Lectin staining of the uterovaginal junction and sperm-storage tubule epithelia in broiler hens

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ABSTRACT Mammalian sperm bind to terminal carbohydrates associated with glycoconjugates on the apical surface of oviduct epithelial cells in the caudal region of the oviduct and undergo cellular and molecular modifications associated with capacitation prior to ovulation. In contrast, chicken sperm are stored for up to 23 d in sperm-storage tubules (SST) localized in the uterovaginal junction (UVJ). Little is known of the cellular and molecular mechanisms that regulate sperm storage in and release from the SST. The purpose of this study was to identify glycoconjugates associated with the SST epithelial cell surface using lectins. Virgin hens and hens of higher and lower fertility in egg production for 6 to 16 wk were used in this study. Sections of UVJ mucosa containing SST were stained with fluorescent conjugated lectins and examined by confocal microscopy. Carbohydrate moieties associated with the UVJ and SST epithelia differed in their lectin binding patterns. No differences in the lectin binding patterns within the 2 epithelia were discernible between the virgin and younger and older hens. Minor differences were observed between the higher and lower fertility hens. Only lectins specific for galactose and N-acetylgalactosamine moieties were localized to the luminal surface of the SST. While resident sperm may be closely apposed to the SST epithelial cell apical microvilli, it is unlikely that sperm binding to the microvilli via terminal carbohydrates associated with glycoconjugates is a requisite for prolonged storage. However, the possibility of SST epithelial cell communication with resident sperm via shedding microvillous vesicles characterized by surface glycoconjugates with terminal galactose and N-acetylgalactosamine moieties is currently being investigated.

Key words: oviduct, sperm storage, broiler breeder, glycoconjugates

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INTRODUCTION

Female birds are capable of prolonged sperm storage following mating or artificial insemination. Following semen transfer, there is an intense sperm selection process within the vagina. The resulting select population of sperm is transported in an adovarian direction to the utero-vaginal junction (**UVJ**) where it enters discrete tubular invaginations of the surface epithelium, the sperm-storage tubules (**SST**). Over the subsequent d or wk, sperm residing in the SST are gradually released from the SST, ascend to the anterior end of the oviduct, and interact with next ovulated ovum. Our current understanding of the possible roles of the SST epithelium, its surrounding loose connective tissue cells, and sperm behavior relative to sperm storage in and release from the SST has been reviewed by Bakst (1993, 2011), Das et al. (2008), and Froman (2003), respectively.

In mammals, glycoconjugates are integral in establishing a sperm reservoir by the selective binding of sperm to the apical surface of the epithelial cells in the isthmus portion of the oviduct [see reviews by Suarez (2008) and Talevi and Gualtieri (2010)]. Ignotz et al. (2001) described 3 functions for the sperm reservoir: to ameliorate the possibility of polyspermy by permitting only relatively few sperm to reach the site of fertilization around the time of ovulation; to sustain sperm viability and fecundity in the interval between semen transfer to the female and ovulation; and to facilitate sperm capacitation and hyperactived motility.

Glycoconjugates associated with the chicken sperm plasmalemma are involved in both sperm selection in and adovarian transport through the vagina to the UVJ (Froman and Engel, 1989; Steele and Wishart, 1996) and in sperm interaction with the inner perivitelline layer (Robertson et al., 2000). Chicken sperm adjacent to the SST epithelium have been described as being closely apposed (Tingari and Lake, 1973) or binding to the SST epithelium (Ahammad et al., 2011). Schuppin et al., (1984) commented that only when the turkey's SST lumen was packed with sperm did they contact the tips of the SST microvilli. Yet, using

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transmission electron microscopy, Bakst and Bauchan (2015) recently observed turkey sperm heads between the microvilli and apposed to the apical portion of the SST epithelial cell microvilli. What has yet to be determined is if this close apposition between sperm and the SST epithelial cells described by the above authors is mediated by carbohydrate-containing glycoproteins and/or glycolipids or are merely apposed due to dense packing of sperm within the SST (Tingari and Lake, 1973). Interestingly, Ashizawa and Nishiyama (1983) co-cultured sperm with tissue explants from different regions of the chicken oviduct and noted "... a close association of spermatozoa with the epithelial cells [SST] was observed, compared with tissue from the other regions." Therefore, the objective of this study was to identify carbohydrate moieties associated with the apical surface of the SST. These observations will serve as baseline information for future studies examining sperm:epithelial cell interactions in the SST lumen. In addition, the distribution of carbohydrate moieties associated with the UVJ and SST epithelia was also described.

