Original article

Prenatal development of the myenteric plexus in human sigmoid colon

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Abstract

Introduction: The enteric nervous system rivals the brain in complexity and can function independently. There are numerous reports on its development in gut but the literature is scant on the development of myenteric plexus in human sigmoid colon which is a site of various congenital and acquired diseases. This study represents a detailed qualitative morphometric analysis of the development of human sigmoid colonic innervation during second trimester. **Material and Methods:** Sigmoid colon from 12 aborted foetuses aged 14-23 weeks of gestation were processed for cresyl violet staining and NADPH-d histochemistry. **Results:** At 14weeks of gestation, both myenteric plexus and submucosal plexus were present and the size of the neurons with thickness of circular muscle was increased from 14-23weeks. Between 14-23weeks, there was remarkable increase in neuropil and nerve fibres in circular muscle. The neurons were more numerous in the mesenteric zone. **Conclusion:** There is an increase in the neuron size from 14-23weeks signifying maturational process. The circular muscle plexus is well developed at 23weeks. This study also revealed that there is correlation between the development of the circular muscle layer and the myenteric plexus. This study supports previous suggestions that nitrergic neurons are the subpopulation of neurons present in myenteric plexus. Such studies may answer important questions regarding the normal and pathologic development of the enteric nervous system.

Keywords: enteric nervous system, myenteric plexus, sigmoid colon, weeks of gestation, submucosal plexus.

1 Introduction

The gastrointestinal tract in vertebrates is an unique biological structure that has an independent nervous system which rivals the brain in complexity, known as the enteric nervous system (ENS). The ENS has been described as a "second brain" (GERSHON, 1998). The second brain contains some 100 million neurons, more than in either the spinal cord or the peripheral nervous system. These neurons helps in breaking down food, absorbing nutrients, and expelling of waste require chemical processing, mechanical mixing and rhythmic muscle contractions that move the contents in the gut. Gershon says that feeling of butterflies in the stomach signifies signaling in the gut as part of our physiological stress response (WADE, GERSHON and KIRCHGESSNER, 1994). Despite of high degree of complexity and its similarities to the CNS, the ENS is derived from the neural crest cells (NCC) which is the source of all branches of peripheral nervous system (LE DOUARIN, 1982).

The sigmoid colon (SC) is a site of various congenital and acquired diseases. The developing enteric nervous system (ENS) has a role in colonic diseases. Some researchers are focused on a particular disorder such as Hirschsprung's disease, while others are doing research on general principles regarding structure and function. It has been seen that the results on small laboratory animals cannot simply be extrapolated to humans (BURNSTOCK and HOYLE, 1989; ERDE and GERSHON, 1981; COSTA, BROOKES and STEELE, 1991). Since the morphological and neurochemical properties of the enteric plexuses are different in various species, the investigation of human development is necessary in order to establish the basic rules of the development of the human ENS. At the same time, clinical studies revealed that congenital malformations of the ENS seriously affect the gut motility, gastric acid secretion, and water and electrolyte transport (UEDA and OKAMOTO, 1967). Since the innervation of the human gut is relatively mature at birth, the study of the development of the ENS requires the use of foetal gut tissue (ERDE and GERSHON, 1981; FURNESS and COSTA, 1987). The development and maturation of the ENS in the sigmoid colon in humans has not been studied. The maturation of myenteric plexus (MP) peaks in second trimester especially from 16-20weeks of gestation (WG) in esophagus (HITCHCOCK, PEMBLE, BISHOP et al., 1992), the present study was designed to evaluate the morphological maturation of the human sigmoid colon during similar time period.

