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RESEARCH ARTICLE

Developmental Changes in Morphology and Distribution of AChE Positive Neurons in the Submucosal Plexus of the Chicken Ileum

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ABSTRACT

The distribution of different subpopulations of enteric neurons in the developing enteric nervous system has been extensively examined in various animals, but up to date, there is no reference found regarding with the normal cholinergic neuronal density and morphology in the submucosal plexus of the developing chicken. In the present study, the fresh specimens were collected at day old, 5, 10, 20, and 40 days chickens respectively, for the whole-mount preparations of the submucosal plexus of chicken ileum. Acetylcholinesterase (AChE) histochemistry was performed. The morphology of the network, cytoplasmic area, the density of the ganglia, and number of the ganglion cells per ganglia was measured. Our results reported that the positively stained neurons have various morphologies and staining intensity. They formed a clear network by ganglia and nerve fibers. The number of ganglia per unit area decreased with age, however the size of positive neurons increased, as the size, in 40 day old group was 3 times than that of the day old group. Change the number of positive neurons per ganglia was dynamic, but the tendency was increased from 10-days old to adult chickens. It was concluded that the significant developmental changes occur in postnatal life of chicken ileal submucosal plexus. The changes of the cholinergic neuronal subpopulation are similar than it has been found in the nitrergic subpopulation in different animal models.

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INTRODUCTION

The diagnosis of Hirschsprung's disease (HD) in children only depends upon the histological observation of the presence of enteric ganglion cells in the submucosa up to date (Martucciello *et al*., 2001; Skaba *et al*., 2006; Piaseczna-Piotrowska, 2007). As our information about the enteric nervous system increases, it is not sufficient to determine only the presence of enteric ganglion cells, but also need to determine their correct number and types of ganglion cells (Montedonico *et al*., 2006). The number of ganglion is counted in the specimen for the confirmatory diagnosis of hypoganglionosis. For the diagnosis of Intestinal Neuronal Dysplasia (IND), the number of ganglion cells increases 5-7 in large ganglion (Kapur, 1999). Pervious data further signifying that the ratio of the different subpopulation of enteric neurons, glial cells may affect the motility disturbance like IBD, diabetes etc (James *et al*., 2004; Gao *et al*., 2008). Find out the comparison between the two most important subpopulation of neurons, the Cholinergic and nitrergic neurons has been found significantly in immature myenteric plexus while less prominent in matured one (Cserni *et al*., 2007). These evidences propose that quantification of the various subpopulations of enteric neurons within the ENS has significance for the understanding motility disorders and leading way in the future for further development in diagnosis.

The distribution of different subpopulations of enteric neurons in the developing enteric nervous system has been extensively examined in various animals (Junquera *et al*., 1998; Maifrino *et al*., 1999; Pauza *et al*., 2002; Greggio *et al*., 2010), but up to date, there is no reference found regarding the normal cholinergic neuronal density and morphology in the submucosal plexus of the developing chicken. However the cholinergic neurons are representing one of the largest subpopulation of the enteric neurons, the submucosal plexus is one of the major

plexuses and the chicken is cheap and easily available for the experiment.

MATERIALS AND METHODS

All apparently healthy broiler chickens of either sex were purchased from a commercial Poultry Farm in Nanjing. Ileum specimens were obtained from a-day (20-30 g), 7 (90-100 g), 15 (400-420 g) and 40 days old (adult, 1800 g) broiler chicken. The 5 broiler chickens of each age group were selected. All the experiments abide by the regulations of the Chinese Committee for Animal Use for Research and Education. Then these segments were washed with 0.1 mol/L phosphate buffered saline (pH 7.3), ligated at the two ends, and fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffered saline (PBS) (pH 7.3) for 1.5-2.5h at 4°C. After being fixed, the segments were cleared with two rinses in 0.1 mol/L of PBS for 10 min, and stored overnight in PBS at 4°C. Lastly, the segments were opened along the mesenteric border, sheared into 10 cm long segments. Samples were pinned flat on a silicone plate, and whole-mount preparations were made under a dissecting microscope (Olympus S261; Japan). Initially, the mucosal layer was scraped out along with the inner submucosal plexus using fine-pointed forceps. After that, the submucosal layer containing the outer submucosal plexus was carefully separated from the muscle layer with a fine forceps starting in one corner of the tissue sample.

