



RESEARCH ARTICLE

Distribution of Acetylcholinesterase Positive Neurons in the Oviduct of Laying Hen

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ABSTRACT

The acetylcholinesterase histochemistry is used to identify the cholinergic nerves in tissue sections. Little is known on localization of cholinergic nerves in the oviduct of laying hens. We have used this technique to localize and compare the acetylcholinergic neurons in different regions through the oviduct in laying hens. The cholinergic neurons were seen as single cells, pairs or three cells arranged together. The cytoplasm and the processes of positive neurons showed strong reaction, with an eccentric nucleus. Morphologically, the neurons were rounded and oval cells of unipolar, bipolar and multipolar shapes. Similar features were seen in the whole mounts. Varicose nerve fibers were present. Cholinergic neurons were commonly seen in the muscularis; the fibers ran along the muscularis, occasionally showed a bifurcation to enter the lamina propria, reaching the secondary and tertiary mucosal folds; the fibers also targeted the blood vessels in the intermuscular region. The regional distribution of cholinergic neurons was highest seen in the infundibulum; medium in the magnum, isthmus and uterus (shell gland), while vagina had significantly lower ($P<0.05$) number; i.e. 8.00 ± 1.00 , 5.33 ± 0.33 , 4.67 ± 0.67 ; 5.67 ± 0.33 ; and 3.67 ± 0.33 , respectively. The local comparison of cholinergic neurons in muscularis and lamina propria showed significantly higher ($P<0.05$) number in muscularis than lamina propria of the isthmus. It was concluded that acetylcholinesterase positive (cholinergic) nerves may have a role in the regulation of the smooth muscle functions and blood supply in the oviduct of chicken.

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INTRODUCTION

The chicken oviduct comprises of the five segments, viz. (from the ovarian end) infundibulum, magnum, isthmus, uterus (shell gland) and vagina (Bacha Jr and Bacha, 2000; Chousalkar and Roberts, 2008; Fig. 1). The muscular region of the avian oviduct contains a special layer consisting of a thick connective tissue interposed between the external longitudinal muscle and the inner circular muscle layers. This layer is called "intermuscular". The intermuscular region of the avian oviduct harbors numerous blood vessels in various arrangements, and a dense nerve plexus. These nervous structures show the paravascular and perivascular

distributions (Costagliola *et al.*, 1997). It draws from the blood or actively synthesizes many molecules added to oocyte, which is then transported through the oviduct and laid down by the active movements of the parietal musculature which is controlled by the autonomic nervous system (Costagliola *et al.*, 1997).

Acetylcholine (ACh) is an excitatory neurotransmitter widely diffused in central, peripheral, autonomic and enteric nervous system (Amenta and Tayebati, 2008). Ganzer *et al.* (2012) using antibody to the vesicular acetylcholine transporter (vAChT) determined that ACh is a parasympathetic neurotransmitter in human prostate. The enzyme acetylcholinesterase (AChE) hydrolyzes the ACh released by the nerve fibers into the synaptic clefts, leaving acetic acid and choline (Murabayashi *et al.*, 2009). Histochemical labeling for AChE reaction is widely used to

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identify the cholinergic nerves. A recent report showed that in nematodes acetylcholine from some neurons may inhibit egg-laying behavior (Bany *et al.*, 2003). In the harderian gland of the eye in chicken, the AChE reactivity was reported within the nuclear membranes, the lumen of the Golgi lamellae and on the plasma membrane of the nerve cells at ultrastructural level. The AChE positive nerve varicosities showed contacts with the endothelium or the smooth muscle fibers of the blood vessels (Hiramatsu and Ohshima, 1999).

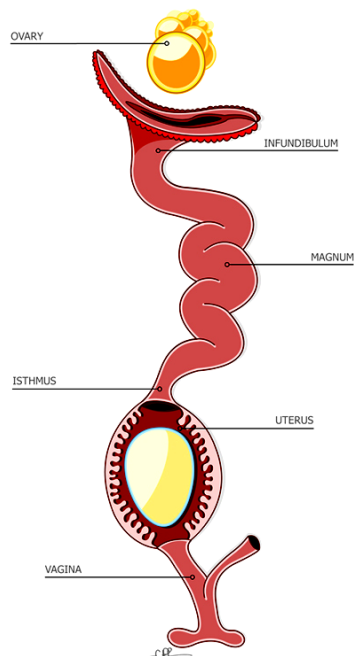


Fig. 1: Stylized depiction of the reproductive system of the hen, containing an incomplete egg in the uterus (Adopted from Hincke *et al.*, 2012).

