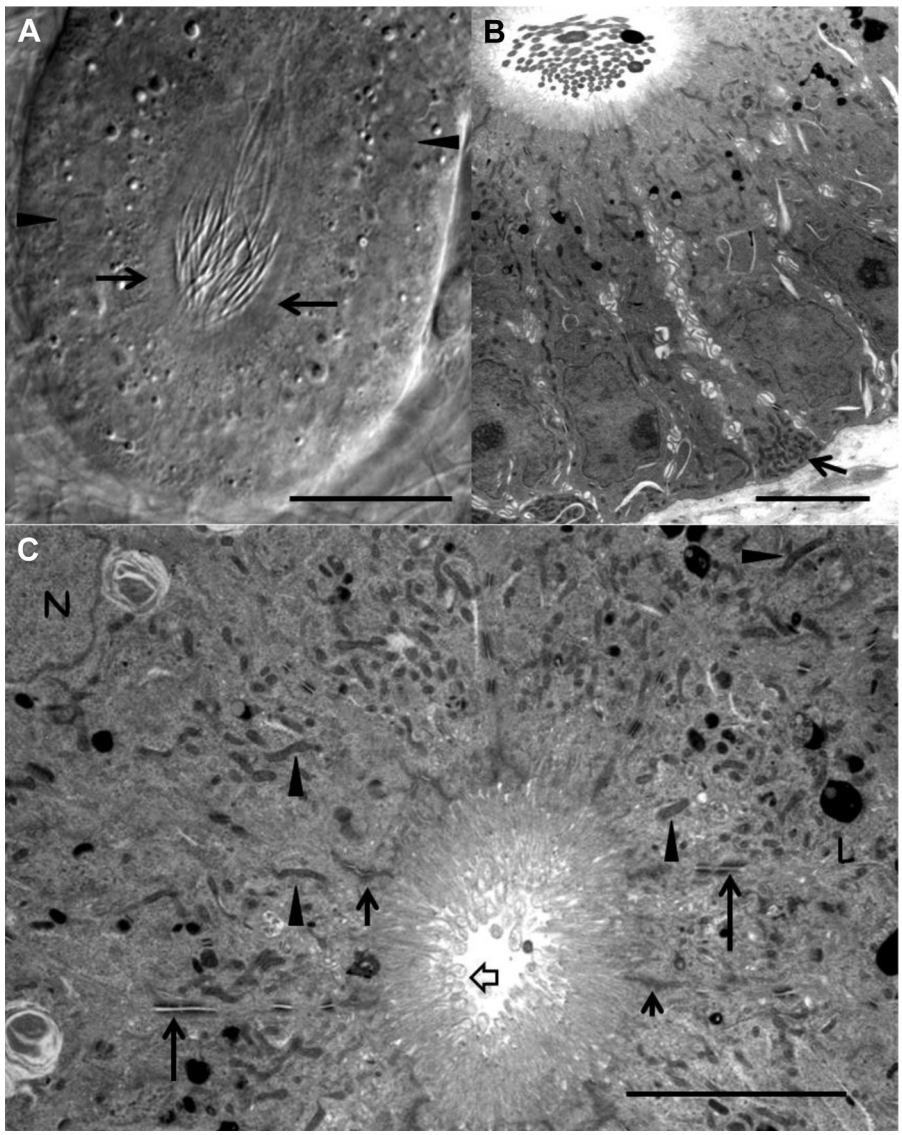

Fig. 1. (A) Observed is a differential interference contrast image of unfixed uterovaginal junction mucosa showing the distal end of a sperm storage tubule (SST) with sperm in its lumen. The cluster of sperm is aligned head to head oriented toward the base of the SST and is apposed to the apical microvilli (arrows). Profiles of nuclei (arrowheads) and numerous lipid droplets populate the SST epithelial cells (scale bar, 20 μm). (B) A partial cross section of an SST with resident sperm viewed by transmission electron microscopy (TEM) reveals wide intercellular spaces between the adjacent epithelial cells. Nuclei are typically in the basal half of the SST epithelium. Mitochondria are scattered throughout the SST epithelial cells and are frequently seen aggregated in the base (arrow) of these cells (scale bar, 5 μm). (C) The apical halves of several SST epithelial cells are observed by TEM. Microvilli form numerous microvillus blebs (arrow outline) that project into the SST lumen. Tight junctions (short arrows) and numerous desmosomes (long arrows) are evident as well as elongated mitochondria (arrowheads), lysosomes (L), partial nucleus (N), and lipid droplets containing swirls of partially extracted lipid (scale bar, 5 μm).

