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PHYSIOLOGY, ENDOCRINOLOGY, AND REPRODUCTION

Serotonin Localization in the Turkey Vaginal but not Sperm Storage Tubule Epithelia[1]

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ABSTRACT Elucidating the cellular and molecular mechanisms regulating sperm selection and transport in the vagina of the hen had been the focus of a limited amount of research over the past decade. New observations indicate the presence of nonneuron endocrine cells in the epithelia lining the lumina of the turkey hen vagina and uterovaginal junction. Although no cells in the vagina or uterovaginal junction surface epithelia exhibited argentaffin staining, typical of cells containing neurosecretory granules, cells restricted to the vaginal and uterovaginal junction but not the sperm storage tubule epithelia were immunoreactive positive to serotonin. We speculate that if released into the vaginal lumen and submucosa, serotonin could augment cilia and sperm tail beat frequencies and facilitate smooth muscle contraction, respectively. If this is the response to sperm at insemination, it would represent the first evidence of a local control mechanism responding to sperm in the turkey vagina.

Key words: avian, poultry, turkey, oviduct, serotonin

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INTRODUCTION

In domestic birds, the vagina serves as a conduit between the uterus and cloaca for the egg mass at the time of oviposition. Upon transfer of semen into the hen either by natural mating or artificial insemination (**AI**), the vagina either actively or passively permits a relatively small percentage of sperm transferred to ascend to the uterovaginal junction (**UVJ**) and enter the sperm storage tubules (**SST**; Bakst et al., 1994). However, the precise cellular and molecular interactions in the vagina responsible for sperm selection and transport after sperm transfer remain unknown.

Sperm that do traverse the vagina and reach the SST do so after intensive sperm selection or by cryptic female choice, or a combination of both. We do know that there is sperm competition within the vagina, because sperm with certain phenotypes are more successful at traversing the vagina and entering the SST than sperm of other phenotypes. One such phenotype is sperm mobility, first defined and measured by Froman and McLean (1996). Sperm from those chicken males with superior mobility, as defined by their ability to penetrate a viscous medium, were most likely to populate the SST and subsequently fertilize the ovulated ova between successive weekly inseminations.

There is also evidence throughout the animal kingdom that after the transfer of sperm into the female, the female then selects those sperm that will ultimately fertilize its ova. This phenomenon is referred to as cryptic female choice (Eberhard, 1996). From a mechanistic perspective, one would expect luminal epithelial cells that detect some signal from sperm or seminal plasma. Such a signal and subsequent response by the epithelial cell sensing the signal would transmit information from the vaginal sperm to the central nervous system. Alternatively, this cryptic female choice can be a local mechanism. For example, when plasmalemma-associated glycoproteins were removed from chicken sperm by neuraminidase or hypotonic media before insemination, such sperm failed to populate the SST. In contrast, untreated sperm traversed the vagina and populated the SST (Bakst et al., 1994; Wishart and Horrocks, 2000). A sperm selection process orchestrated by the vagina is further demonstrated after the transfer of heterologous semen into the chicken vagina. These sperm fail to be transported to the UVJ. Yet, if co-cultured with UVJ explants containing SST, heterologous sperm are observed residing in the SST (Bakst et al., 1994; Wishart and Horrocks, 2000).

Most neural responses are a result of direct stimuli to sensory neurons. In some tubular organs such as the gut, there are neuroendocrine-like cells that serve as sensory receptors. The enterochromaffin cell in the intestinal epithelium of mammals is such a sensory cell. By some mechanism, possibly mechanical or chemical, enterochromaf-

