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RESEARCH ARTICLE

# Histochemical Localization of the Cholinergic and Nitrergic Neurons in the Chicken Ileum

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**ABSTRACT**

Histochemical localization and analysis of the cholinergic and nitrergic neurons in the chicken ileum were investigated by staining with acetylcholine esterases (AChE) and nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), respectively. AChE and NADPH-d activity was demonstrated in neuronal cell bodies and nerve fibers in the chicken ileum. The positive neurons showed irregular or polygonal shape and were mainly present sporadic or clumped in the myenteric and submucosal plexus. The positive nerve fibers frequently surrounded the ileac blood vessels. They were abundantly present in myenteric and submucosal plexus of the ileum forming a network. Some positive nerve fibers traversed the submucosa into the lamina propria mucosae. Fine nerve fibers were found to penetrate into intestinal villi underneath the epithelium. Extensive networks of more intensely staining AChE positive nerve fibers were present in the mucosa as compared to that of NADPH-d positive fibers. Ganglia density of submucosal plexus was markedly bigger than that of myenteric plexus, whereas neurons per ganglion and the number of neurons per $mm^2$ and the size of neurons of submucosal plexus were shorter than that of myenteric plexus. In addition, the number of AChE positive neurons and nerve fibers was more than that of NADPH-d positive neurons and nerve fibers. We concluded that the chicken ileum is characterized by abundance of nerve structures which may play a significant functional role in ileum of the chicken.



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## INTRODUCTION

The enteric nervous system (ENS) comprises of thousands of ganglia and neurons that lie within the walls of gastrointestinal tract (GIT). The nerve fibers that connect these ganglia, and nerve fibers that supply the muscle of the gut wall, the mucosal epithelium, arterioles and other effector tissues, in addition to this ganglion and nerve fibers form an intricate network, capable of reflex control of gastrointestinal muscle activity independent of the central nervous system (VeeremanWauters, 1996). Because of its extent and its degree of autonomy, the ENS has been referred to as a second brain (Wood *et al.,* 1999).

Enteric neurons secrete more than 20 neurotrans-mitters (Nezami and Srinivasan, 2010). One major neurotransmitter produced by enteric cholinergic neuron is acetylcholine. In general, cholinergic neurons that secrete acetylcholine are excitatory, stimulating smooth muscle contraction, increases intestinal secretions, release of enteric hormones and dilation of blood vessels (Harrington *et al.,* 2010; Vincent, 2010). However, nitric oxide is considered to be one of the most important neurotrans- mitters of nonadrenergic and nitrergic inhibitory neurons of ENS (Taguchi *et al.,* 1999), which is responsible for mediating relaxation of smooth muscles and blood vessels (Vincent, 2010; Jing *et al.,* 2011).

It was evidenced that nitric oxide synthase (NOS), the marker enzyme of nitric oxide which catalyzes L-arginine to produce nitric oxide, was a NADPH-d and the localization of nitric oxide could be revealed by NADPH-d histochemistry (Grozdanovic *et al*., 1992). In addition, the marker enzyme of acetylcholine was acetylcholine esterase, and the localization of acetylcholine could be revealed by acetylcholine esterase histochemistry (Maifrino *et al.,* 1997). At present, Cholinergic and nitrergic neurons are best characterized by the histochemistry of the synthesizing enzymes such as acetylcholine esterase (AChE) and neuronal NOS, this

methods were reliable and operable (Ramachandran *et al.,* 2007).

The presence of AChE and NOS in the ENS has been shown in various segments of the gut of many mammalian species (Junquera *et al*., 1998; Maifrino *et al*., 1999; Kluchova *et al*., 2002; Lalatta-Costerbosa *et al*., 2007), but only sporadically in developing birds (Balaskas *et al*., 1995; Bagyánszki *et al.,* 2000) . Therefore the present study was performed to investigate indirectly the occurrence, distribution patterns and morphological features of cholinergic neurons and nitrergic neurons in the chicken ileum by using histochemistry to demonstrate AChE and NADPH-d activity.

## MATERIALS AND METHODS

All protocols were approved by the Chinese Committee for Animal Use for Research and Education.

