Histogenesis of enteric ganglia in human fetal stomach.

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Abstract

Introduction
The Enteric nervous system (ENS) is a network which contains reflex circuits that detect the physiological condition of the gastrointestinal tract, integrate the information, and provide outputs to control gut motility, exocrine and endocrine secretions, microcirculation, immune and inflammatory processes. Elucidation of the mechanisms of ENS development and function allow the development of new approaches to the diagnosis, therapy, and prevention of human disorders of gastrointestinal motility. Therefore it is essential to understand the normal development of the ENS in human during prenatal period. This study henceforth aimed to determine the histogenesis of the enteric neurons.

Materials and methods
This study was done on human fetuses to evaluate the histogenesis of the enteric neurons at various gestational ages (10-28 weeks) by H&E, Masson’s Trichome Silver impregnation and immunohistochemistry for synaptophysin.

Results
The earliest fetus studied in the present study was 10-12 weeks by which most of the neural crest cells have migrated from towards the developing gut. The neural crest derived cells were arranged as a band of scattered neurons in the serosa on the outer aspect of the developing muscularis externa where the myenteric plexus would finally form. The migrating neurons moved from the periphery of the developing muscularis externa towards the submucosal region traversing through connective tissue pathways through the muscle layers. Immunohistochemistry for synaptic marker synaptophysin was positive first at 28 weeks of gestation.

Conclusion
As the stomach progressed through the gestational ages, the cells became aggregated into more organized ganglionic groupings from a scattered collection of neurons at the beginning of the second trimester. Submucosal plexus was seen to secondary to the myenteric plexus. Progressive organization was seen in the myenteric and submucosal plexuses during various gestational ages. By 28 weeks of gestation, the human stomach showed organized enteric neurons positioned at intermuscular and submucosal regions with a positive for synaptic vesicle protein synaptophysin.

Introduction
Disorders of the ENS may result in motor, secretory, and inflammatory and immunologic dysfunction of the gut. The disorders can range from either a deficiency or degeneration of the enteric neurons characterized by disturbances in gastrointestinal transit or propulsion; or pathologic excitation of motor or secretomotor enteric reflexes by toxins and inflammatory mediators which may appear clinically as secretory diarrhoea.¹

One of the common motility disorders affecting 1 in 1500 live births is Hirschsprung disease. Hirschsprung disease is a congenital intestinal aganglionosis which is characterized by complete absence of neuronal ganglion cells from a portion of the intestinal tract. The diagnosis of Hirschsprung disease requires histopathologic demonstration of absence of enteric ganglion cells in the distal rectum. (Parisi MA) Accessory findings include hypertrophic submucosal nerves and/or an abnormal acetylcholinesterase enzyme staining pattern.²

The aganglionosis in a variable length of the distal gut in Hirschsprung’s disease results from the abnormal prenatal development of neural crest cells of the enteric nervous system. Based on experimental studies in mutant mouse strains, an imbalance between the rate of migration of neural crest cells and the rate of differentiation of the mesenchyme of the distal gut has been proposed as an etiologic factor in Hirschsprung’s disease.³

Similar to the CNS, the ENS also follows the same principal of development which is as follows: Proliferation of the precursor neuroblast at the site of origin, migration from their site of origin to final destination, differentiation into neuronal and glial cell population, formation of cell processes and establishment of synapses.⁴,⁵

Cells of the enteric division are also affected by the same pathologic changes that can occur in neurons of the brain. Lewy bodies associated with Parkinson’s disease as well as amyloid plaques and neurofibrillary tangles associated with Alzheimer’s disease have been found in walls of the large intestine. This finding may lead to development of routine rectal biopsies for early diagnosis of these conditions as it is not possible to biopsy the brain. Due to this similarity the possibility of the use of the ENS as a source of neurons for transplantation into the brain in neurodegenerative diseases have been recently postulated. It was concluded in a study that even in the absence of surrounding layers of smooth muscle, enteric ganglia stem cells grafted into the corpus striatum survive and stimulate the production of axonal sprouts from striatal and other

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neurons, which subsequently grow into the grafts.  