2 Materials and Methods

12 fresh foetuses were used for the present study from the foetal repository in the department of anatomy (collected at different times from the labour room of Department of Obstetrics and Gynaecology, All India Institute of Medical Sciences, New Delhi). All the foetuses used in the study were collected in accordance with the protocol approved by the Ethics Committee, All India Institute of Medical Sciences, New Delhi. The foetuses less than 20 WG were obtained from cases where medical termination of

pregnancy was performed for family planning (legalized in India under MTP Act, 1971) while those more than 20WG were stillbirths. None of the mothers suffered from any medical illness during pregnancy and the foetuses used in the study had no congenital anomalies. However, the causes of death of the stillbirths were undetermined. Immediately after arrival in the laboratory, the foetuses were weighed and measured for crown rump length, foot length and biparietal diameter (LACERDA, 1990; SAILAJA, AHUJA and GOPINATH, 1996). Together with above parameters and the clinical history, the foetal age was determined. After making a paramedian incision in the abdomen the foetuses were immersed in 4% buffered paraformaldehyde for proper fixation. Most of the specimens used in the present study were obtained within 6-8 hours after delivery and were preserved at 4 °C to minimize the post-mortem changes. Sigmoid colon was identified by its mesentry and dissected out immediately. A portion of the sigmoid colon was removed and kept in the appropriate fixative for further evaluation.

The Cresyl violet staining of paraffin sections and NADPH diaphorase histochemistry - enzyme histochemistry techniques were used to study the tissue preparations. For Cresyl violet staining, after proper fixation in 4% buffered paraformaldehyde for 7 days, the colon tissue were dissected to make small tissue blocks. The blocks were washed with phosphate buffer for 5-6 times for removing the extra fixative. The tissue was then processed for paraffin embedding. The tissue was sectioned at 7µ thickness using a rotary microtome [Microme (GmBh) microtome]. The sections were hydrated after passing through xylene and descending grades of alcohol. The sections were stained in 0.1% cresyl violet in distilled water for 10 min at 37 °C. The stained sections were rinsed in distilled water and then differentiated in 96% alcohol until only the Nissl substance was stained purple. The differentiation process was controlled visualising under a microscope. The sections were dehydrated rapidly in absolute alcohol, cleared in xylene and mounted in DPX.

For NADPH diaphorase histochemistery, colonic tissue was fixed in fresh 4% buffered paraformaldehyde for 2 hours at 4 °C. They were washed thoroughly after fixation in chilled 0.1 M phosphate buffer and then cryoprotected in 15% and 30% sucrose at 4 °C for 3 hours and 8 hours (till tissue sinks) respectively. The samples were frozen in OCT (optimum cutting temperature) compound (Tissue Tek) and 20µ thick sections were cut using a cryostat (Leica). Frozen sections were mounted onto 1% gelatin coated slides. Slides were kept at –20 °C for further enzyme histochemistry. Cryostat sections on the glass slides were washed several times with 0.1 M phosphate buffer (pH 7.4). NADPH diaphorase activity was rendered visible by incubating the sections in 10 mL 0.1 M Tris-Cl buffer (pH 7.8, adjusted with few micro-litre of concentrated HCL) containing 10mg NADPH, 1mg Nitrobluetetrazolium(NBT) and 0.3% Triton X-100 at 37 °C for 45 min to 1 hour in the incubator in dark. The treatment with TritonX-100, a detergent, is required because it is a good permeabilizing agent (in comparison to Brij-58 and dimethylsulfoxide). The development of the reaction was monitored under a dissecting microscope. Reactions were terminated when the stain was sufficiently intense (45min-60 min) by washing the tissues gently with chilled 0.1 M phosphate buffer and the sections were mounted in a mixture of glycerol and phosphate buffer (4:1) for morphological studies (KIMURA, SINGLE and VINCENT, 1983). The stained sections were examined under a microscope and images were captured using a CCD (charge-coupled-device) camera connected to a frame grabber card in an IBM PC interfaced with a Zeiss binocular microscope. The images were saved as JPEG with minimum compression and maximum quality.