**Tissue preparation:** Ten adult male or female tender chickens with an age of 65 days and weight of 1.5-2.0 kg were purchased from Nanjing Farm product market (Nanjing, Eastern China). For the histochemical demonstration of AChE and NADPH-d, fresh tissues of the chickens were prepared as a series of frozen sections (thickness, 30μm) and whole-mount preparations.

NADPH-d histochemistry: For NADPH-d histochemistry, the section was incubated in 1 $mg.mL^{-1}$ β-NADPH (SIGMA, Dorset, UK), 0.25 $mg.mL^{-1}$ nitroblue tetrazolium (Himedia, Mumbai, India) and 0.3% Triton-X in 0.05 $mol.L^{-1}$ Tris-HCl buffer (pH 7.6) for 48 h. The process was stopped by washing the specimens in PBS (phosphate buffered saline) for 15 min. For specificity, β-NADPH was omitted from incubation medium in control incubations. Staining of neurons was nerve observed in these specimens.

AChE histochemistry: Staining for AChE was based on the method of Karnovsky and Roots as modified by Hanker and colleagues. This involved incubating of the sections in a mixture of two stock solutions, respectively. Stock solution A was made of acetylcholine iodide (BDH, UK) in 0.1 M acetate buffer (pH 6.0), sodium acetate, copper (II) sulphate anhydrous distilled water and tetra isopropyl pyrophosphoramide (Sigma, Germany). Stock solution B consisted of potassium ferricyanide in water. Incubation was performed for 4 h.

**Microscopy and statistics:** All specimens stained with AChE and NADPH-d was initially viewed using conventional light microscopy. Photographs were taken of every section of the specimen. Photographs were taken at 40–400 magnification using a Leica Microsystems microscope. The ganglionic and neuronal densities were calculated as the numbers of positive ganglia or stained neurons per $mm^2$ of the whole-mount area, the number of positive neurons per ganglia and the size of positive neurons were also calculated. All numerical data are expressed as means ±standard deviation (SD).

## RESULTS

In frozen sections, the AChE and NADPH-d histochemistry showed that the NOS positive neurons were blue homogenous sediments, and AChE positive neurons were buffy homogenous sediments. AChE and NADPH-d activity was detected in neuronal cell bodies as well as in nerve fibers at various levels of the wall of the chicken ileum (Fig. 1 A, B). NOS positive nerve fibers showed clear varicosities, whereas varicosities could not be identified in AChE positive nerve fibers (Fig. 2 B,H). We observed large populations of AChE and NOS positive neurons in both myenteric and submucosal plexus. Meanwhile, some positive neurons were also found within longitudinal and circular muscle layer. The positive nerve fibers run along the smooth muscle cells of the longitudinal and circular layer (Fig. 1). Several AChE and NOS positive nerve fibers were located frequently in the vicinity of ileac blood vessels (Fig. 2 C,D,I,J). The neurons at this level were irregular or polygonal in shape with clearly visible processes, and appearing as a sporadic cell or small groups of cells connecting by their process, forming ganglia (Fig. 2 B, E, F, K, L). Nerve fibers that expressed AChE and NADPH-d activity often penetrated into the circular layer from the ganglia of myenteric plexus as a bundle and were connected with positive neurons (Fig. 2 A, G). Histochemically positive nerve fibers for AChE and NADPH-d staining, emerged from reactive cell bodies present in the submucosa (Fig. 1 C, D, F, G). Arrangements of positive neurons in submucosal plexus were similar to those of neurons in myenteric plexus; they were also present as sporadic cells or clumped. Positive nerve fibers penetrated from neuron bodies of the submucosal plexus through the lamina muscularis mucosae into the lamina propria mucosae to form dense mucosal nerve plexus. Several positive nerve processes of the submucosal nerve cells were found underneath the epithelium (Fig. 1 E, H). The distribution of AChE positive nerve fibers was clearly more extended than those of NOS positive nerve fibers in the mucosa (Fig. 1 E, H). In addition, nitric oxide positive neurons were found in the mucosa of the ileum (Fig. 1 E, H).