MATERIALS AND METHODS

Animals

Ross 308 broiler breeders (Aviagen Inc, Huntsville, AL) were raised under recommended husbandry conditions. At 20 wk of age broilers were placed in individual cages in an environmentally controlled poultry facility at the Beltsville Agricultural Research Center and photo-stimulated (14 h light:10 h dark photoperiod) at 21 wk. Hens were initially inseminated at 24 wk of age and inseminated weekly thereafter with 300 million sperm. Eggs were collected daily, stored in an 18°C walk-in cool room, incubated weekly, and true fertility determined at the time of egg candling. For this study, high fertility was 90% or higher and low fertility below 80%. The care and use of the broilers as described in this study were approved by the Beltsville Area Animal Care and Use Committee.

Tissue Collection and Histology

Hens were euthanized by cervical dislocation after 6 (n = 5), 11 (n = 4), 14 (n = 8), and 16 (n = 7) wk of egg production and the uterus and vagina excised as one segment. An additional group of 6 virgin hens were euthanized in the sixth wk of egg production. The connective tissue enveloping the posterior uterus and vagina was removed, the UVJ was exposed, and the SST localized by stereomicroscopy (Nikon SMZ1500, Brighton, MI) (Bakst, 1992). Two to 4 samples of UVJ mucosa containing SST were isolated from each hen and placed in 10% neutral buffered formalin (NBF). After 24 to 48 h fixation, samples were transferred to 70% (v/v) ethanol for 2 changes at 30 min each, moved to an automated system tissue processor (Tissue Tek VIP, Sakura Finetek, Inc., Torrence, CA), and then embedded in paraffin (Paraplast® X-tra, Leica Microsystems Richmond, Inc., Richmond, IL). Four to 6 sections, 4-μm thick, were collected in sequence onto slides, air-dried on a warming tray at 37°C, and stored at room temperature (**RT**) until staining. Staining consisted of hematoxylin and eosin (H&E; Llewellyn, 2009), PAS reaction (Bancroft and Stevens, 1982), and lectin histochemistry (Apichela et al., 2010). A Zeiss Axioskop (Thronwood, NY) equipped with a QICam digital camera operated with NIS Elements Software (Nikon Instruments, Melville, NY) was used for light and fluorescence microscopy.

Lectin Histochemistry

The lectins (Vector Laboratories, Burlingame, CA), their binding carbohydrate moiety, fluorescent conjugate, concentration, and respective carbohydrate inhibitors used in this study are summarized in Table 1. With each set of slides processed, 2 negative controls were prepared: one slide was incubated without a lectin to account for auto-fluorescing cells and another slide was incubated with a lectin and its inhibiting sugar for least 40 min.

Lectin labeling was performed according to Apichela et al. (2010) with slight modifications. Briefly, slides were deparaffinized (2 × 10 min in xylene) followed by rehydration in a decreasing alcohol series (2 × 5 min 100% ethanol, 2 min each 95% and 70% ethanol) followed by several dips in deionizer water to remove excess ethanol. Slides were incubated 10 min in 10 mM Tris-HCl buffered saline (pH 7.4) supplemented (TBS+) as needed (Table 1) with 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, 0.01 mM $MgCl_2$, or 0.1 mM $ZnCl_2$. The TBS+ tissues were then labeled with a single lectin at optimized concentrations (Table 1) for 35 to 60 min; the slides were washed 3 × 10 min using TBS+, and then washed again for 3 min in deionized water to remove excess salts. This procedure was repeated for dual lectin labeling. Cover slips were applied with Prolong Gold (Life Technologies Corp., Grand Island, NY) mounting media. Slides were protected from light after the fluorescence conjugated-lectin labeling had begun. Slides were stored in the dark at RT for 24 h to allow the mounting medium to cure before sealing the edges of the cover glass with nail polish for longer storage at 4°C for observation and imaging. Imaging was completed within a wk of labeling. Sections were either examined using a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY) or by laser scan confocal microscopy using an Axio Observer LMS 780 (Carl Zeiss Microscopy GmbH, Thornwood, NY) with Zen 2012 64-bit image acquisition and processing software. Lectin labeling intensity was visually scored as negative (−) with no fluorescence, (+/−) with inconsistent fluorescence with the same cell type,

Table 1. Lectin conjugates[a] grouped by preferred binding carbohydrate moiety, lectin concentration, and inhibitor used.