3 Results

3.1 Cresyl violet stained sections

Under low magnication, mesentery was noted attached to the superficial surface of the colons of all foetuses studied. Colonic lumen and mucosal folds were clearly visible (Figure1a-f). The colon was covered by thick serosa. Well developed mesentery was present in the colon (Figure 2a). By 14 WG, the colonic wall thickness was more in comparison to the lumen size (Figure 1a). By 14 WG, both the MP and SP were developed in the sigmoid colon (Figure 3A). The myenteric ganglia(MG) was present between inner circular muscle(CM) and outer longitudinal muscle(LM) layer (Figure 2A, a). The submucosal plexus(SP) was characterised by scattered neurons in the submucosa of the colon (Figure 3A). The MG of the MP was spheroidal and elongated in shape (Figure 3A). The various portions of the MG were adjacent to each other and were interconnected by fibers. At mesenteric border, the ganglia were usually spheroidal and elongate in shape at antimesenteric border (Figure 2a). The capsular cells were deeply stained with cresyl violet and surrounded the ganglia (Figure 3B). Neuronal cells were small with round nuclei and occasionally contained two nucleoli representing immature neuroblasts (Figure 3 A, B). Little Nissl substance were present and the neuronal cells had scanty cytoplasm (Figure 3B). Neuronal cells of different shapes were arranged in a compact manner in the ganglia especially on the outer surface of the CM and these cells were appearing larger than the other cells (Figure 2a). Neurons were more numerous at the mesenteric border than the free surface of the gut (Figure 2a). Smaller cell profiles of immature glial cells were also noted (Figure 3A, B). Scattered neurons were present in submucosa on the inner surface of CM. The submucosal neurons were of same size as of the myenteric plexus. The CM was well developed and the LM was thick on the mesenteric attachment (Figure 2a). The LM cells were distinguished by its smaller rounded nuclei (Figure 2a). Large cells that appeared to be undifferentiated mesenchymal cell were present in the submucosa and mesentery. Goblet cells and absorptive cells were present in the epithelium lining the lumen (Figure 2A, 3A). The villi like structures were also noted in the lumen and their core was formed by lamina propria (Figure 2A). The lamina propria and the submucosa were cellular having immature fibroblasts, lymphocytes, undifferentiated mesenchymal cells and rarely mast cells (Figure 2A, 3A). In the submucosa well developed blood vessels were noted (Figure 3A). Muscularis mucosae(MM) was not developed at this age.

At 15 WG, the myenteric ganglia appeared as large collection of cells. The neurons appeared to be larger but had similar features as noted in 14 WG foetus. The cells were pleomorphic in appearance. The colonic wall appeared to be less thick than in the previous age group. The ME was better developed and appeared thicker. The MM were not developed at this age. At the anti-mesenteric border, smaller ganglia with fewer cells were observed.

At 16 WG, the myenteric ganglia size appeared to be increased; however neurons apparently were of the identical in size as in the earlier age group. In the myenteric plexus, the ganglia were found to be elongated in the direction of the circular muscle coat. The CM was well developed and increased in width in comparison to the previous age group.

At 18 WG, the neurons appeared akin to those in the previous age group (16WG). In SP, neurons could be visualized clearly in clusters. It was noted that, the large neurons were present on the outer border of the CM in the ganglia(Figure 4B). The Nissl substance could be clearly seen (Figure 4B). The muscularis externa (ME) increased in thickness (Figure 4A). The taenia could be clearly visible on surface of the colon at this age (Figure 4A). The MM was identified for the first time at this age (Figure 4A). At 20 WG, the neurons were more in number and increased in size within the MG. Within the ganglion, large cells were uniformly distributed. The CM appeared to have increased in thickness.

At 23 WG, the majority of MG were elongated in shape in comparison to the previous age group(Figure 5A, B). In the MG, the neurons of various shapes (often triangular) and sizes were noted (Figure 5A, B). They appeared less in number and reduced in size. At this stage, the neurons were uniformly arranged within the MG (Figure 5A, B). The CM and LM increased in thickness (Figure 5A, B). Some of the neurons showed two nucleoli. The SP was seen in groups containing neuronal, glial and immature cells in the submucosa (Figure 5A). The goblet cells (GC) appeared as unstained areas among the colonic enterocytes (Ec) as noted in previous age groups and MM was present but could not clearly demarcated (Figure 5A).

