Evidence for the innervation of sperm storage tubules in the oviduct of the turkey (*Meleagris gallopavo*)

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The presence of neural tissue and smooth muscle elements in the vicinity of the oviductal sperm storage tubules at the uterovaginal junction was assessed by several modes of light microscopy. Isolated neurones and small ganglia were identified in the uterovaginal junction of the turkey oviduct. The nerve cell bodies were observed in the tunica mucosa by bright field microscopy. Immunoreactivity against neurofilament antibody and recombinant fragment C of the tetanus toxin reacted with nerve fibres and the nuclei of neurones. Fluorescence microscopy and confocal laser scanning microscopy revealed that nerve fibres continued from the base of the tunica mucosa into the plicae. Axons appeared to terminate on, or run immediately

adjacent to, individual sperm storage tubules. Neither phalloidin reacting with F-actin nor the monoclonal antibody against alpha-smooth muscle actin detected smooth muscle fibres in the tissue encapsulating individual sperm storage tubules. In contrast, F-actin was strongly localized in the apical region of the epithelial cells of the sperm storage tubule and in smooth muscle elements in the tunica mucosa and tunica muscularis. These observations present the first evidence for the innervation of the sperm storage tubules. It is suggested that a previously unrecognized neural factor may function in oviductal sperm storage in, and release of spermatozoa from, the sperm storage tubules of hens.

Introduction

The oviductal sperm storage tubules are specialized simple tubular invaginations of the epithelium of the uterovaginal junction in which, depending on the species, spermatozoa can reside for a few days to 10 weeks after mating or artificial insemination. Although the sperm storage tubules have been the subject of numerous studies over the past four decades, the processes involved in sperm uptake into, maintenance within, and release from the sperm storage tubules remain unknown. Neither light microscopy (Fujii, 1963; Fujii and Tamura, 1963; Bobr *et al.*, 1964; Van Krey *et al.*, 1967; Gilbert *et al.*, 1968; Bakst, 1987; Bakst *et al.*, 1994) nor transmission electron microscopy (Schindler *et al.*, 1967; Van Krey *et al.*, 1967; Burke *et al.*, 1972; Tingari and Lake, 1973; Schuppin *et al.*, 1984) have revealed the presence of nerves, smooth muscle or myoepithelial cells associated with the sperm storage tubules. In the present study, contemporary immunocytochemical procedures, specific neurotoxin uptake and modes of light microscopy were used to re-evaluate the possible innervation of sperm storage tubules, and the presence of contractile elements within and around the epithelium of sperm storage tubules.

Materials and Methods

Animals and treatments

Large White turkey hens (*Meleagris gallopavo*; Nicholas Turkey Breeding Farms, Sonoma, CA) of 26–46 weeks of age were maintained under standard husbandry conditions and feed and water were provided *ad libitum*. Hens were photostimulated at 28 weeks of age (14 h light:10 h dark) and were producing eggs by weeks 30–31. Toms were maintained under similar conditions in a separate facility, and were photostimulated at 26 weeks of age, and semen was collected 2 weeks later. Hens were subjected to artificial insemination as described by Bakst (1992). Ten inseminated hens that had been producing eggs for at least 1 week were killed by an i.v. injection of sodium pentobarbital (A. J. Buck & Son, Cockeysville, MD) and the caudal oviduct was perfused intraluminally with 4% (v/v) paraformaldehyde in PBS for 10–15 min.

The oviduct was removed using the method described by Bakst (1987, 1992) and tissue samples from the uterus, uterovaginal junction and vagina were obtained. The location of the sperm storage tubules within the uterovaginal junction was determined by stereomicroscopy (Bakst, 1987). Excised tissue samples were fixed in 4% (v/v) paraformaldehyde (Sigma, St Louis, MO) in PBS. After 24 h, the fixed tissues were transferred to PBS with 30% (w/v) sucrose and stored at 5°C. For histological assessments, some samples were embedded in paraffin wax, sectioned at

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5–7 μm and stained with haematoxylin and eosin. Whole mount, spread preparations were prepared using the procedure described by Bakst (1987). The remaining fixed tissue specimens were subjected to cryomicrotomy using a sliding microtome (American Optical, Buffalo, NY). Sections of about 90 μm in thickness (range 30–120 μm) were required to maintain the integrity of the plicae, which are particularly fragile in the uterovaginal junction and vagina.