In the whole-mount preparations, the ganglia and nerve fiber bundles in intestinal myenteric and submucosal plexus of the chicken ileum could be seen forming clearly meshwork (Fig. 3). The length of nerve fiber bundles were clearly shorter in submucosal plexus so that meshwork looked more close (Fig. 3 B, D). The ganglia were fusiform. In myenteric plexus, the meshwork showed a tendency of parallel arrangement, vertical to longitudinal smooth muscle, and some nerve fibers could be found within longitudinal muscle layer, parallel to longitudinal smooth muscle (Fig. 3 A, C). In submucosal plexus, the meshwork showed also a tendency of parallel arrangement, vertical to circular smooth muscle, and large numbers nerve fibers could be found within circular muscle layer, parallel to circular smooth muscle (Fig. 3 B, D).

In myenteric plexus, the number of AChE positive neurons per ganglion and the number of AChE positive neurons and ganglia per $mm^2$ were 46.59±13.37, 91.65±19.82 and 1.96±0.48, respectively. The number of NOS positive neurons per ganglion and the number of NOS positive neurons and ganglia per $mm^2$ were 30.59±8.16, 78.55±15.29 and 1.82±0.32, respectively. In submucosal plexus, the number of AChE positive neurons per ganglion and the number of AChE positive neurons and ganglia per $mm^2$ were 12.24±6.28, 47.91±11.26 and

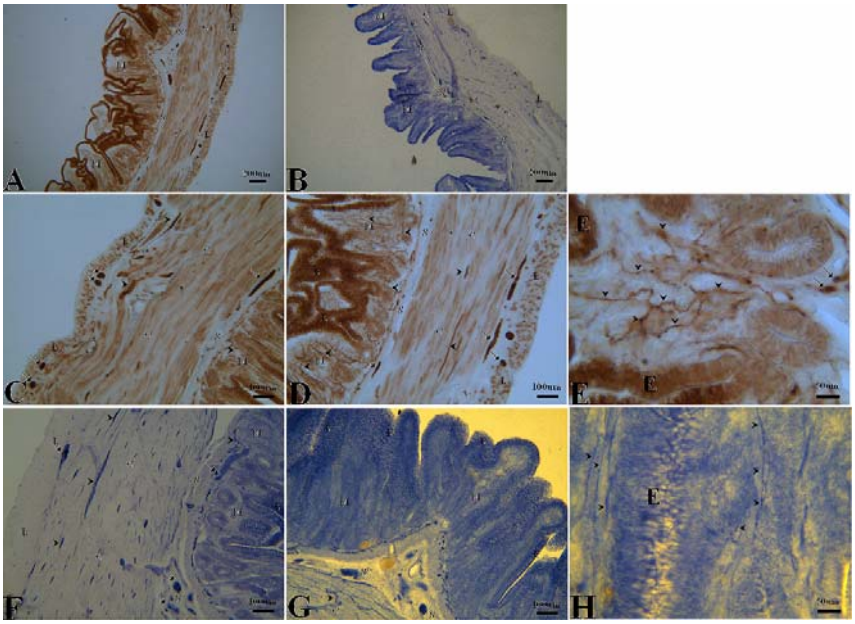

**Fig. 1:** Histochemical localization of AChE and NOS positive neurons and never fibers in the chicken ileum. A,B-AChE and NADPH-d activity was detected in neuronal cell body (long arrow) as well as in nerve fibers (short arrow) in various layers of the wall of the chicken ileum. C, D, F, G-Large amounts of AChE & NOS positive neurons (long arrow) & never fibers (short arrow) were observed in both muscular & submucosal layers. Positive never fibers run along the smooth muscle cells of the circular layer. E, G- Fine positive nerve fibers (short arrows) that form an extensive plexus in the mucosa (m) and are located adjacent to the epithelium (E) of the chicken ileum. The distribution of AChE positive nerve fibers (E) was clearly more extended and strong than that of NOS positive nerve fibers (G) in the mucosae. No positive nerons were found in the mucosa of the ileum. (L: longitudinal muscle layer; C: circular muscle layer; S: submucosal layer; M: mucosa; E: epithelium) Scale bars: A,B: 200µm; C, D, F, G:100µm; E, G: 50µm.