Several studies have reported the localization of cholinergic nerves by using acetylcholine iodide in birds (Masumoto *et al.*, 1999; Atoji *et al.*, 2000). In guinea pigs the glandular nitric oxide (NO) increases the cervical secretions at ovulation (Morlin and Hammarstrom, 2005), and endometrial secretions at the time of implantation (Morlin *et al.*, 2005). Morlin and Hammarstrom (2005) reported that the cholinergic innervation regulates uterine secretions in human females. A previous study reported the localization of nitrenergic and vasoactive intestinal peptide (VIP) positive nerves in the hen oviduct (Costagliola *et al.*, 1997). It has been proposed that the nervous networks may contribute to egg formation, transport, and ovulation in the avian oviduct (Atoji *et al.*, 2000). However, little is known on the identification and localization of the cholinergic nerves in oviduct in laying hens. Moreover, the cholinergic innervation might have some role in the normal functioning of the oviduct in laying birds. We have used acetylcholine iodide as substrate to determine the regional localization of cholinergic nerves in laying hens.

MATERIALS AND METHODS

Ethical approval: All the experimental protocols complied with the regulations of the Chinese Committee

for Animal Use for Research and Education. The birds were sacrificed by cervical dislocation. The oviduct samples collected very carefully, immediately after the sacrifice.

Experimental animals: We used 12 normal laying Chinese Three-Yellow hens between 8 and 12 mo of age and weighing 2.0 to 2.6 kg in the current study. The housing of birds included holding in individual cages under standard uniform experimental conditions and adequate lighting (10 h dark cycle and 14 h light cycle). The birds had free access to food and water. All the birds were normal and healthy.

Sample collection and preparation: The experimental tissue samples included five segments of the hen oviduct, viz., infundibulum, magnum, isthmus, uterus (shell gland), and vagina. The tissue blocks were immediately fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS) (pH 7.5) for 2–4 h. The tissues were washed in 0.1 M PBS, and cryoprotected in sucrose (15%) in the same buffer overnight at 4°C. The samples were embedded in OCT medium (Ted Pella Inc., Redding-USA). Frozen tissue sections of 5–10 µm thickness were used. The sections were mounted on 3-aminopropyltriethoxysilane (APES) coated slides, a 2% solution in acetone.

Histochemical labeling for acetylcholinesterase (AChE) positive neurons: The sections were left at room temperature for 5-10 min before performing the histochemistry. The oviduct samples were processed for acetylcholinesterase (AChE) activity according to the prescribed procedure (Karnovsky and Roots, 1964). The reagents in the incubation medium consisted of 0.49 mM sodium citrate, 2.9 mM copper sulfate, 1.25 mM potassium ferricyanide and 10^{-5} M iso-OMPA (tetra-isopropyl pyrophosphoramidate) as an inhibitor of non-specific cholinesterase, and 1.7 mM acetylcholine iodide as the substrate for cholinesterase. The slides were preincubated in a medium omitting the substrate for 30-45 min at 37°C. Then the sections were incubated with the incubation medium for 1 h at 37°C. The reaction was stopped by rinsing with 0.1 M PBS. The sections were coverslipped with a mounting medium containing PBS/glycerol (1:1).

Statistical analysis: The number of positive neurons in each segment was counted from the equal number of microscopic fields under the same magnifications. The data were analyzed using the SPSS software. The values were shown as mean±SEM. The significance was determined using the student's t-test. The values less than ($P < 0.05$) were considered significant.

RESULTS

The number of acetylcholinesterase positive neurons was variable in each segment of chicken oviduct. The cells were seen as single cells, pairs or three cells lying together. Although the single cells were common, however, the tendency was to be arranged in the form of more than one cell. The intensity of labeling was variable;

the strong reaction was seen in the perikaryon and the processes of neurons. The nucleus was displaced eccentrically towards one pole of the cell. The neurons appeared round or oval in shape. Similar features were seen in whole mounts. The polar characteristic appeared to be unipolar, bipolar, and sometimes multipolar neurons. Varicose nerve fibers were also observed. The distribution of the positive neurons in lamina propria and muscularis was the highest seen in infundibulum, followed by magnum; while in the isthmus, uterus (shell gland) and vagina the lamina propria showed lower populations than muscularis. The cholinergic neurons in the isthmus were significantly higher ($P < 0.05$) in the muscularis than the lamina propria.

The infundibulum showed the highest population of AChE positive cholinergic neurons with intense staining. The neurons were commonly seen in the intermuscular region. Thin fibers were seen in the sub-serosal connective tissue. The positive fibers also targeted the blood vessels. Thick cholinergic fibers travelled along the intermuscular region, and bifurcated near the lamina propria of mucosal folds. Several thin fibers were seen in the primary, secondary and tertiary mucosal folds (Fig. 2 a-f).