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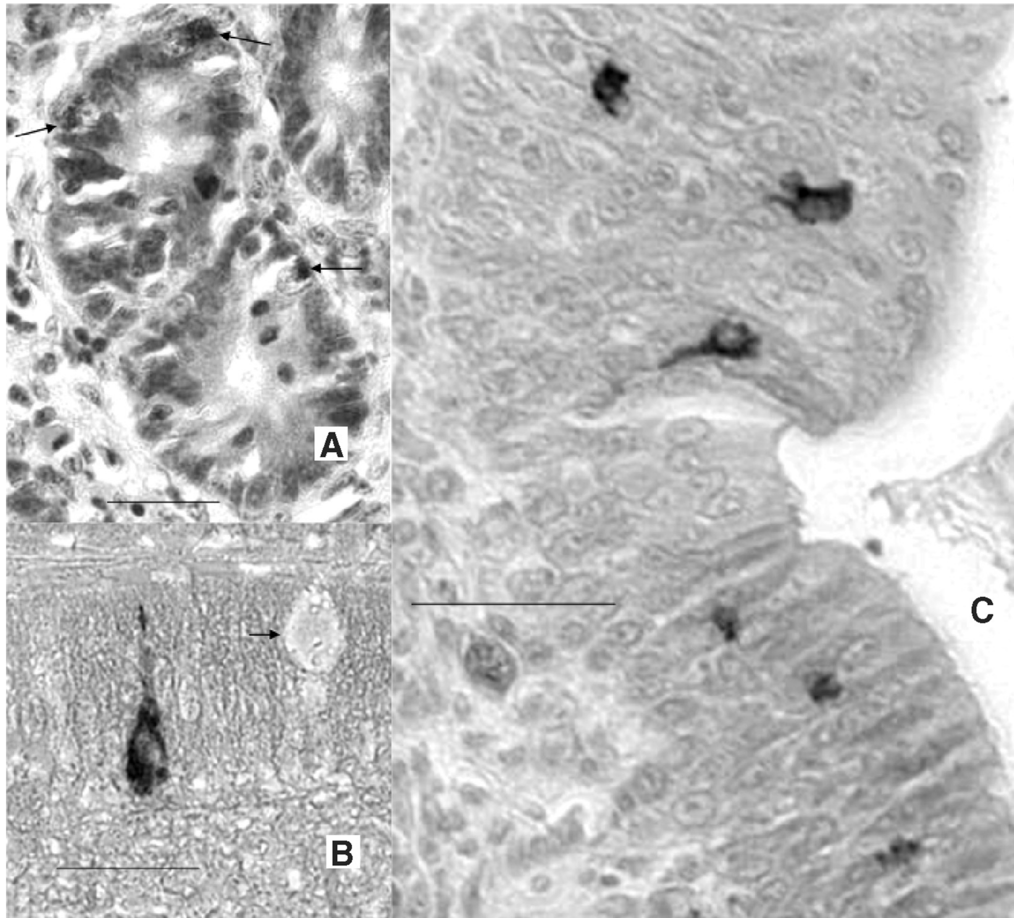

Figure 1. (A) A section of the duodenum mucosa revealing cross-sections of glands with argentaffin-positive cells (arrows). Bar = 20 μm. (B) A section of the duodenum luminal surface lining epithelium revealing a single serotonin-positive cell. The narrowed neck portion reaches the lumen, and the more dilated portion of the cell is associated with the basement membrane. The arrow is pointing to a goblet cell. Bar = 20 μm. (C) A section of the epithelium lining the luminal surface of the dorsal wall of the proctodeum revealing several argentaffin-positive cells. Bar = 20 μm.

fin cells are stimulated to release the neurotransmitter serotonin. Serotonin reaches complementary receptors on terminals of sensory neurons located in the gut lamina propria, which then results in these neurons initiating a locally controlled peristaltic reflex. This sequence is part of what has been described as the enteric nervous system.

The movement of the egg mass through the oviduct is a result of peristaltic activity initiated by local distention of the oviductal smooth muscle layer (Arjamaa and Talo, 1983). Alternatively, how sperm are transported through the vagina to the UVJ-SST is not understood. Furthermore, the mechanism of sperm release from the SST and whether they are released in small numbers over the course of the ovulatory cycle or in large numbers associated with oviposition remains a contentious issue. Observations by Freedman et al. (2001) revealed that SST were innervated and the SST epithelium possessed a vast actin network just subjacent to the apical microvilli. It was speculated that SST innervation and the actin network play a role in the egress of resident sperm from the SST. In the following study, we attempted to determine if an enterochromaffin-like cell is present in the vaginal and UVJ mucosa that may exert some local control over sperm selection and transport in the vagina. We were particularly interested in the neurotransmitter serotonin, because it has been previously detected spectrofluorometrically in the mature chicken oviduct in segments anterior to the uterus (Rzas et al., 1991).