Lectin, common name, abbreviation	Concentration (μg/mL)	Labeling time (min)	TBS supplement ion[b]	Inhibitors
Fucose binding lectin				
Lotus tetragonolobus, Winged pea, LTL	1.2	60	Ca^{++}, Mn^{++}	L-fucose
Galactose/N-acetylgalactosamine binding lectins				
Erythrina cristagalli, Coral tree, ECL	1.25	60	Ca^{++}, Mn^{++}, Zn^{++}	Lactose
Arachis hypogeae, Peanut, PNA	1.25	60	Ca^{++}, Mg^{++}	Galactose
Psophocarpus tetragonolobus, Winged bean, PTL	1.25	35	–	N-acetylgalactosamine
Ricinus communis, Castor bean, RCA-II	1.0	60	–	Galactose; lactose
Glycine max, Soybean, SBA	1.25	60	Ca^{++}, Mn^{++}	N-acetylgalactosamine
Wisteria floribunda, Japanese wisteria, WFL	1.0	60	–	N-acetylgalactosamine, acetic acid
Mannose binding lectin				
Canavalia ensiformis, Jack bean, Con-A	1.0	60	Ca^{++}, Mn^{++}	α-methylmannoside/α-methylglucoside mixture
N-acetylglucosamine binding lectins				
Griffionia (Bandeiraea) simplicifolia, GSL-II	1.0	60	Ca^{++}, Mn^{++}	N-acetylglucosamine
Triticum vulgaris, Wheat germ, WGA	1.25	60	Ca^{++}	N-acetylglucosamine with acid or salt N-acetylglucosamine with acid or salt
N-acetylneuraminic acid binding				
Sambucus nigra, Elderberry bark, SNA	1.0	35	–	Lactose with acetic acid

[a]All lectins were conjugated with fluorescein (FITC) except SBA and Con-A which were conjugated with rhodamine (TRITC).
[b]Tris-buffered saline (10 mM TBS, pH 7.4) was supplemented as needed using 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.01 mM $MnCl_2$, and/or 0.1 mM $ZnCl_2$.

positive (+) when fluorescence was clearly discernible, and (++) with bright, consistent fluorescence.

RESULTS

Although the UVJ surface epithelium is pseudostratified consisting of columnar secretory and ciliated cells, the SST, which originate as tubular invaginations of the UVJ surface epithelium, are lined by a non-secretory, non-ciliated, columnar epithelium. Loose connective tissue enveloping the SST and subjacent to the UVJ epithelium contains various cell types and connective tissue fibers (Figures 1a, b). The SST are primarily localized to the upper portion of the mucosal folds but are occasionally observed in the basal mucosa connecting adjacent folds (Figure 1b). Sperm clusters within the SST, densely packed groups of sperm aligned nearly parallel to the long axis of the SST with their heads oriented toward the base of the SST, were sporadically observed in the hens examined (Figure 1a).

Lectin binding affinities for the epithelia lining the UVJ and SST are summarized in Table 2. Because relatively few sperm were observed in the SST, sperm lectin binding affinities were not included in this paper. No differences in the lectin binding patterns were discernible between the virgin and inseminated younger and older hens, and only minor differences limited to ECL (see Table 1 for lectin abbreviations) were observed between the higher and lower fertility hens (addressed below). Differences in lectin binding between the apical surfaces of the 2 epithelia were evident. N-acetylglucosamine containing glycoconjugates, identified with GSL-II and WGA, had nearly identical binding characteristics. There was strong binding of WGA to the apical surfaces of both the UVJ and SST epithelia (Figures 1a, 2a–d). In top-down views of the apical surface of the SST lumen, WGA appeared to bind specifically to the SST apical microvilli (Figure 2b). GSL-II binding was limited to the SST epithelial cell cytoplasm and intermittent binding to the apical surface of the SST and the cytoplasm of the UVJ epithelium (Figure 2c).

Evidence of galactose and N-acetylgalactosamine containing glycoconjugates varied with the 6 lectins examined (Table 2) and, to some extent, between the higher and lower fertility hens. WFA binding to the SST epithelial cells was similar to that of WGA except the SST microvilli did not uniformly bind WFA with the same intensity as WGA (Figure 3a–c). Binding of ECL (Figure 3d) and SBA (Figure 2c,d) to the apical surface of the SST epithelial cells were either negative or positive suggesting a differential expression of galactose and N-acetylgalactosamine moieties by individual SST epithelial cells. WBA-I bound to the apical surface and to a lesser extent the basement membrane of the UVJ epithelial cells but like LTL, did not bind to the SST epithelial cells. Only ECL showed slight but detectable differences in binding patterns between higher and lower fertility hens. In higher fertility hens, ECL bound intermittently to the SST microvilli with some SST epithelial cells strongly fluorescent and others negative (Figure 3d). ECL did not bind to the SST microvilli in the lower fertility hens and failed to bind to the apical surface of the UVJ epithelium from higher and lower fertility hens.