In magnum, a similar pattern of cholinergic fibers and somata was observed, although the number of the positive neurons was less as compared to the infundibulum. Thin positive fibers of light staining were seen under the epithelium in the mucosal folds. Positive neurons were found in the intermuscular region and in the lamina propria, in the later, the fibers were arranged into thicker bundles. The positive somata were also observed near the blood vessels. The neurons in the whole mounts seen arranged as three cells together (Fig. 3 a-e).

The cholinergic neurons were few in the isthmus as compared to the magnum. Positive fibers in the intermuscular tissue showed very strong reaction with long fibers showing similar dense staining. Thin fibers also ran in the lamina propria of the mucosal folds. Several thin fibers were seen around the blood vessels (Fig. 4 a-c).

In the uterus (shell gland) the cholinergic neurons again showed higher population. The cells were arranged as single large cells targeting the blood vessels in the intermuscular region, or in pairs in the muscularis near lamina propria. The fibers in the intermuscular region were seen as varicose fibers of varying number and thickness (Fig. 5 a-d).

In vagina, very few positive neurons were observed, more commonly in the intermuscular region, but also in the lamina propria. These cells were variable in size from small cells with long fibers, to large unipolar single cells (Fig. 6 a & b).

Cholinergic neuronal populations in the hen oviduct appeared to be higher in the proximal regions. The highest number found in the infundibulum; medium in the magnum, isthmus and uterus (shell gland), the vagina showed significantly lower ($P < 0.05$) number; i.e. 8.00 ± 1.00 , 5.33 ± 0.33 , 4.67 ± 0.67 ; 5.67 ± 0.33 ; and 3.67 ± 0.33 (mean \pm SEM) respectively. There was no significant difference ($P < 0.05$) among the positive populations in the infundibulum and uterus; similarly in the magnum, isthmus and shell gland (uterus); while the

number of cells in the vagina was significantly lower than other segments. The distribution of acetylcholinesterase positive nerves and the staining intensity in fibers in different regions of the chicken oviduct are summarized in Table 1.

DISCUSSION

Studies have been performed on the localization of various neurotransmitters in the avian oviducts in past (Costagliola *et al.*, 1997; Atoji *et al.*, 2000; Costagliola *et al.*, 2004; Yoshimura *et al.*, 2006). In pigeon oviduct, the preferred localization of cholinergic neurons was reported in the muscularis, and near the blood vessels in the intermuscular region. The cholinergic fibers were observed as single fibers or collected in small bundles (Atoji *et al.*, 2000). Sjöberg *et al.* (1997) reported that autonomic innervation of mammalian female reproductive tract comprises of three different nervous systems, the adrenergic, the cholinergic, and the peptidergic, and that these innervations chiefly supply both, vascular and non-vascular smooth muscle cells. In the present study, we have determined the variations in the population of cholinergic neurons in different regions of the oviduct in laying hens. We observed very low number of AChE positive cholinergic neurons in the vagina. Contrarily, in another study (unpublished data) we found highest number of nitric oxide synthase (NOS) positive nitrergic neurons in vagina of hen oviduct. It seems likely that this segment is highly innervated by the inhibitory nerves. Freedman *et al.* (2001) investigated the presence of neural tissue and smooth muscle in oviductal sperm storage tubules (SSTs) at the uterovaginal junction in turkey. They observed axons terminating on, or running adjacent to individual sperm storage tubules; but no smooth muscle fibres detected using antibodies against the F-actin and alpha-smooth muscle actin in the tissue encapsulating the sperm storage tubules. They suggested that a previously unrecognized neural factor may be responsible for the storage in, and release of, spermatozoa from SSTs.

In the present study, the highest population of the AChE positive cholinergic neurons was found in the infundibulum. This might be, in part due to the key role that it has to engulf the released ovum. In our study, we found that there was a higher population of the cholinergic neurons in the anterior oviduct. Because the developing egg takes longer time to go through these two segments, and majority of the important components are added in the magnum and shell gland- the egg white (albumen) added in the former segment, while this time the egg undergoes rotations on its longitudinal axis; and the shell gland wherein the egg shell is deposited. It seems likely that these segments might be under the influence of the excitatory neurotransmission. Conversely, in adult rat uterus the AChE positive innervation was reported to be poor at the tubal end but increased toward the cervical end (Melo and Machado, 1993). Changes in cholinergic innervation of the human oviduct during pregnancy were studied by Kraus and Gombos (1990). They reported that the AChE positivity of the oviduct was not substantially changed. The authors showed that the positive fibers in the mucosa increased in second and third trimesters.