sperm plasmalemma was generally tightly apposed to the acrosomal membrane (Fig. 3B) but may be ruffled and distended overlying the nucleus and midpiece (Figs. 2B and 3A). Notwithstanding, at points along the length of the nucleus and midpiece, the plasmalemma of adjacent sperm was closely apposed (Fig. 3A). Membrane fragments, small spherical vesicles (30–130 nm in diameter), and oblong vesicles were associated with the sperm plasmalemma overlying the head and midpiece (Fig. 3A, B). Fusion of the small spherical vesicles with the sperm plasmalemma was rarely observed (Fig. 3C). However, convexities about 40 to 80 nm in length, possibly the result of small vesicle fusion with sperm, were observed seemingly contiguous with the plasmalemma overlying the head and midpiece of a small number of sperm (Fig. 3A).

4. Discussion

The question of whether MvB formation and release were artifacts of fixation was raised by Schuppin et al. [13] and Bakst et al. [8]. Other than the partial extraction of lipid from the large lipid droplets, there was no evidence of

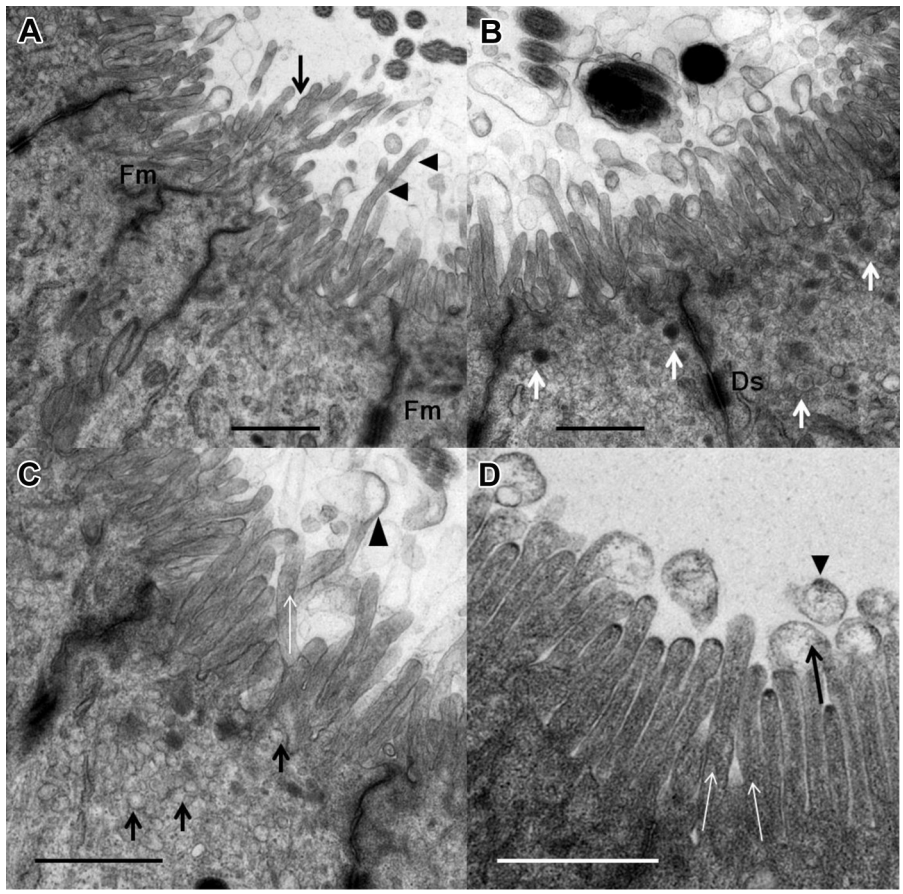

Fig. 2. (A) Microvilli of varying lengths have formed both spherical and oblong microvillus blebs (MvBs). Slight constrictions (arrowheads) of an elongated microvillus are observed as is a projection of the apical cytoplasm and accompany microvilli (arrow). Scattered filaments in the core of the microvilli and tufts of fine filaments (Fm) are observed. Relatively few vesicles are observed in the apical cytoplasm. Dense tight junctions and scattered rough endoplasmic reticula are present in the apical zone. Cross sections of sperm tails as well as empty unilaminar oblong vesicles and spherical vesicles containing a flocculent material are in the sperm storage tubule lumen (scale bar, 1 μm). (B) This micrograph is similar to A, but the apical zone contains numerous electron dense vesicles and sparse numbers of electron opaque vesicles (arrows). Tight junctions (zonula occludens), intermediate junctions (zonula adherens), and a desmosome (Ds) are observed (scale bar, 1 μm). (C) The apical zone contains a few electron dense vesicles and numerous electron opaque vesicles (black arrows). Within the microvillus core are small vesicles (white arrow). One side of a spherical MvB appears to be