MATERIALS AND METHODS

All materials and methods used in this study were approved by the Beltsville Area Animal Care and Use Committee according to the US Animal Welfare Act. Large White turkey (*Meleagris gallopavo*) poults (Nicholas, Lewisburg, WV) were raised under recommended commercial husbandry conditions. At 28 and 30 wk of age, males and hens, respectively, were photostimulated by increasing light exposure to 14L:10D. Two weeks after the onset of photostimulation, males were subjected to manual semen collection, and the semen was evaluated. By 3 wk after the onset of photostimulation, the semen was collected, diluted with Beltsville Poultry Semen Extender (Continental Plastics, Delavan, WI; 1:1, vol/vol), and used within 1 h of collection for AI. At 32 wk of age,

hens were inseminated twice 72 h apart with about 300 $\times 10^6$ sperm and then inseminated once weekly thereafter.

All hens used in this study (n = 14) were from 38 to 44 wk of age, euthanized by cervical dislocation, and had a hard-shelled egg mass in their uterus. The uterus, vagina, and cloaca, including the proctodeum (most caudal compartment of the cloaca), were isolated, and the vagina and uterus were stripped of connective tissue to access the UVJ. The UVJ containing SST, confirmed by stereomicroscopy, and vaginal samples from the anterior, mid, and distal portions were fixed in neutral buffered formalin. Specimens were embedded in paraffin, sectioned at 5 μm, and stained.

Oviduct tissue samples were also fixed for 2 h in 2.5% glutaraldehyde in 0.15 *M* phosphate buffer at 4°C. After fixation, specimens were rinsed several times in PBS and stored until embedding. Specimens were dehydrated in ethanol, embedded in epoxy, and sectioned with a microtome at 1 to 2 μm thick using glass knives.

Positive control tissues for the localization of enterochromaffin cells and serotonin-containing cells were isolated from the midregion of the duodenum and the dorsal wall of the proctodeum. These were fixed and embedded in paraffin as described above.

Histochemistry

Nonneuron Endocrine Cell Localization. The kit, DiazoMethod for Staining Argentaffin Granules (Eng Scientific Inc., Clifton, NJ), was purchased and used as instructed. Briefly, slides were deparaffinized, rehydrated in distilled water, incubated for 1 min at 40°C in a solution of brentamine fast red B and lithium carbonate, and then transferred to Mayer's acid alum hematoxylin for 3 min. The slides were then washed for 5 min in warm tap water, rinsed in 2 changes of 95% ethanol, dehydrated rapidly in absolute ethanol, and cleared in 2 changes of xylene before coverslips were applied. Positive reaction product was rust red, background was pale yellow, and nuclei were blue.

Serotonin Localization. Immunocytochemical localization of serotonin-containing cells was accomplished using rabbit primary antibody (Ab8882, Abcam Inc, Cambridge, MA) and DakoCytomation EnVision System-HRP (AEC, Dako North America Inc., Carpinteria, CA) reagents. Dewaxed slides were washed in Dako Wash Buffer (**DWB**) before flooding the sections with Dako peroxidase blocking solution for 5 min at room temperature (**RT**). The slides were washed again with DWB for 5 min at RT. Primary antibody to serotonin was diluted 1:2,500, added to the sections, and incubated either 2 to 4 h at RT or for 18 h at 4°C. After incubation, slides were washed 3 times at 5-min intervals with DWB, and then the secondary antibody was applied to the sections. The secondary antibody was either labeled polymer-HRP antirabbit or dual-labeled polymer-HRP antirabbit + antimouse. Slides with the secondary antibody added were incubated 30 to 90 min at RT and then washed 3 times at 5-min intervals with DWB before addition of the AEC+ substrate-chromogen solution. Color development was visually monitored (5 to 30 min) and when sufficient, slides were washed in DWB 3 times at 5-min intervals. Counterstain was Mayer's hematoxylin (1 min), followed by a wash with tap water before applying aqueous mounting medium and coverslips.