The preparations were then put in PBS to prepare for Acetylcholine esterase (AChE) histochemistry: Staining for AChE was based on the method of Cserni and colleagues (Cserni *et al*., 2009a). This involved incubating of the sections in a mixture of two stock solutions, respectively. Stock solution A was made of acetylcholine iodide (Sigma, Germany) in 0.1 M acetate buffer (pH 6.0), sodium acetate, copper (II) sulphate anhydrous distilled water and tetraisopropyl pyrophosphoramide (Sigma, Germany). Stock solution B consisted of potassium ferricyanide in water. Incubation was performed for 4h.

The strongly buffy labeled cells and weak positive were regarded as positive cells. The cells have bodies of same color in the background, cell out lines were seen dimly even the nucleus was obvious, were confirmed as negative cells. However, data were presented only for the positive cells. A standard line was calibrated to scale on the glass slide, and the pictures were taken at different magnifications (the total length=1mm; the smallest scale=10μm). These pictures were then imported to the Image-Pro Plus 6.0 software (IPP version 6.0). This was used as a standard to measure the different size parameters, such as the cell body diameter, microscopic field etc. All statistical data were obtained by the software of IPP as the method of Yang and colleagues (Yang *et al*., 2011; Yang *et al*., 2013).

Statistical analysis: The analysis of variance test was used to compare ganglia density, number of ganglion cells per ganglia, and ganglion cell size among the different segments with the Student-Newman-Keuls test being used for pair wise comparisons. The P value of less than 0.05 was considered statistically as significant. All statistical tests were performed using a commercially available software package (SPSS 16.0 Statistical Analysis Software, Chicago, IL).

RESULTS

AChE positive neurons and nerve fibers were spread widely distributed in the submucosal plexus of the chicken ileum. Positive ganglia and positive nerve fibers formed irregular network. Ganglia appeared long shuttle shaped with different sizes. Positive neurons send nerve fibers formed positive nerve fibers, a nerve fiber direction and longitudinal muscles beam vertically. Nerve fiber bundle branched continuously to form secondary and tertiary bundles (Fig. 1). AChE positive neurons showed larger nuclei, less coloration, cytoplasmic staining shades. Neurons appeared round, oval, triangular and irregular in shape. In the 40 days of age group, about above 2/3 neurons were strong coloring, other were generally or lower. With the age increased, the proportion of the more strong staining were found. We define the color has the obvious difference in the background is positive cells.

The network morphology of AChE positive ganglia and nerve fibers in the submucosal plexus is different with ages (Fig. 2). The density of AChE positive ganglia showed a decreasing trend with increasing age, and the network morphology looks more and more loose. Lowest density of ganglia was found in 40 days of age group and highest in 1 day old group (Table 1). However, the size of AChE positive neurons showed a increasing trend with age (Fig. 3). Lowest size of neurons was found in 1 day of age group and largest in 40 days old group (Table 1). With the growing age, the change of the number of AChE positive neurons per ganglia did not follow a fixed tendency in the submucosal plexus. In 1 day old of age, the number was 3.87±1.29 neurons per ganglion, then the number up to 8.29±3.78 neurons per ganglion in 5 days of age, in 10 days of age the number was reduced to 5.34±2.61 neurons per ganglion. From the beginning of 10 days age, the tendency of the number of AChE positive neurons per ganglion was increasing gradually (Table 1).