dilated and about to rupture (arrowhead; scale bar, 1 μm). (D) Inactive microvilli possess a dense cap on their apical tips. Active microvilli forming spherical MvBs contain an amorphous cytoplasmic substance that may include a large single vesicle or smaller vesicles (black arrow). A dense cap may or may not be associated with the MvBs (arrowhead). Small vesicles are observed in the core of several microvilli (white arrows; scale bar, 1 μm).

fixation artifacts in the SST epithelial cells, i.e., mitochondria were elongated and contained a homogenous matrix with longitudinal cristae. Furthermore, more recent publications had addressed the formation and multiple roles of shedding microvesicles from different cells in general [17], the mammalian epididymal and prostatic epithelial cells [14], and the intestinal epithelial cell microvilli in particular [18]. Finally, if MvB formation was an artifact, it would be unlikely that active microvilli and inactive microvilli would be observed adjacent to each other and exhibit subtle transverse constrictions along their lengths. Such constrictions have been reported in the vesicle forming brush border microvilli in the chicken enterocytes [19] and the vesicle-generating microvilli of epithelial cells grown *in vitro* [20].

Therefore, on the basis of aforementioned publications and observations reported herein, it is concluded that SST epithelial cells do actively form and secrete MvBs. Using Cocucci's et al. [17] classification of types of small vesicles shed by apocrine secretion from the surface of various cells, the MvB would be classified as a variation of a "shedding vesicle" as it buds directly from the plasma membrane into the extracellular space.

Similar type of vesicles released by epididymosomes and prostasomes in humans, and other mammals have been shown to be rich in proteins and lipids. As a result of their fusogenic properties, epididymosomes and prostasomes are capable of transferring such macromolecules to sperm thereby altering the sperm's cholesterol:phospholipid ratio, sperm function relative to their maturation, motility properties, and antioxidant capabilities [14,21,22]. Prostasomes in particular, with their high cholesterol content, have been proposed to inhibit capacitation and the acrosome reaction [15].