Immunofluorescent staining

After permeabilization with 0.3% (w/v) Triton-X100 and blocking in 2% (v/v) normal goat serum (Sigma) in PBS–0.4% polyvinyl pyrrolidone (PVP; w/v) (Sigma), tissue sections were rinsed in PBS–PVP and incubated for 1 h at 37°C in the dark with a 1:1000 or 1:5000 dilution of either SMI 31 (Sternberger Monoclonals Inc., Baltimore, MD) or Neurotag TM Green (Boehringer Mannheim, Indianapolis, IN). SMI 31 is a mouse monoclonal antibody that reacts strongly with phosphorylated neurofilaments. Neurotag TM Green, a recombinant non-toxic fragment C of the tetanus toxin conjugated to tetramethylrhodamine isothiocyanate (TRITC), was used to detect nerve cell bodies and fibres. The tissue sections were rinsed and blocked in 2% goat IgG in PBS–PVP, and incubated with fluorescent anti-mouse secondary antibody or antibodies at a 1:128 dilution of the stock solution (Sigma T-5393 or Sigma F-0257). Oregon Green (Molecular Probes, Eugene, OR), a goat anti-mouse IgG conjugate, was also used (1:40 dilution) to visualize the primary antibodies, as directed by the manufacturer. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vectashield Mounting Medium with DAPI; Vector Laboratories, Inc., Burlingame, CA) or *bisbenzimidazole* (Hoescht 33342; Molecular Probes).

Localization of smooth muscle actin was attempted with monoclonal anti- α smooth muscle actin (Sigma A-2547). Sections were incubated at 37.5°C for 15–30 min at a 1:400 dilution of the stock solution. F-actin was localized using either rhodamine phalloidin or Oregon Green 488 phalloidin (Molecular Probes). Sections were exposed to 80% acetone for 5 min at –20°C before incubation with rhodamine phalloidin (1:800 dilution) for 30 min in the dark at ambient temperature.

The following controls were used in each immunocytochemical procedure: when the primary antibody was goat anti-mouse, a secondary antibody of goat anti-rabbit conjugated to fluorescein isothiocyanate (FITC) or TRITC at a 1:40 dilution of the stock solution was used; the specimens were assayed simultaneously in the absence of primary antibody; the specimens were assayed simultaneously in the absence of secondary antibody; and a control tissue section (specimen in which the presence of the target antigen is not normally observed).

Specimens were viewed with appropriate filter sets using an Olympus IMT-2 inverted microscope equipped with a reflected-light fluorescence attachment. In addition,

immunolabelled whole mounts and cryosections were also observed using a Zeiss LSM 410 (laser scanning confocal microscope). Fluorochromes were excited using appropriate combinations of excitation and barrier filters and an Argon–Krypton laser for FITC, TRITC conjugates and phalloidin or UV laser (Inova 90.5, Coherent Inc., Palo Alto, CA) for excitation of *bisbenzimidazole*. Each fluorochrome was scanned individually and digitally recombined into colour images using Zeiss 410 LSM software. Digitalized images were recorded on optical disks and printed using a colour dye-sublimation printer (Codonics NP-1600 Photographic Network Printer; Codonics Inc., Middleburg Heights, OH).

Results

Standard histological staining with haematoxylin and eosin and immunohistochemistry revealed many isolated neurones and small ganglia in the mucosa of the uterovaginal junction. Neurones were identified primarily by their distinct euchromatic nuclei that contained prominent nucleoli. The perikarya (nucleus and surrounding cytoplasm) of these neurones ranged from 10 to 15 μm in diameter (Fig. 1a,b), and their nuclei were eccentrically positioned, as is typical of autonomic neurones. Isolated or paired neurones were observed most frequently in the lamina propria portion of the tunica mucosa at its interface with the tunica muscularis. Occasionally, individual perikarya were observed in the plica relatively close to the sperm storage tubules. (Fig. 1b: the single neurone observed is about 183 μm from the lumen of the sperm storage tubules.) Ganglia containing several perikarya (Figs 1a and 2b) were identified only occasionally in the tunica mucosa.