DISCUSSION

It is important to understand the normal morphology of enteric nervous system during postnatal period and to analyze the change trend of ganglion and neuron density in the diagnosis of gastrointestinal diseases (Wester *et al*., 1999; Bagyanszki *et al*., 2000; Heanue and Pachnis, 2007). Generally, the initial biopsy of the mucosa and submucosa is used for the identification of early mammalian development of chronic constipation and bowel obstruction (Meier-Ruge *et al*., 1995; Sjölund *et al*., 1997). In addition, the submucosal plexus display is the main clinical cause of the development of Hirschsprung's disease, intestinal neuronal dysplasia and other intestinal motility abnormalities (Fukudo *et al*., 2014). But in the case of a chicken intestinal nervous system, relationship between gastrointestinal diseases and enteric nervous system is potentially weak. Different techniques have been used to quantify ganglia and neurons in the enteric nervous system. In the clinical practice the most popular staining methods for motility disorders are hematoxylin & eosin staining (HE), AChE and NADPH-d enzyme-

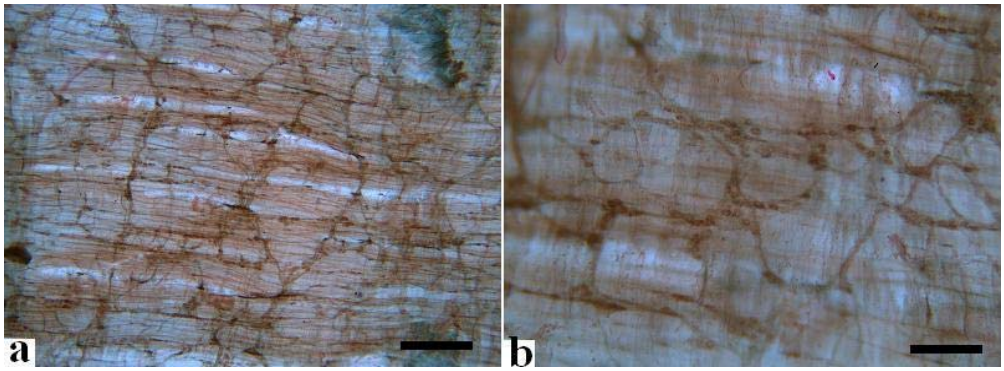

Fig 1: Whole-mount preparations of the submucosal plexus of the chicken ileum; (a) A mesh of nerve bundles with ganglia containing AChE positive ganglion cells at the intersections is clearly seen (Bar=200µm). (b) Typical AChE positive submucosal ganglia (Bar=100µm).