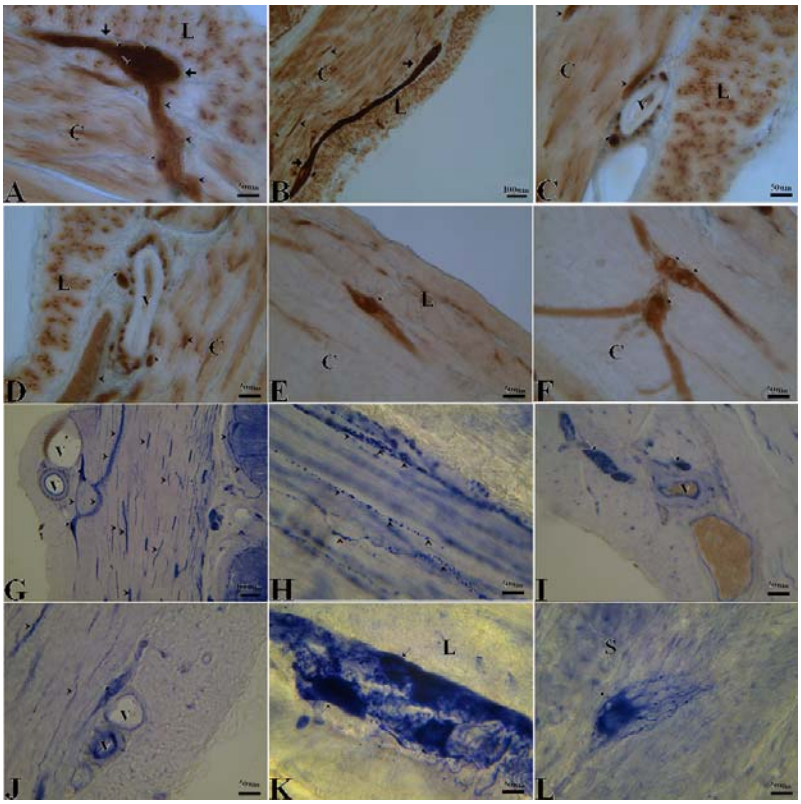

**Fig. 2:** A, G -clumped AChE and NOS positive neurons (long arrow) of myenteric plexus which are connected with nerve fibers (short arrows) penetrating the circular muscle layer (C) of chicken ileum. B-clumped AChE positive neurons (long arrow) connecting by their process form a ganglia in myenteric plexus. C, D, I, J-A lot of AChE & NOS positive nerve fibers were frequently in the vicinity of ileal blood vessels (V). E-shows a AChE positive neurons in myenteric plexus. F-shows clumped AChE positive neurons within circular muscle layer (C). H-NOS positive nerve fibers had clear varicosities (short arrow). K- shows clumped NOS positive neurons connecting by their proces form a ganglia in myenteric plexus. D shows a NOS positive neuron in submucosal layer. (L: longitudinal muscle layer; C: circular muscle layer; S: submucosal layer) Scale bars: A, C, D, E, F, H, I, J, K, L: 50µm; B, G:100µm.