Standard haematoxylin and eosin staining did not allow visualization of the nerve fibres. Alternatively, immunoreactive nerve fibres were apparent in frozen sections and squash preparations of the tunica mucosa (Fig. 2a,c–g). Nerve fibres were obvious in the plicae containing sperm storage tubules and in the contiguous plicae at the anterior end of the vagina, which lacked sperm storage tubules. Nerve bundles, composed of fibres running tightly parallel with each other (Fig. 2d), and individual fibres coursed from the abluminal margin of the tunica mucosa into the base of the plicae. Axons continued into the plicae, often accompanying blood vessels, but many others coursed freely, paralleling the long axis of each plica. In appropriately oriented preparations, nerve bundles and individual axons were observed leaving nerve bundles to course to the sperm storage tubules. These fine nerve fibres either continued along the external surface of a sperm storage tubule for a considerable distance (Fig. 2c–e), crossed the sperm storage tubules obliquely or appeared to terminate directly within the fibrous and cellular layer enveloping the external surface of the sperm storage tubules (Fig. 2f,g).

Scattered nuclei embedded in the nerve fibres indicated the presence of Schwann cells (Fig. 2e). The continuous narrow fibrous layer and scattered fibroblast-like cells on the external surface of the sperm storage tubules were

Fig. 1. (a) A haematoxylin and eosin stained paraffin wax section of the mucosa of the turkey uterovaginal junction showing a relatively large ganglion (arrow) consisting of at least six neurones. Note the prominent nucleoli in the neurones. Pseudostratified columnar ciliated epithelium (arrowhead) lining the uterovaginal junction is also observed. (b) A haematoxylin and eosin stained paraffin wax section of the mucosa of the turkey uterovaginal junction showing a neurone (arrow) situated about 183 μm from spermatozoa in the lumen of a sperm storage tubule (arrowhead). A blood vessel (bv) is also observed. Scale bars represent (a) 58 μm and (b) 60 μm .

apparent by light and confocal microscopy. Immunocytochemically, the cytoplasm of these fibroblast-like cells was negative for F-actin and anti- α smooth muscle actin. In contrast, when the respective antibodies were used, there was an intense immunoreactive response for F-actin and smooth muscle actin in the cells forming the mucosal arterioles and venules, and the smooth muscle cells in the tunica muscularis.

The apical portion of the epithelial cells of the sperm storage tubules, presumably the microvillus core and terminal web, were strongly immunoreactive for F-actin (Fig. 2a,g,h). When viewed by either light or confocal microscopy, the actin layer appeared to form a fluorescent band around the lumen of the sperm storage tubules. Numerous small openings due to the absence of fluorescence were evident in tangential views of this band (Fig. 2h). The epithelium of the sperm storage tubules failed to react with the probe for anti- α smooth muscle actin.

Discussion

This study is the first to demonstrate that sperm storage tubules are innervated. Other attempts to identify nerves or contractile elements associated with sperm storage tubules have been unsuccessful (Gilbert and Lake, 1963; Van Krey *et al.*, 1967; Gilbert *et al.*, 1968). Similarly, transmission electron microscopy (Schinder, 1967; Van Krey *et al.*, 1967; Burke *et al.*, 1972; Tingari and Lake, 1973; Schupp *et al.*, 1984; Bakst, 1987) failed to reveal the presence of nerve terminals in, or nerves coursing near, sperm storage tubules. Furthermore, no evidence has been obtained for the presence of smooth muscle, myoepithelial cells or nervous tissue elements within or adjacent to sperm storage tubules.

At least some of the nerve fibres that traversed the plicae originated from the small intrinsic neurones located in the lamina propria at the interface of the tunica mucosa and tunica muscularis. Other nerve fibres may have originated from the neurones contained in the tunica muscularis (Biswal, 1954; Gilbert and Lake, 1963; Costagliola *et al.*, 1997) or neurones in peripheral ganglia on the exterior surface of the caudal oviduct (Freedman and Sturkie, 1963; Gilbert and Lake, 1963). It is also possible that afferent fibres originated from lumbosacral dorsal root ganglia.