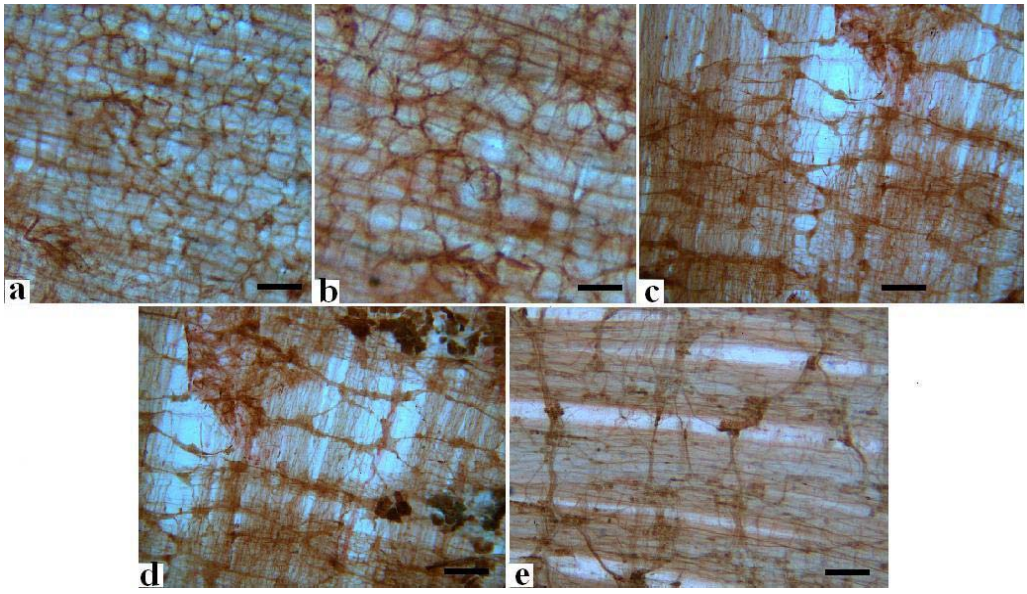

Fig 2: Whole-mount preparations of the submucosal plexus of the chicken ileum in different age groups; (a) 1-day old group. (b) 5-days old group. (c) 10-days old group. (d) 20-day old group. (e) 40-days old group. Bar=200µm.

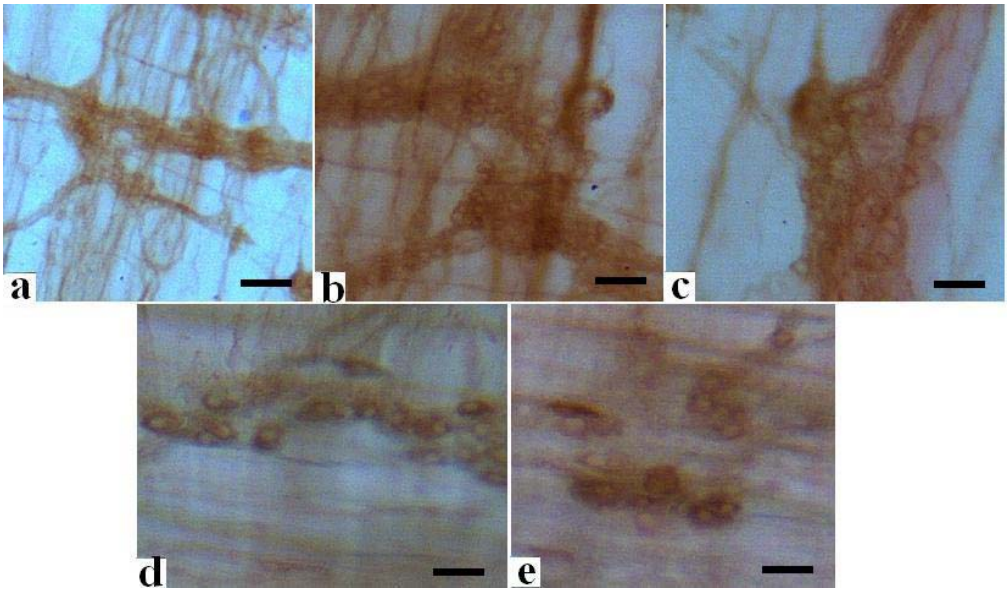

Fig 3: AChE positive neurons in the submucosal plexus of the chicken at different ages AChE positive neurons increase their size throughout life predominantly because of an increase in cytoplasm.(a) 1-day old group. (b) 5-days old group. (c) 10-days old group. (d) 20-days old group. (e) 40-days old group. Bar=30µm.

Table 1: Number of AChE positive neurons per unit area and the neurons per ganglia, and the size of the AChE positive neurons the ileum of different age groups

Age (Days)	Neurons/ mm^2	neurons/ ganglion	Size of neurons (μm^2)
1	65.78 ± 11.27^{a}	3.87 ± 1.29^{a}	66.78 ± 8.38^{a}
5	37.42 ± 5.42^{b}	8.29 ± 3.78^{b}	90.29 ± 15.86^{b}
10	15.32 ± 2.89^{c}	5.34 ± 2.61^{ab}	121.76 ± 18.37^{bc}
20	10.25 ± 1.27^{d}	10.89 ± 3.98^{bc}	159.24 ± 19.65^{c}
40	6.89 ± 0.84^{e}	12.24 ± 6.28^{bc}	209.73 ± 21.51^{d}