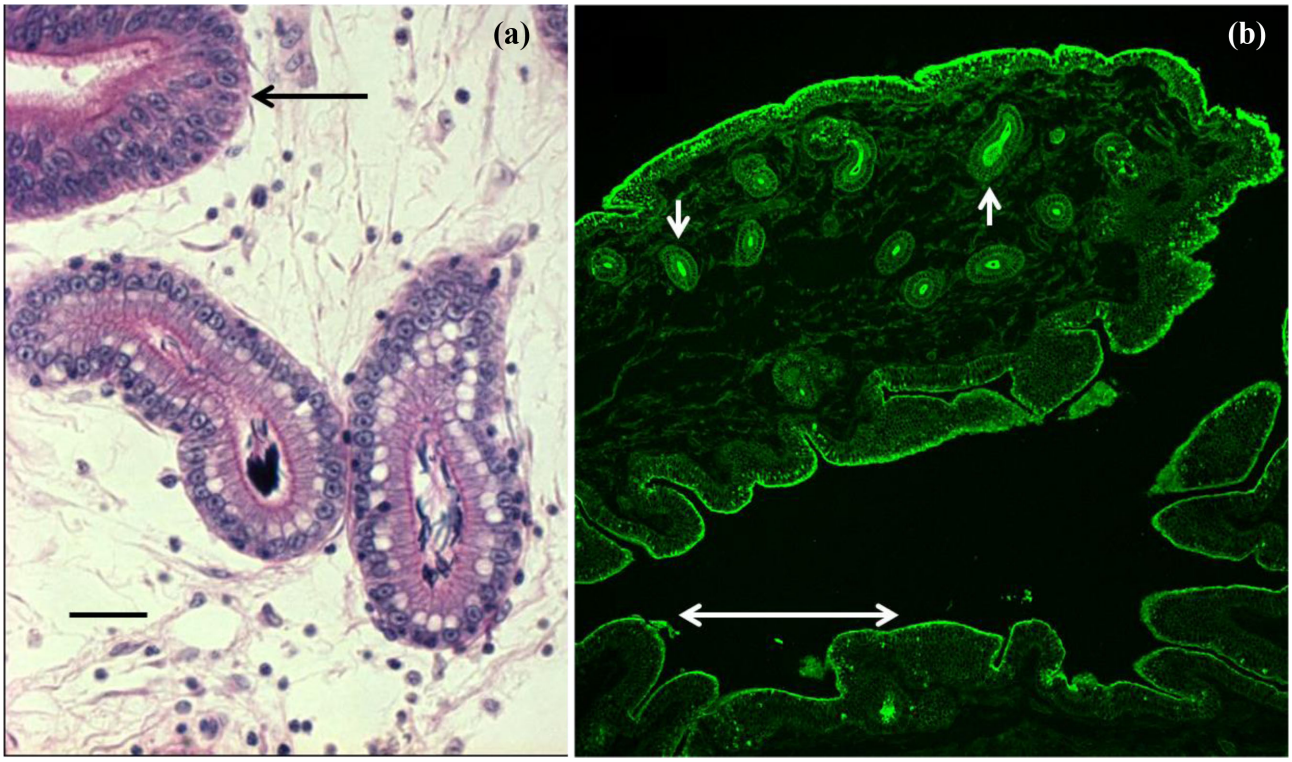

Figure 1. (a) A light micrograph of the uterovaginal junction (UVJ) mucosa reveals the pseudostratified, columnar, ciliated epithelium (arrow) lining the UVJ lumen and the underlying loose connective tissue and cells. Cross sections of 2 sperm-storage tubules (SST) reveal the non-ciliated, columnar epithelium, with basally located nuclei cells and clear vacuole-like structures in the supranuclear cytoplasm, a remnant of lipid droplets that were extracted during tissue preparation. A sperm-bundle is observed in the left SST and more dispersed sperm are observed in the lumen of the SST on the right. Bar = 20 μm (b) The apical surfaces of the epithelia covering both the UVJ mucosal fold, the space between the mucosal folds (double-headed arrow), which typically lack SST, and the epithelium lining the SST (arrows) are strongly positive for WGA. Double headed arrow = 234 μm.