Biswal (1954) and Costagliola *et al.* (1997) documented the presence of small ganglia containing one to three neurones in the tunica muscularis of the chicken vagina. Although Costagliola *et al.* (1997) observed neurones and nerve fibres in the oviductal mucosa, the present study is the first to report ganglia with more than three neurones in the tunica mucosa of the oviduct, specifically in the uterovaginal junction. Costagliola *et al.* (1997) and Biswal (1954) did not specify whether the fibres extended into the plicae and did not report on the innervation of the uterovaginal junction. However, Costagliola *et al.* (1997) suggested that mucosal neurones in the vagina frequently had a long axon. If this observation is correct, the mucosal neurones in the uterovaginal junction represent a possible source of the fibres that innervate the sperm storage tubules.

Gilbert and Lake (1963) found that nerve fibres and nerve cell bodies were present in the caudal oviduct of chickens. Neurones were present in the tunica muscularis of both the uterus and uterovaginal junction and the largest concentration of nerve cell bodies was in the plexus associated with the uterovaginal junction. A subsequent investigation by Gilbert *et al.* (1968) indicated that although many nerves were contained in the tunica muscularis and the serosa,

Fig. 2. Immunohistochemical staining of the turkey uterovaginal junction and sperm storage tubules. (a) A fluorescing (Oregon Green) nerve fibre is observed in the vicinity of some sperm storage tubules. Bisbenzimidazole stained nuclei (blue) highlight the surface epithelium (se) lining the lumen of the uterovaginal junction and the epithelium of the sperm storage tubules. The phalloidin-reactive (magenta) actin-rich apical region of the epithelium of the sperm storage tubules (arrow) is evident in one sperm storage tubule. This confocal image was reconstructed from 1.7 μm thick images (total image thickness is 19 μm) from a frozen section about 90 μm thick. (b) A ganglion in the mucosa of the uterovaginal junction is highlighted

none were discerned in the vicinity of the sperm storage tubules. Similarly, these authors reported that nerve cell bodies were not situated in the tunica mucosa, whereas they were detected in the tunica muscularis and serosa.

In the present study, the ability to document nerves innervating the sperm storage tubules and surrounding tunica mucosa was due to the marked sensitivity of current immunocytochemical techniques. However, the presence of nerve cell bodies in the tunica mucosa was also apparent with the standard haematoxylin and eosin staining procedure. The detection of neurones in the tunica mucosa of the uterovaginal junction can be attributed to fortuitous inspection of appropriate tissue sections. It is suggested that sampling errors were the primary reason for the past failure to detect evidence of neurones and their fibres ultra-structurally. The minute surface area of sections obtained from an ultramicrotome may be another reason why nerve fibres have not been identified previously.

The observation that nerve fibres are associated with sperm storage tubules implies that some aspect of the function of the sperm storage tubule may be influenced by a neural-based mechanism. Considering that the terminal web is capable of constricting *in vitro* (for a review, see Mamajiwalla *et al.*, 1992), the F-actin rich terminal web in the epithelium of the sperm storage tubules may be involved in a co-ordinated contraction or squeezing of the sperm storage tubules, liberating resident spermatozoa closest to the openings of the sperm storage tubules. Careful examination of the fluorescent F-actin band revealed the presence of both punctate gaps and more elongated gaps. The elongated gaps were more apparent in transverse sections of the sperm storage tubules, in which the F-actin rich terminal web had a coiled appearance indicating a contractile function. Tingari and Lake (1973) upon noting the abundance of tonofibrils in the epithelial cells of the sperm storage tubules suggested that a hormonal or chemical signal might initiate contraction of a sperm storage tubule, effecting the release of resident spermatozoa.

The mobility of spermatozoa residing within the sperm storage tubules may be altered by cholinergic neurotrans-

mitters liberated from adjacent nerve terminals. Acetylcholine enhances the motility of chicken spermatozoa *in vitro* (Atherton *et al.*, 1980). These authors used histochemical studies to localize cholinesterase activity in the spermatozoa and subsequently suggested that the motility of avian spermatozoa may be regulated by a system similar to that of the cholinergic neurotransmitter system. Whether the release of spermatozoa from the sperm storage tubules is a result of an acetylcholine-induced increase in sperm motility remains to be determined.