The present study aimed at evaluating the histogenesis of enteric nervous system in the stomach of human foetuses ranging from 10–28 weeks of gestation in a sequential manner from after their colonization into the stomach to their attainment of final position among the layers of the stomach wall. This was a baseline study to observe the light microscopic features of the development and organization of the enteric neurons in relation to the layers of the developing stomach wall. There is a paucity of well documented literature on this basic aspect of the neurogenesis of the human enteric nervous system. Therefore it is essential to understand the normal development of the ENS in human during prenatal period.

**Materials and methods**

**Specimen collection**

The study was conducted on 10 aborted foetuses, procured from the Department of Obstetrics and Gynaecology, Lok Nayak Hospital, New Delhi. Fetuses more than 24 weeks were obtained from spontaneous abortion and fetuses below 24 weeks were obtained from cases coming for medical termination of pregnancy. The fetuses belonged to 10 weeks to 28 weeks. Determination of gestational age was done using CRL, CHL, BPD, FL, and weight as summarized in table 1. The stomach of the foetuses were dissected, formalin fixed and paraffin embedded.

**Processing of specimen**

A median incision was made on the anterior abdominal wall and the foetus was immersed in 10% formalin for 24-48 hours to accomplish immediate fixation of the gut. After the initial fixation, the stomach with both oesophageal and pyloric ends was identified and dissected out; preserved in fresh fixative for 1 to 2 weeks. Stomach that showed any degree of autolysis was not considered in the study.

The specimen was labelled and processed for paraffin embedding keeping the long axis of the stomach on the cutting surface. Serial sections of 7 µm were generated on a rotary microtome. Each longitudinal tissue section had cardiac end, fundus, body and pyloric end. Every 5th section was stained with Haematoxylin and Eosin stain to observe the layers of the stomach, and the appearance of the enteric nerve plexuses. Masson’s Trichome stain was used to observe the differentiation of the muscle layers. Modified Schofield’s silver impregnation method was used to demonstrate the nerve fibers and the neurons.

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immunoreactivity was done as a marker for synaptogenesis. Sections were then examined under the BX61 motorized microscope and the images were captured with the Olympus DP71 camera. Processing of images was done with ImagePro Plus MC6 software.

**Results**

The histogenesis of enteric nervous system in the stomach of human foetuses ranging from 10–28 weeks of gestation was studied in a sequential manner. The structural organization was studied by H&E staining. The differentiation of the various layers was elicited by Masson’s Trichrome staining. The maturation and arrangement of the enteric plexus was conducted by silver staining. Synaptophysin immunoreactivity was demonstrated in the developing neurons as a synaptic vesicle marker. The summary of the results have been tabulated in table 2.

These are the following findings:

**10-12 weeks**

The serosa consists of very loose mesenchymatous tissue. In the muscularis externa, the mesenchymatous tissue started condensing into bundles of fibres. A huge collection of neurons along the serosal aspect of the developing muscularis externa which arranged as a continuous row of scattered neurons. There was no morphological variation in the neurons.

**16-18 weeks**

The developing muscle showed further organization in a continuous circular layer. The scattered neurons had organised together to form large circumscribed collections on the periphery of the developing circular muscle coat of muscularis externa. Neuron collections were still seen to be migrating through the thin connective tissue strands between the developing muscle fibres. Submucosa studded with single neurons.

**18-20 weeks**

There was further organization of the developing circular muscle and condensation of strips of longitudinal muscle coat on the serosal aspect of the circular muscle layers but still appeared discontinuous. (Figure 1) The latter was seen to be more developed on the cardio-esophageal junction as compared to the body and pylorus. The myenteric ganglia became well defined, elongated, encapsulated groups, surrounded by a collagen fibres and fibroblasts (Figure 2). These ganglia appeared to be larger at the cardio-esophageal junction. The cells in the ganglion were of 2 types- large and small. The large cells were about 3-6 cells per plexus and displayed a round nucleus with prominent nucleolus, pink granular cytoplasm with multiple processes. The small cells were seen to have a small oval, vesicular nucleus and eosinophilic cytoplasm with 1-2 fine processes. Single neurons found in the submucosa.