RESULTS

Positive Controls

Argentaffin-positive (Figure 1A) and serotonin-positive (Figure 1B) enterochromaffin cells [nonneuron endocrine cells (**NEC**) located in the gut luminal epithelium] were observed in the glandular epithelium and to a lesser extent in the surface epithelium of the duodenum. These cells either possessed a pyramidal (glandular) or bowling pin configuration with a narrow neck region extending to the gut lumen. Argentaffin-positive cells were also observed in the surface epithelium but not in the subepithelial glandular epithelium of the dorsal wall of the proctodeum (Figure 1C).

Vaginal and UVJ Epithelia

Based on cell morphology, a NEC reminiscent of the enterochromaffin cell observed in Figure 1B was observed in the vaginal epithelium (Figure 2A). However, after using the diazo method, which is generally specific for intracellular neurosecretory granules associated with enterochromaffin cells, there were no positive-staining cells in the vaginal and UVJ mucosae.

Alternatively, serotonin reactive cells were observed scattered throughout the vaginal and UVJ epithelia but not in the SST epithelium (Figures 2B and 3A). The distribution of the reaction product indicated that serotonin was primarily localized to the basal half of the epithelial cells. Although serotonin-positive reaction product was also observed in the apical half of some cells, it never was observed throughout the complete cell, as seen in the serotonin-positive enterochromaffin cell (Figure 1B). Therefore, the precise morphology of the serotonin-positive cells in the vaginal and UVJ epithelia could not be discerned. However, given the distribution of the reaction product, these serotonin-positive cells appear to resemble bowling pin morphology of the serotonin-positive cells observed in the turkey duodenum (Figure 1B). No reaction product was observed associated with any other cell type including thrombocytes [platelet-like cells in birds that are a source of serotonin (Adamson and Campbell, 1988)] or intrinsic neurons in the lamina propria between the tunica muscularis and tunica mucosa. There was no unique spatial relationship between the location of the intrinsic neurons and the serotonin-positive vaginal epithelial cells. No reaction product was observed in vaginal or UVJ (Figure 3B) epithelia in the absense of primary antibody to serotonin.