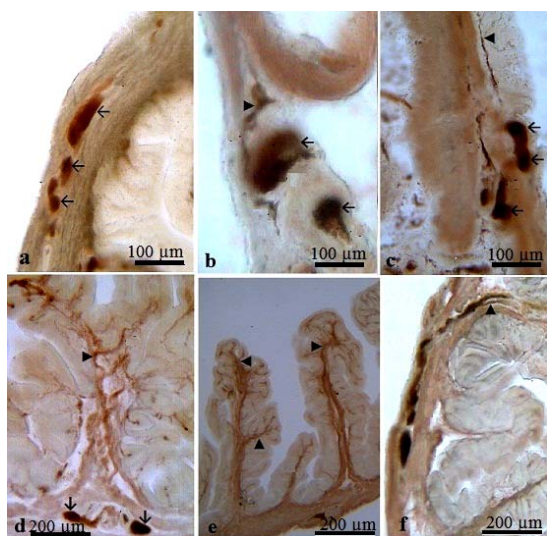


Fig. 2: AChE positive cholinergic neurons in the infundibulum of hen oviduct. (a) Cholinergic nerve cell bodies (arrows) between the outer longitudinal and inner circular muscle layers. Note the arrangement of the cells in groups. (b) A large cholinergic neuronal cell body in the muscular layer and another positive cell (arrows), and the emerging nerve fiber (arrow head). (c) A group of 2 cholinergic neurons and another singlet neuron (arrows). Note the varicose fiber (arrow head). (d) Cholinergic neuron cell bodies (arrows). Cholinergic nerve fibers also run along the lamina propria to the mucosal folds (arrow head). (e) Cholinergic nerve fibers innervating the mucosal folds (arrow heads). (f) A tract of neuronal cells and fibers in the muscularis bifurcating near the lamina propria (arrow head). Note the most frequent appearance of the neuronal structures in the infundibulum.

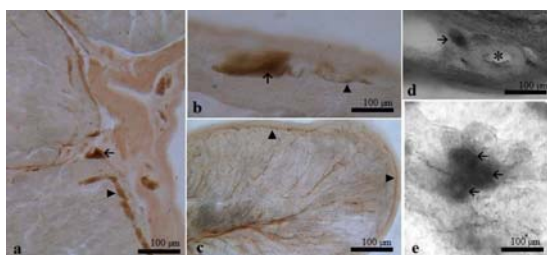


Fig. 3: AChE positive cholinergic neurons in the magnum of hen oviduct. (a-c) The positive nerve structures in the lamina propria and muscularis of the oviduct. (d) A light positive nerve cell body (arrow) in the close proximity with blood vessel (asterisk). (e) Cholinesterase activity in a whole mount from the magnum, a group of three light staining positive cell bodies (arrows) is seen.

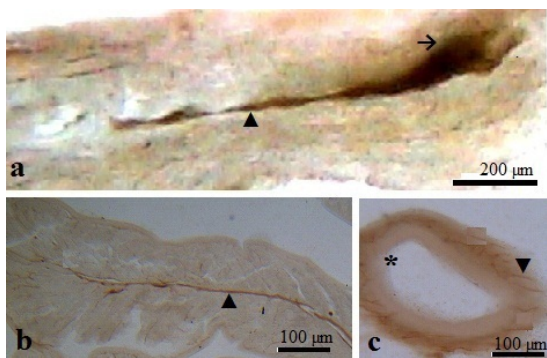


Fig. 4: AChE reactive cholinergic neurons in the isthmus of hen oviduct. (a) A large multipolar AChE positive neuron (arrow) with one long nerve fiber (arrow head) in the intermuscular tissue. (b) AChE positive nerve fiber in the lamina propria of the mucosal fold (arrow head). (c) Several thin AChE fibers (arrow head) targeting a blood vessel (asterisk).