**20-22 weeks**

The myenteric plexus has become more organized with larger elongated knots breaking up into smaller round to oval groups (Figure 3). The ganglia appeared smaller and more spaced out.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>CRL (cm)</th>
<th>BPD(cm)</th>
<th>Foot length (cm)</th>
<th>Number collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-12</td>
<td>5.8</td>
<td>2.2</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>16-18</td>
<td>10.8</td>
<td>3.0</td>
<td>2.2</td>
<td>1</td>
</tr>
<tr>
<td>18-20</td>
<td>14.4</td>
<td>3.8</td>
<td>2.4</td>
<td>1</td>
</tr>
<tr>
<td>20-22</td>
<td>15.6</td>
<td>4.0</td>
<td>3.1</td>
<td>1</td>
</tr>
<tr>
<td>22-24</td>
<td>16.4-17.2</td>
<td>4.2-4.5</td>
<td>3.3-3.5</td>
<td>2</td>
</tr>
<tr>
<td>24-26</td>
<td>20.5-21.7</td>
<td>4.6-4.8</td>
<td>3.6-3.8</td>
<td>2</td>
</tr>
<tr>
<td>26-28</td>
<td>24.1</td>
<td>5.0</td>
<td>3.9</td>
<td>1</td>
</tr>
<tr>
<td>28</td>
<td>25</td>
<td>5.3</td>
<td>4.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 3: Haematoxylin and eosin staining of the myenteric ganglia at 1000x magnification. Arrows show large neurons and arrow head showing a small neuron. This is seen in a 20-22 week fetus.
The number of cells in one ganglion is reduced in number. The submucosa contained scattered single along with collection of neurons.

22-24 weeks
The esophageal and the cardio-esophageal region have started showing the formation of muscularis mucosae while the body and pyloric region there was still just condensation of connective tissue was seen. The plexuses have become more organized with increase in the proportion of large neurons. In the serosa few ganglionated plexuses could be seen. Increased organization was seen in the submucosal plexus where they were capsulated (Figure 4).

24-26 weeks
The longitudinal muscle was observed in most of the body of the stomach except the pyloric region. The pyloric region showed an increase in the circular muscle thickness. The oblique muscle layer was observed in the fundus and the body in discontinuous strips inner to the circular layer of muscularis externa. The myenteric plexuses were observed to have reduced in number as well as reduced in size. The submucosal plexus also showed reduction in the number and the size of the ganglia. The number of cells was found to be 5-8 cells per ganglion.

28 weeks
The myenteric plexus had become more organized and lay between well defined circular and longitudinal muscle coats of the muscularis externa. In the pyloric region it was still present on the outer aspect of the circular muscle layer due to the reason that the longitudinal muscle in the muscularis externa had still not developed. The submucosal plexus also showed reduction in the number and size of the ganglia. Synaptophysin immunohistochemistry for the myenteric neurons showed a faint expression for the first time in a 28 week foetus.

Discussion
The human ENS derives from migrating neural crest cells and is structured into various plexuses embedded in the gastrointestinal wall. Myenteric plexus is well recognized as an important regulator of the gastrointestinal motility while the submucosal plexus influences the glandular secretion and:

Table 2: Correlation of the neuronal density and migration pattern of the enteric nervous system in the stomach of human fetuses at various age groups