3.2 The nitrergic myenteric neurons

At 14 WG, the MP appeared as a continuous band of small neuronal cells (Figure 6A). Binucleate neurons were often observed. Large oval to round nuclei were there with little cytoplasm characterised the neurons (Figure 6B,C). Neuronal processes were observed emerging from the surface of the soma. Among the neurons only simple neuropil was present at this age (Figure 6C). Very few nerve fibres ran parallel to and closely associated with the circular muscle fibres. Occasional bundles of fibres were seen in the entire gut (Figure 6A). Scattered neuronal cells were present in the submucosa towards inner border of the CM (Figure 6A). At 15 WG, continuous band of cells divided the elongated compact ganglia into several subdivisions. These subdivisions were connected to each other via internodal strand. Ganglia and neuronal cells were larger in comparison to the previous age group as the cytoplasm of neuronal cells increased. There was obvious increase in the neuropil also. The nerve fibers were seen to colonize inside the circular muscle layers which were parallel to long axis of muscle fibres. The SP was noted close to the CM with 2-3 neurons. Nerve fibres were less abundant in the submucosa though occasional thin fibres were visible in lamina propria. Nerve fibres were also noted in mesentery. At 16 WG, the subdivisions of MP were noted very close to each other and connected by internodal strands. The neuronal size and the neuropil was equivalent to the previous age group. The nerve fibres in the CM were increased in comparison to the previous age group. The SP was well developed at this age (Figure 7A, B). Nerve components were noted in the LM but was not apparent because of their longitudinal orientation.

At 18 WG, Ganglia were well organized. Neuronal cells were apparently bigger and the neuropil could be clearly visualized. The neurons were pleomorphic with oval to round nuclei. At 20 WG, Neuronal cells were further

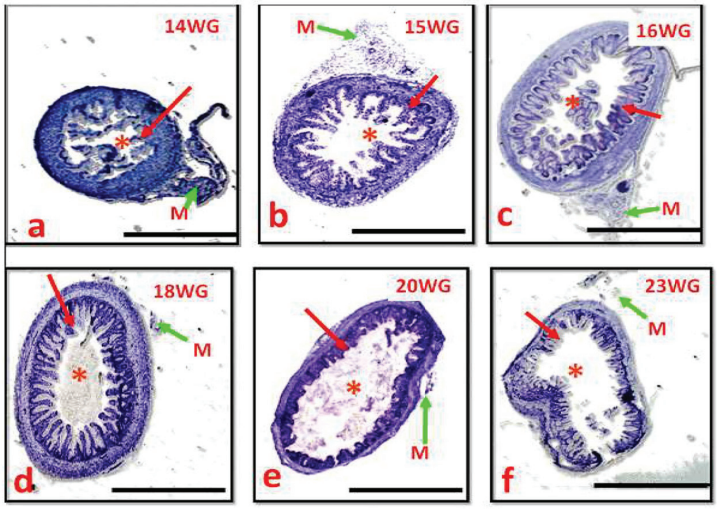

Figure 1. (a, b, c, d, e, f). Photomicrograph of the cresyl violet stained sections of the sigmoid colon of foetuses at various ages showing mesenteric attachment (M with green arrow), lumen (asterix), mucosal folds (red arrow), and well developed gut wall. Scale bar 500µ (a, b, c) and 1000µ (d, e, f).

differentiated in comparison to the previous age group as evident by the increase in size of the cells. Well developed ganglia were noted. Nerve fibres in the CM were increased with increase in the thickness of the neurons (Figure 7C).

At 23 WG, the MG was increased in size (Figure 7D). Neurons increased in size due to increase in the amount of cytoplasm (Figure 8B). Neuronal cell was irregular and processes were lengthened and thickened (Figure 8B). There was remarkable increase in the neuropil and nerve fibres in the CM (Figure 7D, 8A). There was an increase in the thickness of the nerve fibres and the varicosities could be clearly seen (Figure 8A). Within the CM, some of the neurons were oriented along the axis of nerve fibres that was identical to the axis of circular muscle fibres (Figure 8A). These neuronal cells had oval nuclei (Figure 8B). The CM had denser innervation than the longitudinally oriented taenia (Figure 8A). In the SP, large neuronal cells with thick fibres were noted.