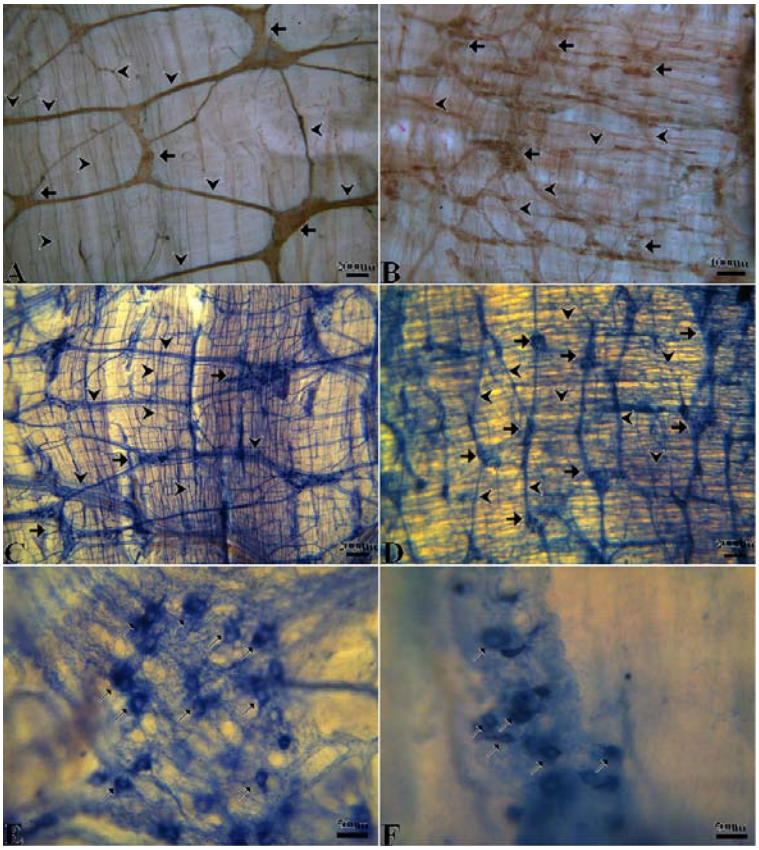


**Fig 3:** shows whole-mount preparations of the myenteric and submucosal plexus of chicken ileum after AChE and NADPH-d staining. The meshwork of nerve bundles with ganglia containing positive neurons is clearly seen. The ganglia with different size (long arrows) are interconnected by short nerve fiber strands (short arrows). A,C-whole-mount preparations of the ileac myenteric plexus after AChE and NADPH-d staining. B, D-whole-mount preparations of the ileac submucosal plexus after AChE and NADPH-d staining. E-the myenteric ganglia contain NOS positive neurons (thin arrows) in the chicken ileum, NOS positive neurons have many thin long processes. F-the submucosal ganglia contain NOS positive neurons (thin arrows) in the chicken ileum. Scale bars: A, C, D: 20μm; B: 100μm; E, F: 50μm.

**Table 1:** Distributions of AChE and NOS positive neurons and nerve fibers observed in the chicken ileum as demonstrated by histochemistry for AChE and NADPH-d activity (densities of activity for AChE and NADPH-d were rated as follows: +++ high, ++ moderate, + low, – absent; M mucosal layer, SP submucosal plexus, CM circular muscle layer, MP myenteric plexus, LM longitudinal muscle layer)

| | LM | MP | CM | SP | ML |
|---|---|---|---|---|---|
| NOS positive neurons | + | +++ | + | +++ | – |
| NOS positive Nerve fibers | + | +++ | ++ | +++ | + |
| AChE positive Neurons | + | +++ | ++ | +++ | – |
| AChE positive Nerve fibers | ++ | +++ | ++ | +++ | ++ |

6.89±0.84, respectively. the number of NOS positive neurons per ganglion and the number of NOS positive neurons and ganglia per $mm^2$ were 10.12±4.27, 33.69±11.51 and 7.18±0.96, respectively. The sizes of AChE and NOS positive neurons in myenteric plexus were 270.37±40.51 $\mu m^2$ and 279.18±50.42 $\mu m^2$, respectively and were 209.73±21.51 $\mu m^2$ and 218.49±29.32 $\mu m^2$ in submucosal plexus, respectively (Fig. 3 E,F).

The distribution patterns of two positive neurons and nerve fibers in the ileum are summarized in Table 1 to make clear the differences and similarities among the different portions.

Morphologically, the AChE and NOS positive neurons and nerve fibers appeared similar at all levels in the chicken ileum. However, the number of AChE positive neurons was more than NOS positive neurons, meanwhile, AChE positive nerve fibers were wider and more extensive than NOS positive nerve fibers.

## DISCUSSION

Since the excitatory cholinergic innervation and the inhibitory nitrergic innervation are major regulators of gut motility. Histochemical staining techniques with acetylcholine esterase (AChE) and NADPH-d offer the best option to study the enteric cholinergic and nitrergic neurons of the gut (Maifrino *et al*., 1999; Ramachandran *et al*., 2007; Vincent, 2010). We used this technique to investigate the detailed distribution of the neurons and nerve fibers in the chicken ileum for the first time. Moreover, the morphology and size of neurons and and neuronal density in the myenteric and submucosal plexus has also been demonstrated.