<table>
<thead>
<tr>
<th>Age (in weeks)</th>
<th>Serosal collection of neurons</th>
<th>Myenteric plexus</th>
<th>Submucosal plexus</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-12</td>
<td>+++</td>
<td>Continuous belt on the periphery of developing muscularis externa</td>
<td>Scattered neurons</td>
</tr>
<tr>
<td>16-18</td>
<td>++</td>
<td>Large circumscribed collections on the periphery of the circular muscle layer</td>
<td>Small groups on the submucosal aspect of the circular muscle layer</td>
</tr>
<tr>
<td>18-20</td>
<td>+++</td>
<td>Well defined, elongated, encapsulated groups</td>
<td>Whorls, groups, single neurons seen</td>
</tr>
<tr>
<td>20-22</td>
<td>++</td>
<td>Large elongated knots breaking into smaller units; 2 types of neurons seen: large and small seen</td>
<td>Increase in number; Solitary, whorls or groups seen</td>
</tr>
<tr>
<td>22-24</td>
<td>++</td>
<td>Large neurons- eosinophilic, granular cytoplasm, round euchromatic nucleus with a prominent nucleolus; Small neurons showed euchromatic nucleus and eosinophilic cytoplasm, without a prominent nucleolus</td>
<td>Encapsulation seen</td>
</tr>
<tr>
<td>24-26</td>
<td>+</td>
<td>Predominantly large type of neurons; neuropil appeared as an eosinophilic meshwork.</td>
<td>Reduction in frequency and size</td>
</tr>
<tr>
<td>26-28</td>
<td>+</td>
<td>Reduced in number</td>
<td>Reduction in frequency</td>
</tr>
<tr>
<td>28</td>
<td>+</td>
<td>Plexus now between the circular and longitudinal</td>
<td>Reduction in number</td>
</tr>
</tbody>
</table>

Figure 4: Haematoxylin and eosin staining of the submucosal ganglia (arrow) at 1000x magnification. This is seen at 22-24 weeks of gestation.
The myenteric plexus at 18-20 weeks became more organized and encapsulated; from a continuous belt in earlier weeks it had been rearranged as distinct and regularly spaced ganglia during later stages.

At 20-22 weeks progressive organization was seen in the myenteric plexus; each ganglion displayed two distinct morphologic types of cells: round cells with large, euchromatic nucleus with eosinophilic, granular cytoplasm and fusiform cells with a heterochromatic nucleus. The round cell was the developing neurons and the fusiform cells were the developing glial cells. The neurons ranged in size from small to large and the large neurons displayed processes. Neuropil was present. The classic study done by Dogiel with intravital methylene staining, distinguished four different types of enteric ganglion neurons but these were based on their size and neurite patterns. In another study, 8 different types of enteric neurons in murine duodenum and jejunum using immunohistochemistry for various neurotransmitters like Ach, Serotonin, NO, VIP etc. were described. This could not be correlated as it was a functional classification rather than morphologic differentiation.

The submucosal plexus also showed increased organization as some of ganglia developed encapsulation around them but they had lesser number of cells; hence they lagged behind the development of the myenteric plexus. The results of our study are comparable with a study conducted on the adult human stomach using Cresyl violet staining and NADPH-diaphorase histochemistry which showed that submucosal plexus contained far less neurons than the myenteric plexus. Submucosal ganglia are absent or extremely rare in the stomach of Wistar rat embryos aged 13-21 days. The first neuronal bodies and their processes containing synaptophysin-immunoreactive were observed on embryonic day 13 which correspond to 26 weeks in human embryos. In another study done on human foetal small intestine it was observed that the expression of synaptophysin was very strong at 10 week which reduced by 30 weeks.

These findings are at variance with our observations on the human fetal stomach where the expression of synaptophysin was observed at 26 weeks. Though the observations on murine stomach model were very similar to our findings, our results were not comparable with immunostudy on developing human oesophagus.

Conclusion
Progressive maturation of ENS was evidenced by:
- Organization of the neurons into distinct, regularly spaced ganglia.
- Encapsulation of the ganglia
- Appearance of two subpopulation of neurons
- Appearance of glial cells
- Increase in size of the neurons
- Appearance of multiple neuronal processes
- Appearance of neuropil
- Apoptotic cells indicating programmed cell death to stabilize the ganglion density and ganglion cell density

References