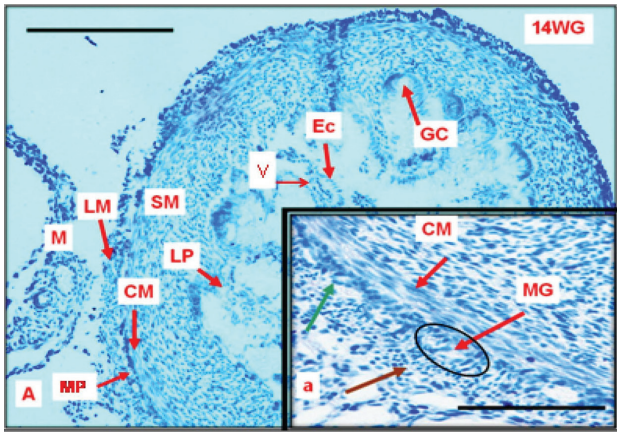

Figure 2. A: Photomicrograph of cross-section of human foetal sigmoid colon at 14WG. Showing well developed epithelium with Goblet cell (GC) and absorptive enterocytes (Ec). Submucosa (SM) and lamina propria (LP) very well seen. Myenteric plexus (MP) seen between inner CM and outer LM. Villi (V) like structures are present. Inset (a) Higher magnification shows that at mesenteric attachment, myenteric neurons (green arrow) are numerous and appear larger in spheroid shaped myenteric ganglia (MG, oval figure) and lying on outer surface of CM. Outer LM layer appear thick in the same region (brown arrow). LM- Outer longitudinal muscle layer ; M- mesentery. Scale bar 200 µ (A), 100µ (a).

4 Discussion

To our knowledge, this study provides for the first time, the morphology of the developing human sigmoid colon during second trimester in Indian population. In this study we observed the differentiation of the neurons, glial cells and other morphological parameters in foetal sigmoid colon from 14-23WG. There are numerous reports available on the development of ENS in various segments of gut like oesophagus, small intestine etc. but the literature is scant on the development of MP in sigmoid colon of humans, which is a site of various congenital and acquired diseases.

In the present study, it was noted that by 14WG, both MP and SP were well developed in the human foetal sigmoid colon. Scattered neurons were also present in submucosa mainly on the inner surface of the CM. The CM was well developed and LM was thick on the site of the mesenteric attachment. The muscularis mucosae were not visible at this age. It has been mentioned earlier by many authors that MP, SP, CM, LM and MM are well developed along the entire length of the gut by 14WG and the foetal gut gives a mature appearance by 14WG (FU, TAM, SHAM et al., 2004; UEDA and OKAMOTO, 1967; WALLACE and BURNS, 2005). In the present study, the MM was not clearly visible in all the foetuses studied. The MM could be identified at 18WG. This can be explained as MM can be obscured by the cells of LP and submucosa. So, a different stain (a connective tissue stain) will differentiate various components of the MM. Hence, MM cannot be always be demarcated well in all foetuses in the cresyl violet stained sections.