Previous ultrastructural studies failed to observe the presence of smooth muscle and myoepithelial cells in sperm storage tubules. Gilbert *et al.* (1968) were also unable to verify the presence of myoepithelial cells using histochemical staining, which is in agreement with the findings from the present study. The presence of smooth muscle components of the sperm storage tubules examined in the current study is precluded by the lack of a positive staining reaction to anti-alpha smooth muscle actin. Therefore, neither smooth muscle nor myoepithelial cell contraction can be involved directly in sperm release from the sperm storage tubules.

Sykes (1953, 1955) and Burke *et al.* (1972) suggested that there are sensory oviductal nerves and found that placing a suture through the wall of various oviductal segments interfered with egg production. Lake and Gilbert (1964) also suggested a sensory nerve component with regard to oviduct function after noting a decrease in the number of spermatozoa in the sperm storage tubules of hens that had had a thread placed in their uterus. Bobr *et al.* (1965) found that intrauterine, as opposed to intravaginal, artificial insemination often caused premature expulsion of eggs and also delayed ovulation. These findings were attributed to direct stimulation of the uterovaginal junction sphincter, but Bobr *et al.* (1965) also implicated the possible involvement of the nervous system. The participation of the sensory nervous system is also indicated by the co-ordinated rapid muscular contractions that occur in the passage of an egg from the uterus into and through the convoluted segments of the vagina during oviposition.

Costagliola *et al.* (1997) also proposed a similar role for

with Neurotag. This spread preparation was prepared from a 120 µm thick section. The fluorescing nuclei average about 6 µm in diameter. (c) A spread preparation reveals blue-green fluorescing (Neurotag) anastomosing nerve fibres coursing in the vicinity of several sperm storage tubules (arrows). The nuclear stain bisbenzimidide imparts a light blue fluorescence to all nuclei. (d) Observed in this spread preparation of uterovaginal junction mucosa is a nerve bundle containing at least three green fluorescing (Neurotag) nerve fibres in the vicinity of a sperm storage tubule (arrow). The nuclear stain bisbenzimidide renders all the nuclei blue. (e) In a preparation similar to those shown in (c) and (d), periodic nuclei (arrows) are observed embedded in nerve fibres. This indicates the presence of Schwann cells. The larger arrow highlights the profile of a sperm storage tubule. (f) In this frozen section (about 90 µm thick), a nerve fibre (green-yellow; SMI 31) appears to terminate (arrow) on a sperm storage tubule. (g) In this confocal image, a nerve fibre positive for Neurotag (green) appears to terminate on a sperm storage tubule. The phalloidin-reactive (magenta) actin-rich apical region of the epithelium of the sperm storage tubules is clearly evident. The nuclei of the sperm storage tubules are stained (blue) with bisbenzimidide. This confocal image was reconstructed from nine 1.75 µm thick images (total image thickness is 15.75 µm) from a frozen section about 90 µm thick. (h) In this confocal image, the phalloidin-reactive actin-rich (orange-magenta) luminal region of the epithelium of the sperm storage tubules is apparent. Close examination of this actin-rich band, which is arranged around the circumference of the sperm storage tubules, shows small gaps in the fluorescence. Nuclei are stained (blue) with bisbenzimidide. This confocal image was reconstructed from nine 1.5 µm thick images (total image thickness is 13.5 µm) from a frozen section about 90 µm thick. Scale bars represent (a,d,f) 30 µm, (b,h) 15 µm, (c) 80 µm, (e) 20 µm, (g) 50 µm.

nerves in the tunica muscularis in the uterus and vagina. Furthermore, these authors provided evidence that vaso-active intestinal peptide (VIP) positive and nitrergic neurones are present in the uterus and vagina of hens. This finding indicates that chickens and possibly turkeys follow the same basic autonomic and peptidergic innervation seen in all other animals that have been investigated. Therefore, it is conceivable that afferent nerve fibres also play a role in co-ordinating the timing of release of spermatozoa from the sperm storage tubules during the daily ovulatory cycle. In conclusion, the finding from the present study that the sperm storage tubules are innervated indicates a possible neurochemical role in sperm storage, particularly in the release of spermatozoa from the sperm storage tubules.

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