Previous studies demonstrated that the histochemical localization of AChE and NOS activity can be exactly applied for detection of neurons in the wall of intestine of mammalian species (Junquera *et al*., 1998; Liu *et al*., 2007; Li and Ru, 2009). But they focused on the distribution of AChE and NOS positive neurons and ganglia in the myenteric plexus and the submucosal plexus, Little attention were paid to a detailed and comprehensive regional localization of neurons and ganglia. The study of ENS should be based on

coordination of neurons in different planes. Thus, we tried to provide a better study on ENS connecting conventional sections and whole-mount preparations.

Junquera *et al,* (1998) reported that the AChE and NOS positive neurons varied from irregular or polygonal in shape in rabbit intestine, in addition, the size of AChE and NOS positive neurons were different with other animals (An *et al*., 2003; Montedonico *et al*., 2006; Cserni *et al*., 2007; Liu *et al.*, 2007). Some studies revealed small intestine has a well-developed plexus and ganglia are aligned along the longitudinal axis of the gut and have a highest density in ileum (Liu *et al.,* 2007). Li *et al.* (2009) also found the density of cholinergic, SP, VIP-Peptidergic and nitrergic neurons were highest in ileum, and the density of cholinergic neurons was highest as compared to others. It could be concluded that precise functions the cholinergic and nitrergic neurons play in ileum was different among species. This also may be due to different ileum structure among species, or it is possible for the eating habit and diet composition. Comparing with other species, the number of neurons and ganglia in rabbit ileum was relatively low whereas was high in the mandarin vole (An *et al*., 2003). Our study showed a fairly moderate neuronal density in chicken ileum. This indicates that the morphology of AChE and NOS positive neurons and arrangement of the ganglia are variable among species.

Balaskas *et al.* (1995) revealed distribution of NADPH-d activity in the embryonic chicken gut and found NADPH-d activity could be detected in neuronal cell bodies as early as embryonic day 5.5 in the foregut, whereas NADPH-d positive fibers were found at embryonic day 9.5. In addition, they pointed out NADPH-d positive submucosal neurons were first detected at embryonic day 11.5 in chicken. A similar study was also carried out by Bagyanszki *et al.* (2000). Thus the functional integration of the ENS is important, both, in the embryonic age and the adult life for the normal regulation of the gut motility functions.

The present study showed AChE and NOS positive nerve fibers of submucosal ganglia in the chicken ileum penetrate the lamina muscularis mucosae into the lamina propria mucosae frequently along vessels forming the mucosal nerve plexus. This is similar to the previous findings (Van Ginneken *et al.,* 1998; Thippeswamy and McKay, 2005). Meanwhile, we found the distribution of AChE positive nerve fibers was clearly more extensive and stronger than that of NOS positive nerve fibers in the mucosae. It can be concluded that the excitatory cholinergic innervation play a more significant role in the mucosae than the inhibitory nitrergic innervation and both have regulatory functions in the blood flow.

The present study also showed the distribution pattern of AChE and NOS positive neurons and nerve fibers was basically same in the muscular and submucosal layers of the ileum. This distribution corresponds to the function of gut that the myenteric plexus innervates the longitudinal and circular muscle layers, and also has neural connections with the submucosal plexus and the mucosa. The submucosal plexus innervates the mucosa, submucosal vessels, and the circular muscle layer, as well. Previous studies showed that AChE and NOS positive neurons could cooperatively regulate the functions of the gut and it can be concluded that the gut motility depend on precise cooperation of different types of neurons both excitatory and inhibitory (Wood *et al.,* 1999; Lomax and Furness, 2000).

At present, morphological and structural abnormalities in the innervation of the gastrointestinal tract have been widely researched in mammalian (Ramachandran *et al*., 2007; Rolle *et al*., 2002). On the other hand, though there are many disease conditions of the gastrointestinal tract related to the disorders of ENS (Hafez, 2011), but these conditions have rarely been investigated.

In conclusion, the present study provides the qualitative information on the distribution pattern of AChE and NOS positive neurons in the wall of chicken ileum. It also offers valuable insights into comparative distribution of the two major effector subpopulations of the ENS in the ileum of the adult chicken.

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