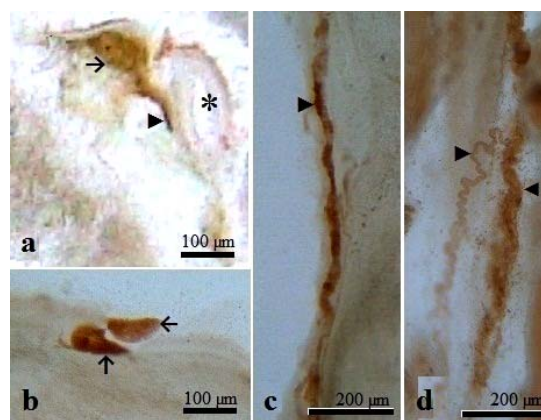


Fig. 5: AChE positive cholinergic neurons in the uterus (shell gland) of hen oviduct. (a) A bipolar neuron in the muscularis of uterus (shell gland) that propagates its fiber (arrow heads) to innervate a blood vessel (asterisk). (b) Two positive neurons (arrows) in the muscularis of uterus (shell gland). (c) Long positive nerve fibers arranged into bundles (arrow head) lying in the subserosal connective tissue region of the uterus (shell gland). (d) Thick varicose nerve fibers in the intermuscular region (arrow head; left-hand) of the uterus (shell gland). Several thin fibers are closely arranged to form thick varicose bundles (arrow head; right-hand).

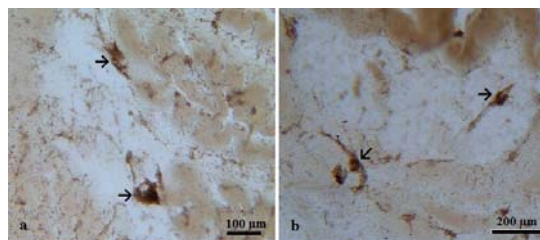


Fig. 6: AChE positive cholinergic neurons in the vagina of hen oviduct. (a) Two positive neurons in the intermuscular tissue of the vagina (arrows). (b) AChE positive neurons (arrows) in the lamina propria of vagina.

In another study on the localization of the nitroergic and VIP positive nerves in hen oviduct, the two types of nerve fibers found as isolated or grouped in small bundles, running beneath the serosa, in the intermuscular space and lamina propria of the mucosa. The intermuscular space showed the majority of nerves surrounding blood vessels. The vaginal segment showed the fibres forming an intricate network encircling bundles of smooth musculature (Costagliola *et al.*, 1997). Prieto *et al.* (1994) also reported similar distribution of cholinesterase positive nerves in the equine ureter, forming muscular, subepithelial and perivascular plexuses. In our study, we found very similar results on the distribution patterns and the regional localization of the AChE positive (cholinergic) nerves in the hen oviduct. The populations of the AChE positive nerves also followed a similar variation in the number to that of nitroergic and VIP positive nerves in the hen oviduct (Costagliola *et al.*, 1997).

Renegar and Rexroad (1990) reported in sheep uterus that adrenergic and cholinesterase positive fibers in myometrium and endometrium innervated the blood vessels, while the smooth musculature exclusively supplied by the adrenergic fibers during anestrus. The fibers of both types were closely associated with endometrial glands; the adrenergic fibers supplied the

Table 1: The comparison of distribution of acetylcholinesterase positive nerves and fibers in the chicken oviduct

Segment of the oviduct	Positive structures in lamina propria		Positive structures in muscularis	
	Proportion of positive neurons	Staining intensity	Proportion of positive neurons	Staining intensity
Infundibulum	++++	+++	++++	++++
Magnum	+++	+	+++	+++
Isthmus	+	++	+++	+++
Uterus (Shell gland)	+++	++	+++	+++
Vagina	+	++	++	++

Proportion of positive neurons: ++++ Very numerous; +++ numerous; ++ moderate; + few; Staining intensity in nerve fibers: ++++ Very intense; +++ intense; ++ moderate; + weak.

submucosal connective tissue. Tomita *et al.* (2010) determined the functional differences of enteric innervation between jejunum and ileum in normal humans and reported that cholinergic nerves are mainly involved in regulation of the jejunum rather than the ileum. While, the ileum was more strongly innervated by non-adrenergic non-cholinergic (NANC) inhibitory nerves than the jejunum. Sugasawa *et al.* (2002) correlated the changes at different stages of the reproductive cycle in the innervation patterns of uterine NO and AChE positive nerves to the adrenergic and cholinergic controlling mechanisms of uterine function regulation in Japanese long-fingered bat. Thus it is clear that variations in the innervations are common in different organs/systems, and at various stages of the reproductive cycle.

It has been reported that in the chicken vas deferens single cholinergic nerve fibres penetrate between the epithelial cells (Sadanowicz *et al.*, 2004). We also found such thin cholinergic fibers that ascend to innervate the primary, secondary or even tertiary mucosal folds in the infundibulum, magnum and isthmus of hen oviduct.

Conclusion: The present study provides the qualitative evidence on the distribution patterns of AChE neurons in different regions; in addition, the number is also variable in the lamina propria and muscularis even within the same region of the oviduct in laying hens. The regional variations in the population of cholinergic effector system might be indicative of the different roles in the process of the egg formation and transport through the hen oviduct.

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