Mannose containing glycoconjugates were identified with Con-A (Table 2) and were most consistently positive in the loose connective tissues and cells in the SST submucosa. In the 2 epithelia examined, Con-A binding was inconsistent or positive and was found to provide background contrasting other lectins when performing dual staining (Figures 3a, b, d) Glycoconjugates containing N-acetylneuraminic acid moieties were localized with SNA (Table 2). SNA was distributed throughout the UVJ epithelial cells, absent from the lateral cell membranes of the SST epithelium, and intensely bound to the SST apical microvilli. While LTL (fucose affinity) was localized to the cilia on the epithelial cells of the UVJ, LTL failed to bind either to the apical surface, cytoplasm, or the lateral or basement membranes of the SST epithelial cells (Table 2).

DISCUSSION

Intra- and extracellular carbohydrate moieties associated with glycoconjugates of the UVJ and SST epithelia were identified, and differences in lectin binding patterns between the 2 epithelia were observed. However, no differences in the lectin binding patterns within the 2 epithelia were discernible between the virgin and younger and older inseminated hens, and only minor differences were observed with ECL binding in the SST epithelium between the higher and lower fertility hens. Differential binding with both WFA and ECL on the apical surface of contiguous SST epithelial cells suggests that SST epithelial cells do not uniformly synthesize glycoconjugates with terminal carbohydrate moieties specific for these lectins.

Three lectins (ECL, SBA, and WFL) with affinities for carbohydrate structures containing galactose and N-acetylgalactosamine and one lectin (WGA) with an affinity for carbohydrate structures containing N-acetylglucosamine were strongly associated with the microvilli on the SST apical surface. SNA, with specificity for N-acetylneuraminic acid, revealed minimal binding to the apical surface of the SST. Indirectly, this would support observations by Steele and Wishart (1996). These authors incubated chicken sperm with neuraminidase, inseminated hens with the neuraminidase treated sperm, and subsequently found few sperm in the SST. Yet, when neuraminidase-treated sperm were co-incubated with UVJ-SST explants, sperm were observed in the SST. Therefore, it can be assumed that terminal neuraminic acid in glycoconjugates on the surface of the sperm plasmalemma is not required for sperm binding to the apical microvilli on SST epithelia cells. A similar study in which

Table 2. Lectin labeling[a] in chicken UVJ and SST grouped by preferred oligosaccharide moiety.

	UVJ			SST			
Preferred sugar lectin[b]	Apical surface	Cytoplasm	Basement membrane	Basement membrane	Cytoplasm	Lateral membranes	Apical surface
Fucose							
LTL	+	−	+/−	+/−	−	−	−
Galactose / N-acetylgalactosamine							
ECL	−	−, +/−	+/−, +	+/−	−, ++	−, +	+/−, ++
PNA	+/−	+/−	+/−	+/−, +	+	+/−, +	−, +/−
RCA-II	+/−	++	+	+	+/−	+/−, +	+/−
SBA	+	++	+/−	+/−	+	+/−	+/−, ++
WBA-I	+	−	+/−	+/−	−	−	−
WFL	−	−	−	+/−, ++	++	+/−, ++	++
Mannose							
Con-A	+/−	+/−, +	−	−, +/−	+/−, +	+	−, +/−
N-acetylglucosamine							
GSL-II	+/−	+/−	−, +/−	−, +/−	++	−	+/−
WGA	++	+	−, +/−	+/−, ++	+	−, +/−	++
N-acetylneuraminic acid							
SNA	+/−	+/−	+	−, +/−	+/−, +	−/+	−

[a]Lectin labeling intensity was visually scored as negative (−) with no fluorescence, (+/−) with inconsistent fluorescence with the same cell type, positive (+) when fluorescence was consistently discernible, and (++) with intense, consistent fluorescence.
[b]See Table 1 for full lectin names.

galactose and N-acetylgalactosamine moieties will be enzymatically removed from the chicken and turkey sperm plasmalemma and then artificially inseminated and co-incubated with UVJ mucosa containing SST is planned. This will demonstrate the role, if any, of these carbohydrates in sperm binding to the SST epithelium and sustained hen fertility. Galactose and N-acetylgalactosamine have been shown to be involved in sperm binding to the epithelial cells lining the isthmus in the llama (Apichela et al., 2010) and pig (Talevi and Gualtieri, 2010).