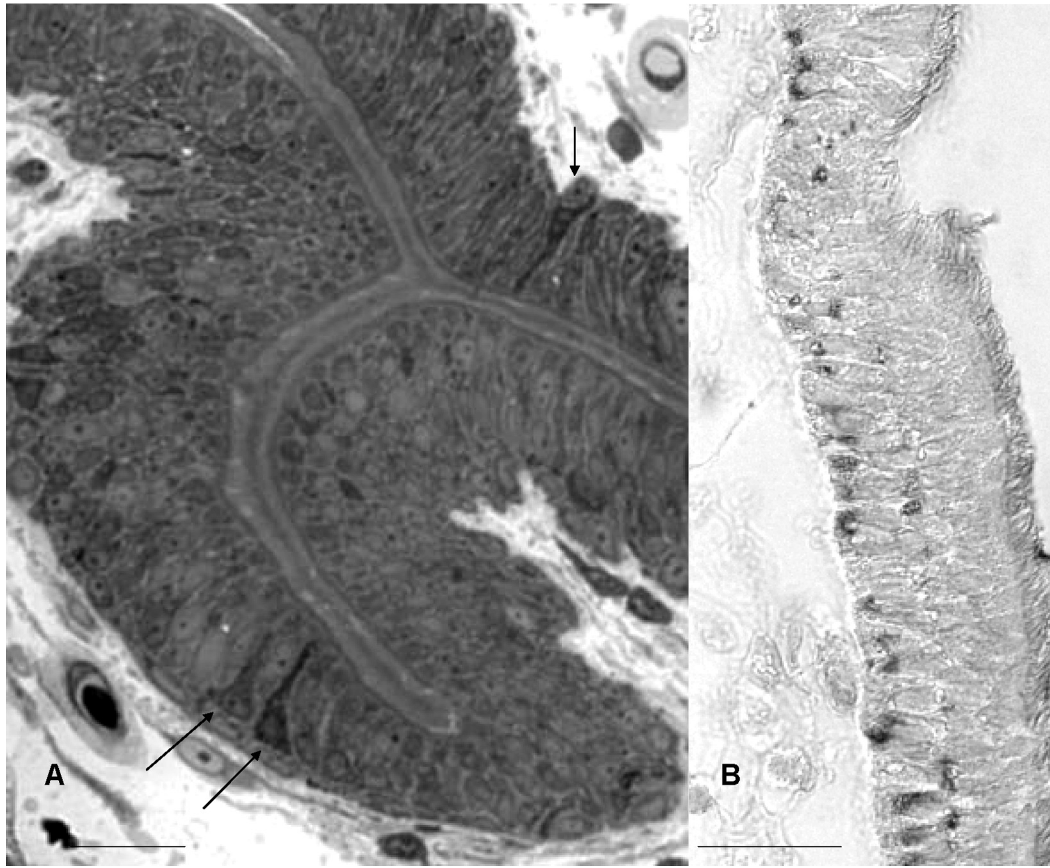

Figure 2. (A) A 1- to 2-μm thick plastic section of the densely ciliated epithelium lining the deep folds of the vaginal mucosa. Several presumptive nonneuron endocrine cells with a dilated basal region and a more narrow apical neck extending to the lumen are observed (arrows). Bar = 20 μm. (B) A section of the ciliated epithelium lining the luminal surface of the vagina revealing several serotonin-positive cells. Bar = 20 μm.

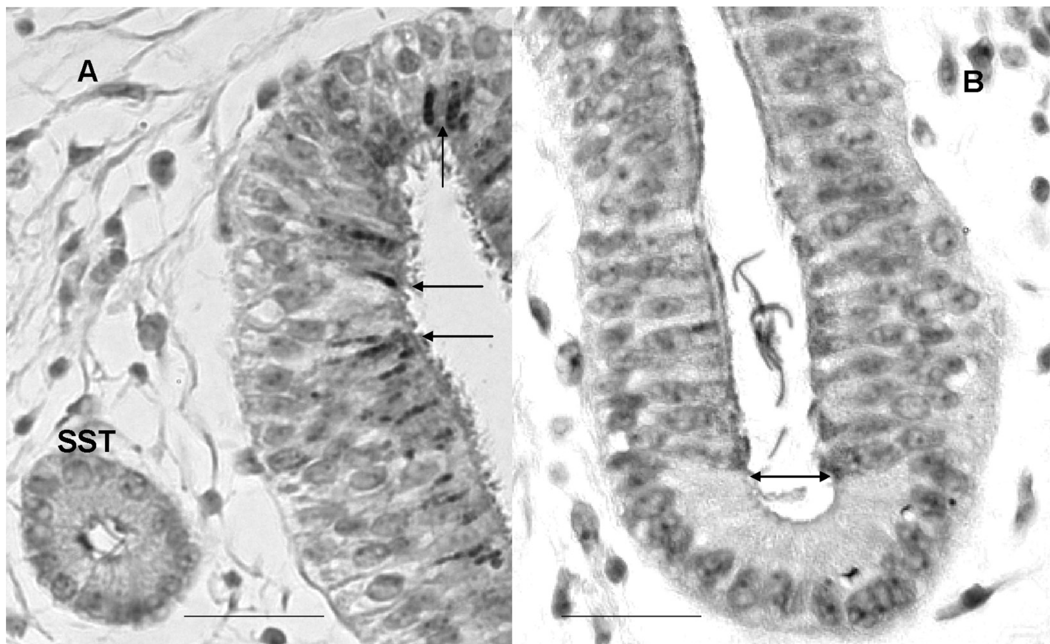

Figure 3. (A) A section of the ciliated epithelium lining the luminal surface of the uterovaginal junction (UVJ) revealing several serotonin-positive cells (arrows). A cross-section of a single sperm storage tubules (SST) shows epithelial cells lacking immunoreactivity to serotonin. Bar = 25 μm. (B) A control section of the UVJ mucosa that lacks immunoreactivity to serotonin but clearly shows the abrupt transition (double-headed arrow) between the pseudostratified columnar ciliated epithelium and the simple columnar epithelium of the SST. Sperm are observed in the UVJ lumen between apposed folds. Bar = 25 μm.