Values (mean±SD) bearing different superscript in a column differ significantly ($P<0.05$).

histochemistry. The HE staining is non-specific as it stains all neurons and glial cells in the ganglia, and it is mostly used by experienced pathologists to assess the presence of ganglia for example, to diagnose HD (Meier-Ruge *et al*., 1995), but this staining is not suitable to quantify neurons. NADPH-d staining is believed to stain nitrergic neurons. Nitrergic neurons are representing one of the largest subpopulation of enteric neurons. NADPH-d histochemistry is used in the clinical practice and was very popular in different studies to quantify numbers of neurons, probably because it gives a nice sharp contrast image. However the staining intensity depends upon the NADPH-d content of the cells and incubation time (Cserni *et al*., 2009a) and motor neurons and interneurons as well as glial cells may be stained.

AChE staining is also popular in the clinical practice to diagnose HD and IND (Qualman *et al*., 1999; Tomita *et al*., 2003). It is rarely used in research studies for quantification, probably because it results in a less sharp contrast image compared to NADPH-d staining. However the cholinergic neurons represent a very important and large subpopulation of neurons, similar to the nitrergic neurons.

There are certain limitations in the observations of ENS of tissue sections, like the neurons are cells with cytoplasmic projection; it's hard to track their processes and nerve fibers. Moreover, the ENS neurons often appear as clumps and observed in the form of giants, making difficulty in quantitative analysis. The muscle layer and the submucosa preparations of neurons can be largely overcome these difficulties, at the same time, application of IPP significantly reduced the error due to the traditional method, so the experiment results are more objective and accurate. We use the chicken as the experimental animal, firstly as chick is a good experimental model to study the origin, and development of ENS on the chick embryo has been referred by the animal researchers, but little research has been done on embryo development, the postembryonic development is similar to other mammals giving the good indicators of the chick embryo model. Secondly chicken has great economic value, there are some differences between the mammalian intestinal structure and chicken, chicken ENS to clarify the gastrointestinal diseases and species diversity. There is a great significance. AChE histochemical staining is recognized as the effective method of ACh display. AChE is a ACh hydrolase, enhanced AChE activity represents increased ACh release, cholinergic neuronal excitability, there are reports that AChE positive neurons accounted for about 45% of all neurons in ENS.

The statistical results show, the submucosal plexus network obviously become sparse density and ganglia becomes smaller gradually with ages. The changing trend of nerve in the other species of myenteric and submucosal plexus, or bladder and esophagus are also found (Doxey *et al*., 1995; Hryhorov *et al*., 2004; Montedonico *et al*., 2006; Cserni *et al*., 2009a). One possible explanation is that intestinal luminal growth causes the nerve networks to expand and thus the ganglia density decreases.

The number of ganglia per unit area decreased with age, however the size of positive neurons increased, like the size in 40-days-old group was 3 times than that of the day old group. The change of number of positive neurons per ganglia was dynamic, but the tendency was increased from 10-days-old to adult. These results are similar to the nitrergic neurons revealed by other researchers along the development (Montedonico *et al*., 2006; Sri Paran *et al*., 2008; Cserni *et al*., 2009b) in different species. It suggests that the relation of the major subpopulations of neurons is not changing significantly. It is also remarkable that these changes are similar in the myenteric and submucosal plexuses and they are quite uniform in different species from pig to chicken.

In the chicken intestinal submucosal layer, the neural growth and development is dynamic from birth period to adulthood that is linked to the clinical period, which is more prone to early born enteric nervous system disease. If the neurons have some developmental problems it will lead to a series of gastrointestinal disorders, such as intestinal neuronal dysplasia and Hirschsprung's disease (Kapur, 1999; Heanue and Pachnis, 2007). Our studies give the picture of developmental changes of submucosal plexus in chicken, provides the certain criteria in gastrointestinal tract of chickens caused by nervous disorders.

Author's contribution: QC and PY conceived and designed the experiment. PY, YL, YW and NA performed the experiment and analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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