The cellular and molecular interactions between the SST epithelium and resident sperm remain largely unknown. While Ahammad et al. (2011) suggested that chicken sperm acquire the ability to bind to the SST epithelium in a specific ligand-receptor interaction during passage through the male reproductive tract, the significance of such binding relative to sperm storage and sustained viability can only be speculated. As suggested for mice (Fazeli et al., 2004), sperm bound to the SST epithelium may initiate signal transduction pathways that could modify the gene expression of the SST epithelial cells. This possibility is currently being explored in this laboratory as the transcriptome profiles from SST epithelial cells isolated from virgins, higher fertility, and lower fertility broilers are being deciphered.

Bakst (1992) reported that the sperm in a sperm-bundle exhibit a synchronized, slow, undulating movement in squash preparations of UVJ mucosa while dispersed sperm in the SST lumen do not. The signaling mechanisms synchronizing the sperm undulation between dozens to hundreds of sperm is not known. Tingari and Lake (1973) reported that within a sperm-bundle, the plasmalemma overlying the sperm heads of adjacent and parallel sperm appeared to adhere. They referred to this as sperm agglutination. Schuppin et al. (1984) also adapted this terminology in describing apposed plasma membranes of adjacent turkey sperm in a sperm bundle. However, this is a misleading term as there has been no evidence to date suggesting that this is an immunological based agglutination of sperm, but rather tightly apposed sperm membranes. More recently, Bakst and Bauchan (2015) observed that within sperm-bundles in the turkey SST the sperm plasmalemma overlying the nuclei and midpieces were ruffled except in areas where the 2 plasma membranes were tightly apposed. Though not resembling a cell junction, the tightly apposed plasma membranes of adjacent sperm may function in sperm-to-sperm communication, particularly in the synchronization of the slow, undulating movement of sperm in a parallel alignment within a sperm-bundle.

The possibility exists that sperm binding to the SST microvilli via a carbohydrate ligand has little, if any, role in prolonged oviductal sperm storage. Unlike mammals where each sperm binds to an epithelial cell in the isthmus, sperm more centrally located in a sperm-bundle do not appear to have direct contact with the SST epithelium. Considering that an estimated 392 chicken sperm could be packed in a sperm-bundle within a SST (Zavaleta and Ogasawara, 1987) it is apparent that only those sperm adjacent to the SST epithelium would bind to and communicate with the SST.