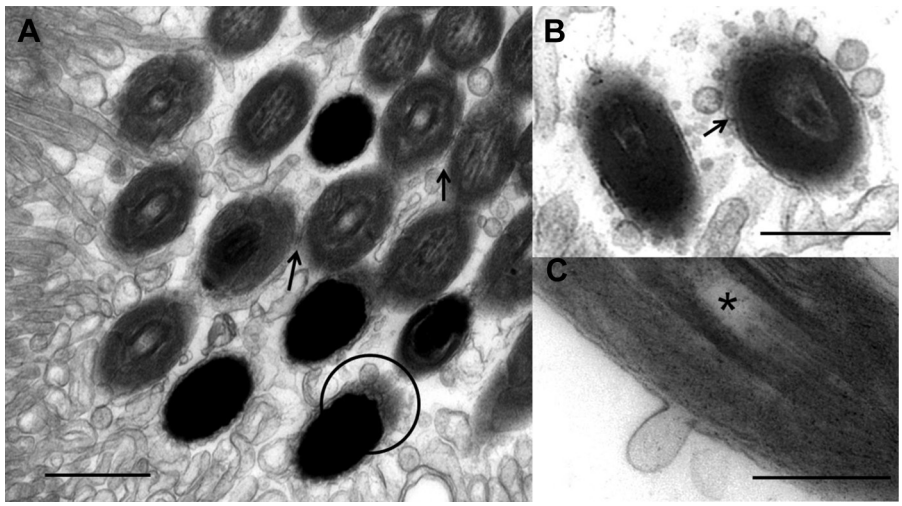

Fig. 3. (A) Microvilli and/or secreted microvillus blebs and membrane fragments are closely associated with a partial bundle of sperm in the SST lumen. The plasmalemma of adjacent sperm is closely apposed (arrows). Intact vesicles ranging from 90 to 250 nm are also observed closely associated or possibly fused (within circle) with the sperm plasmalemma (scale bar, 1 μm). (B) Vesicles ranging from about 30 to 130 nm in diameter are observed closely associated with the plasmalemma overlying the acrosome of two sperm. Several vesicles appear to have fused with the plasmalemma overlying one of the acrosomes (arrow; scale bar, 500 nm). (C) A small spherical vesicle is observed fused with the sperm plasmalemma overlying its midpiece at the level of the distal centriole (*; scale bar, 200 nm).

How sperm survive within the SSTs and retain their fertilizing capacity has been addressed by several authors [2,3,8,10,23]. On the basis of observations of these authors, what is known about the structure and function of epididymosomes and prostasomes, and observations from this study, it is proposed that SST MvBs have three primary functions that include serving as a source of metabolic substrates used by resident sperm, serving as a source of macromolecules that alter sperm functions associated with fertilization (decapacitation factors?), conferring protection against oxidative stress, and acting as transport vesicles moving fluid from the SST epithelial cells to the SST lumen.

Froman [10] stated that endogenous long-chain fatty acids fuel sperm motility within the vagina, whereas exogenous long-chain fatty acids support sperm motility within the SST lumen. Within the SST, clusters of sperm have been observed in a relatively slow synchronous movement [8], presumably to maintain their position against a fluid flow directed toward the SST orifice [10]. This fluid may be generated by aquaporins that have been immunocytochemically localized in the apical zone of SST epithelial cells [24] and by the release of the contents of MvBs within the SST lumen. Given the tight junctional complexes and a prominent band of actin filaments in the SST epithelial cell apical zone that circumscribes the SST lumen [25], it is unlikely that fluid secretion is *via* an intercellular route.

Pons-Rejraji et al. [15] incubated human sperm under capacitation conditions with prostasomes and reported that sperm plasma membrane integrity improved and that markers indicative of sperm capacitation decreased concurrently with the numbers of capacitated and acrosome-reacted sperm. Considering that chicken and quail sperm neither require a period of capacitation to hydrolyze the IPL nor to fertilize an ovum *in vitro*, one would expect a cellular or molecular process within the SSTs that would render resident sperm functionally quiescent. It is suggested that macromolecules associated with the MvBs interact with sperm residing in the SSTs and achieve a decapacitated state. The MvBs described in the present study may stabilize the plasmalemma of SST sperm in a manner similar to the human prostasomes by augmenting the cholesterol:phospholipid content and altering the phospholipid content of the sperm plasmalemma in a manner similar to that described by Pons-Rejraji et al. [15].

This study reports MvBs are not artifacts of specimen preparation and, in fact, are prevalent in the turkey SST. It is suggested that the MvBs promote prolonged survival within the SSTs either by fusing with resident sperm or by some mechanism, the secretory products released by the MvBs are incorporated on or into the resident sperm rendering them to a decapacitated state until sperm egress from the SST.

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