DISCUSSION

Serotonin-positive epithelial cells appear to populate the turkey vaginal and UVJ surface mucosa but not the SST epithelium. Based on the pattern of distribution of the reaction product, the serotonin-positive cells can be construed to have a morphology similar to the serotonin-positive cells in the lining of the duodenum surface epithelium. We could not locate argentaffin-positive cells in the same epithelia. This was generally considered uncharacteristic for serotonin-positive cells. However, Portela-Gomes et al. (1987) noted that rat antral (stomach) mucosa had 1 cell population that was serotonin positive but argentaffin negative and another cell population that was positive for serotonin and argentaffin. We assume that the argentaffin-positive enterochromaffin cells found in the turkey duodenum contain serotonin. Because we did not perform any double-labeling staining techniques, we could not with any certainty state that the argentaffin-positive cells are serotonin positive as well, because all enterochromaffin cells are not argentaffin positive (Civantos, 1986).

Whether serotonin-positive cells synthesized the serotonin or they sequester and store exogenous serotonin, as some enteric neurons have been observed to do (Goodrich et al., 1980), is not known at this point. The lack of both argentaffin-positive cells and other serotonin-positive cells in the vaginal and UVJ mucosae would preclude local synthesis by the intrinsic neurons. Most likely, the serotonin is synthesized elsewhere and reaches the vagina and UVJ from its vascular bed. Serotonin has been detected spectrofluorometrically in the mature chicken oviduct in segments anterior to the uterus (Rzas et al., 1991). These authors did not propose the origin (endogenous or exogenous) of the serotonin but suggested that serotonin may help regulate blood flow to these segments.

The question that must be addressed here is, what, if any, is the role of serotonin observed in the surface epithelial cells in the vagina and UVJ? Serotonin presumed to originate from the chicken enterochromaffin cell is thought to be a factor in the contraction of the gastrointestinal tract (Kitazawa et al., 2006). From a broader perspective, enteric NEC have been proposed to provide an afferent link between the gut and the central nervous system (Flemstrom and Sjoblom, 2005). If the same is found to be true for the vaginal-UVJ serotonin-positive cells (NEC?), then presumably, the female is capable of playing a more selective role in oviductal sperm transport and selection, and ultimately paternity, than previously assumed by poultry biologists.

Avian biologists investigating reproductive physiology, behavior, and breeding strategies in nondomestic birds have postulated that after insemination, there is a mechanism that controls sperm selection in the female. This postinsemination phenomenon is referred to as cryptic female choice (Eberhard, 1996). For example, Fossoy et al. (2006) observed that female birds engaged in extrapair copulations did not necessarily have extrapair offspring. Fossoy et al. (2006) cited several possible explanations for the absence of offspring derived from extrapair mating including female cryptic choice as a source postcopulatory bias in paternity. More in line with the thinking of poultry biologists, Denk et al. (2005) downplayed sperm genotype as a selective factor driving cryptic choice of the female and suggested that superior sperm motility was the primary criteria predicting paternity.

If the vaginal-UVJ serotonin-positive cells are NEC capable of functioning like the enterochromaffin cell, how would this influence the process of vaginal or UVJ sperm selection either by cryptic female choice or differences in sperm motility? We speculate that sperm elicit a paracrine response from the vaginal-UVJ epithelia. More specifically, viable sperm apposed to vaginal epithelial cells would trigger the release of serotonin either by some chemical signal, a pH differential, or differentials in CO_2 concentration, or all three. Serotonin could stimulate sperm tail and epithelial cilia beat frequency by activating intracellular adenylate cyclase in oviductal sperm and the ciliated columnar epithelial cells (Stephens and Prior, 1992). Increased concentrations of serotonin in the lamina mucosa may act indirectly on sperm transport by causing the intrinsic neurons between the laminae muscosa and muscularis to signal the onset of peristaltic activity by the lamina muscularis. Hypothetically, the cumulative effect is more efficient sperm transport of those sperm that are capable of eliciting a paracrine response from the serotonin-producing cells.

To summarize, sperm expressing unique molecular or metabolic signals may trigger the activation of an NEC-like cascade affording to these fit sperm more efficient transport to the UVJ-SST. If this hypothesis proves to be true, then both female cryptic choice and sperm competition act synergistically facilitating the transport of selected sperm to the SST. Identifying these signals and in some manner incorporating them into the semen dilution and storage process may augment the numbers of sperm reaching the SST after AI. Finally, given the observations presented in this study, future research will seek to determine the effect of serotonin on turkey sperm mobility and the beat frequency of cilia on cells isolated from the vagina.

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