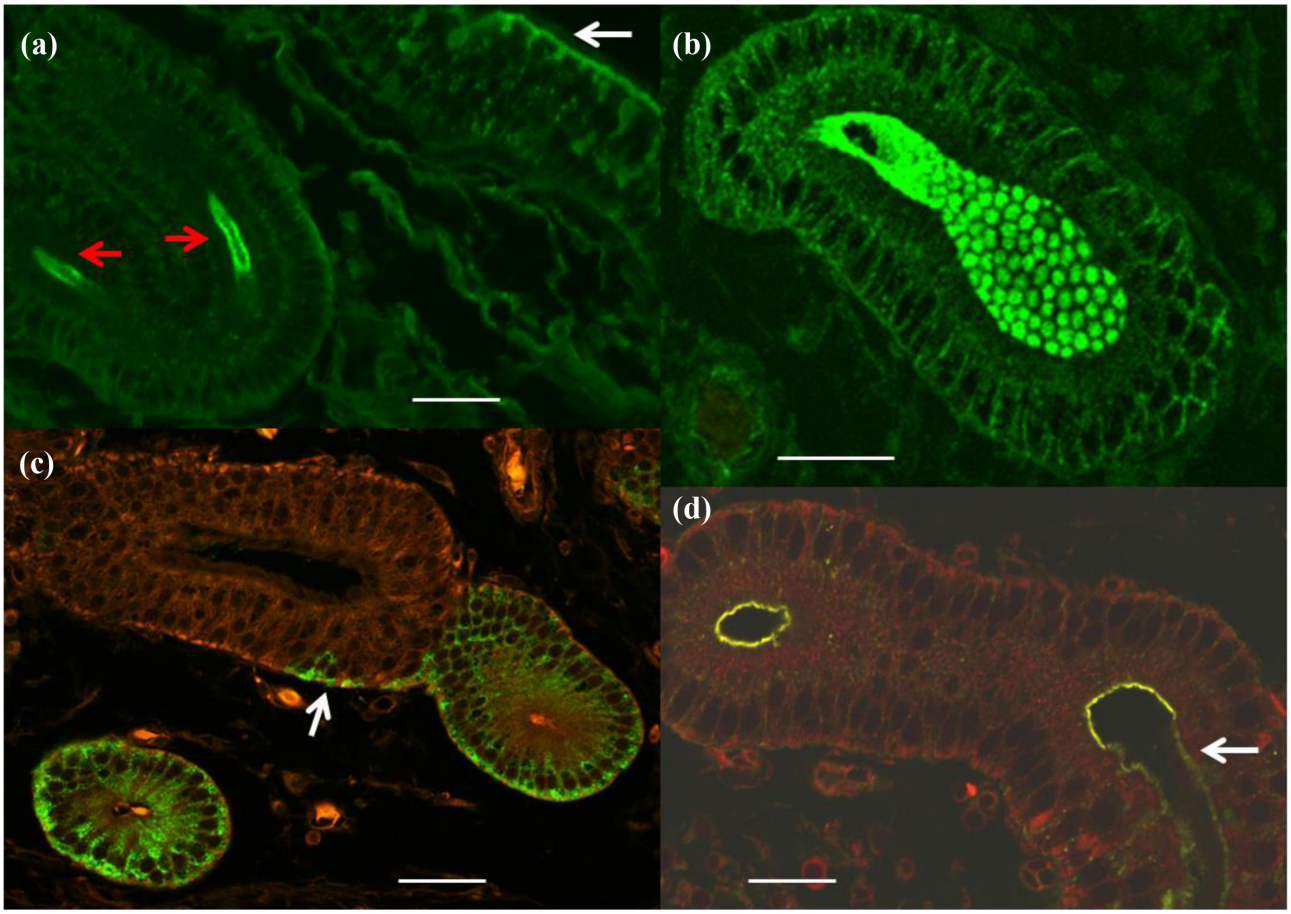

Figure 2. N-acetylglucosamine moieties were observed with the lectins WGA and GSL-II and galactose and N-acetylgalactosamine moieties were observed with SBA. All micron bars are 20 μm. (a) The apical surface and cytoplasm in the UVJ epithelium (white arrow) were positive for WGA. The SST microvilli projecting into the lumen of the SST (red arrows) also bound WGA. (b) A Z-stack image shows intense WGA binding associated with the SST apical microvilli (cobblestone like appearance) and less intense binding on their lateral borders. (c) In this dual stained image GSL-II binding is limited to the SST epithelial cell cytoplasm (green) and one or 2 cells in the UVJ epithelium (arrow). SBA uniformly binds to the UVJ epithelial cells and to a lesser extent the apical cytoplasm and microvilli in the SST epithelium. (d) In this dual stained image both WGA and SBA binding with the SST microvilli had merged producing the yellow fluorescence. WGA (green) is just discernible on the apical surface of the UVJ epithelium WGA is less intense on the (arrow). The SST and UVJ epithelia lightly bind SBA (red).

Froman (2003) proposed a model describing sperm storage in and their release from the SST that is uniquely a function of sperm motility and does not involve direct communication with the SST epithelium. Briefly, Froman's model entails sperm entering the SST and remaining in the SST as long as their swimming velocity is such that it allows them to maintain their position in the SST against a fluid current generated from the base of the SST to its orifice. Sperm are carried out of the SST when their swimming velocity falls below that of the SST fluid flowing toward the UVJ lumen. While Froman's model of sperm storage does not include a role for sperm binding to the SST epithelium, it does provide some insight into the formation of sperm-bundles and the subsequent dispersion of sperm in the SST. As described by Froman (2003), sperm enter the SST with a swimming velocity that exceeds the fluid current flowing out of the SST. It is proposed here that these sperm traverse the SST until they aggregate at the base of the SST lumen. Sperm continue to align parallel to the long axis of the SST with their heads toward the base of the SST, forming a slowly undulating sperm-bundle. This sperm-bundle is maintained until the swimming velocity of individual sperm decreases to that of the fluid current moving through the SST lumen. Those sperm with reduced velocity become disengaged from the bundle and are dispersed within the SST or vented from the SST. If sperm are subject to signals from the UVJ epithelium it may be through the fluid originating from the SST epithelium or microvillous blebs generated on and released from the apical tips of SST epithelial cells (Bakst and Bauchard, 2014).

To conclude, galactose and N-acetylgalactosamine moieties associated with glycoconjugates on the apical surface of the SST epithelium have been described. Whether these or other yet to be identified SST surface glycoconjugates play a role in the storage in and release of sperm is currently being investigated.