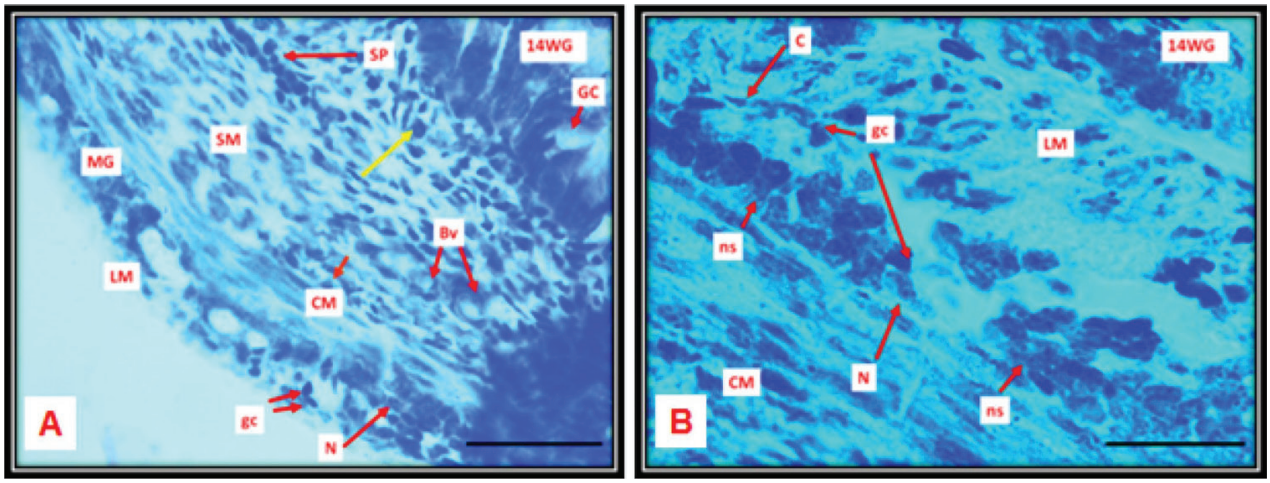

Figure 3. Cross-section of cresyl violet stained sigmoid colon of human foetal sigmoid at 14WG. (A) Showing spheroid and elongated shaped myenteric ganglia (MG) with neurons (N) and glial cells (gc). Nucleus with one or two nucleoli present in a neuron. Submucosal plexus (SP) present in in the submucosa (SM). Blood vessels (Bv) and large cells (yellow arrow) present in SM. Well developed CM and LM are seen. Goblet cell (GC) are also present. (B) Nissl substance(ns) is visible. Double nucleoli in a neuron can also be seen. Capsular cell (C) is lining the MG. CM –circular muscle, LM – longitudinal muscle. Scale bar - 50µ (A), 20µ (B).

In the developing gut studied here, scattered neurons were present in submucosa on the inner surface of the CM at 14WG and then groups of neurons were present as age advanced. Similar neurons were observed in oesophagus by Hitchcock, Pemble, Bishop et al. (1992). In the present study it was observed that there was a gradual increase in the size of ganglions as foetal age advanced. Fu, Tam, Sham et al. (2004) also documented that as gut wall is formed, the ganglion plexus increased in size with more neurons and glia, and the formation of intra-plexus nerve fascicle, so our findings supports the available literature reports and shows normal growth pattern. It has been seen