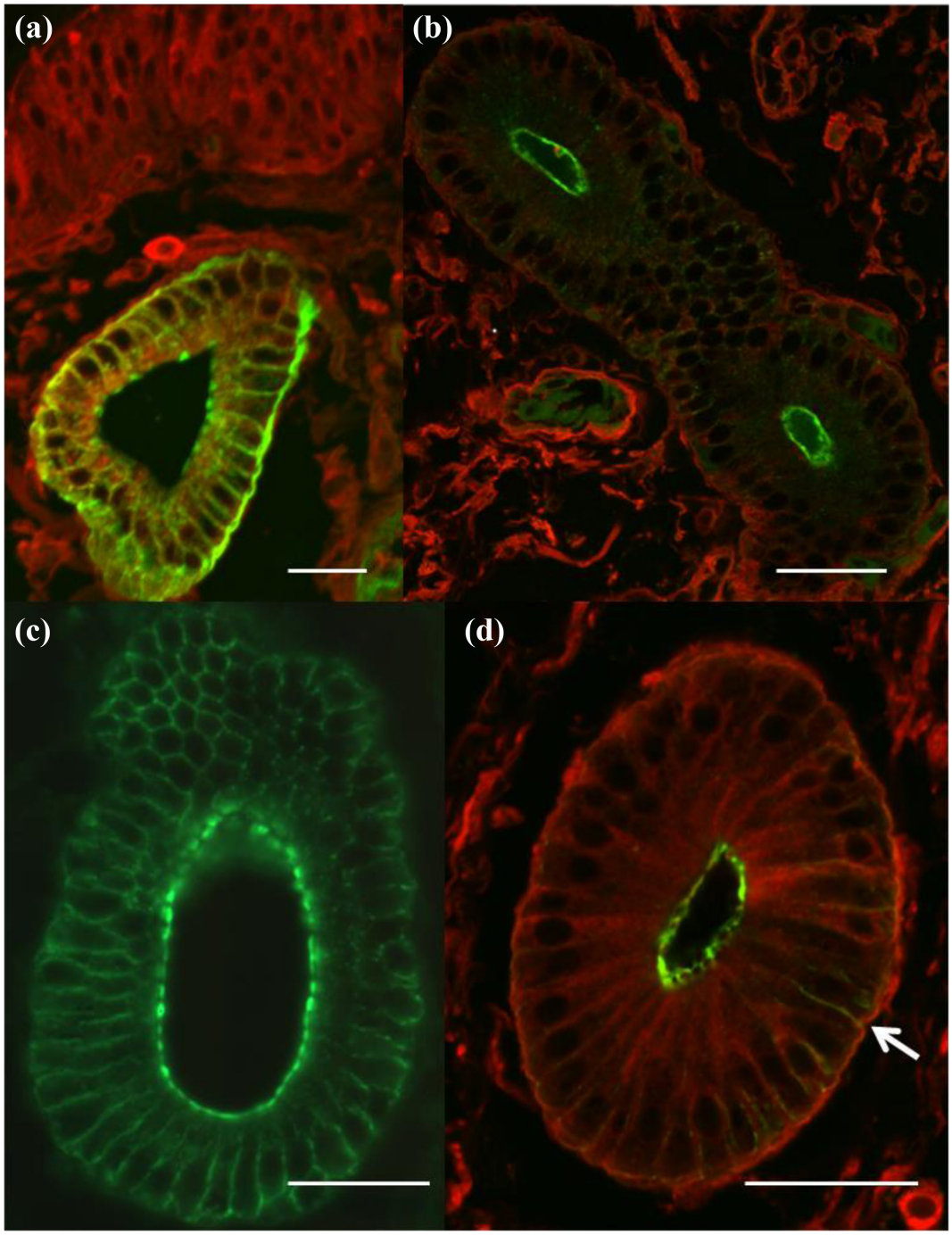

Figure 3. Galactose and N-acetylgalactosamine moieties were observed with the lectins WFA and ECL and mannose moieties were observed with Con-A. All micron bars are 20 μm. (a) In this dual stained image, Con-A binds to the UVJ epithelial cell cytoplasm and apical cytoplasm in the SST epithelial cells. WFA does not bind to the UVJ epithelium, but is observed strongly bound to the SST basement membrane, lateral cell walls, and the apical surface of some but not all of the SST epithelial cells. (b) Unlike Fig. 3a, WFA does not bind strongly to the SST lateral walls but does bind strongly to the SST microvilli. Con-A binding is limited to the loose connective tissue and the basement membrane of the UVJ epithelium. (c) WFA binding to the SST epithelial cells is similar to that of WGA except the SST microvilli do not uniformly bind WFA with the same intensity. (d) ECL binding was intermittent on the SST apical microvilli and sparse on the SST lateral cell membranes (arrow). Con-A bound lightly to the loose connective tissue and the SST apical cytoplasm and basement membranes.

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