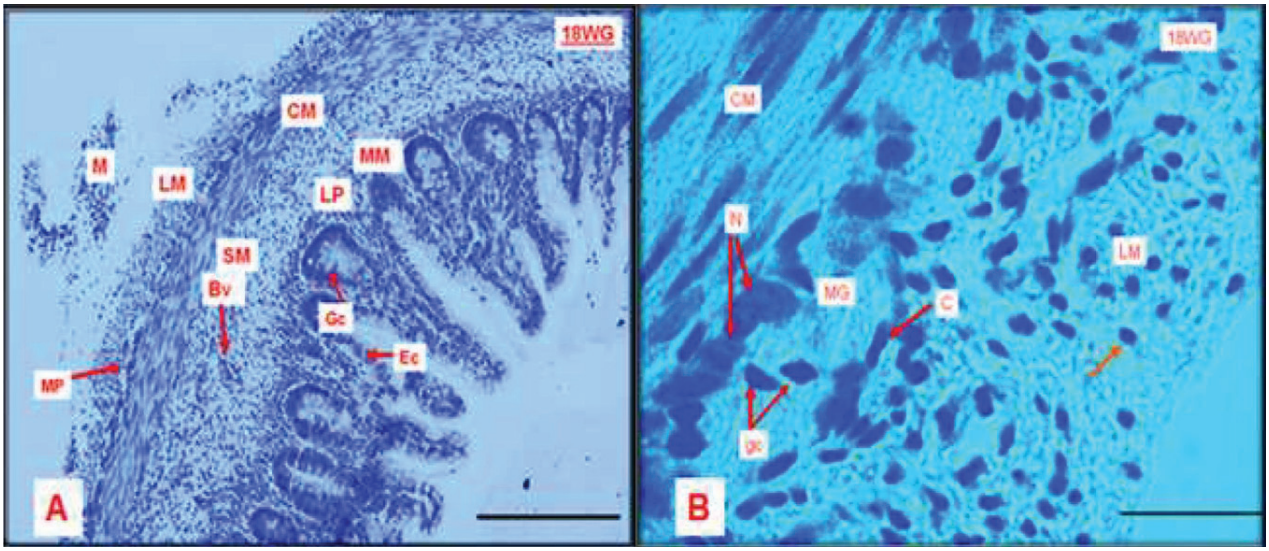

Figure 4. Cross-section of cresyl violet stained human foetal sigmoid at 18WG (A, B). (A). Showing all the layers of sigmoid colon. MP is sandwitched between inner CM and Outer LM. Muscularis Mucosa (MM) is seen. Thick outer LM representing taenia coli is present along mesenteric attachment. CM has increased in thickness compared to 14 WG. (B) At higher magnification, neurons (N) with glial cells (gc) are present in the MG. Round to oval nucleus with nucleoli and Nissl substance visible in N. Capsular cells are lining the MG. Outer LM cells are identified by rounded nuclei (orange arrow). More number of neurons are lying on outer surface of CM. M- Mesentery, MP- Myenteric plexus, MG – myenteric ganglia, MM – muscularis mucosa, LP- Lamina propria, Bv- Blood vessel. CM – inner circular muscle, LM – outer longitudinal muscle, Ec – absorptive enterocyte, Gc – goblet cell. Scale bar - 200µ (A), 20µ (B).

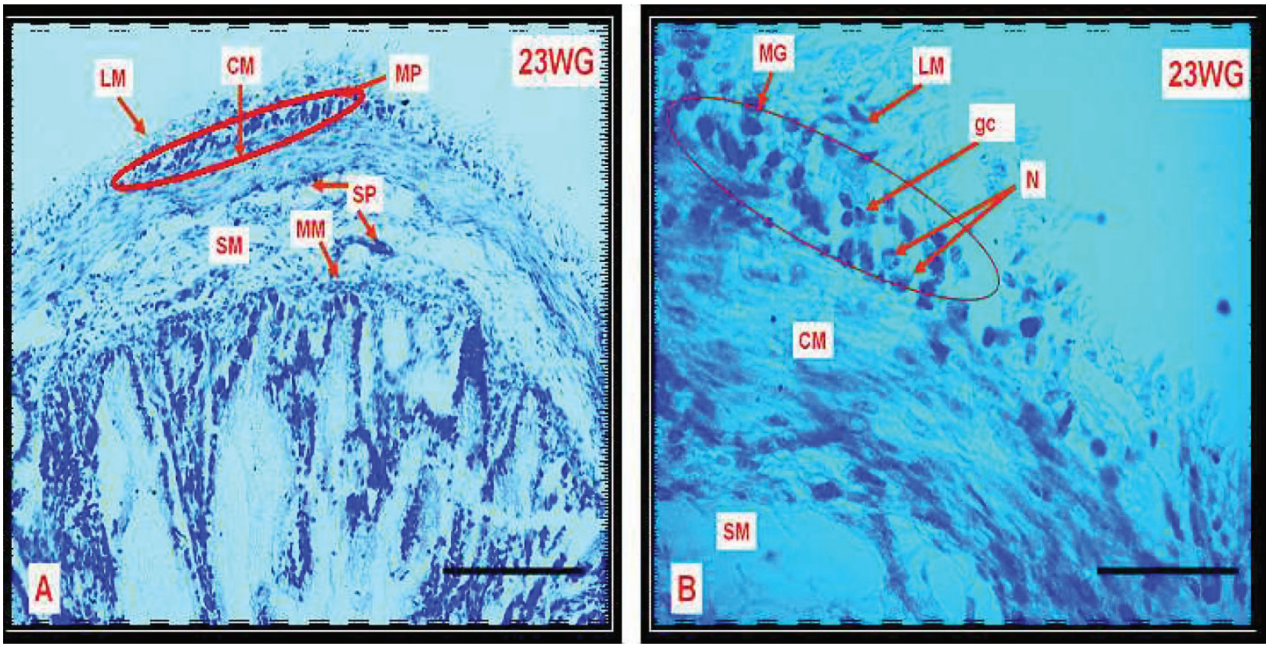

Figure 5. (A) At 23 WG, Myenteric plexus (MP) and Submucosal plexus (SP) are visible in the sigmoid colon. CM layer is increased in thickness. Neurons (N) are uniformly distributed with in elongated shaped myenteric ganglia (MG, red oval figure). Well developed epithelium is present. Muscularis mucosa is well developed. (B). At higher magnification, Neurons (N) with glial cells (gc) are present in the MG (red oval figure). Nissl subatance is visible within the cytoplasm of neurons. CM – inner circular muscle, LM – outer longitudinal muscle. SM - submucosa. Scale bar – 200µ (A), 50µ (B).

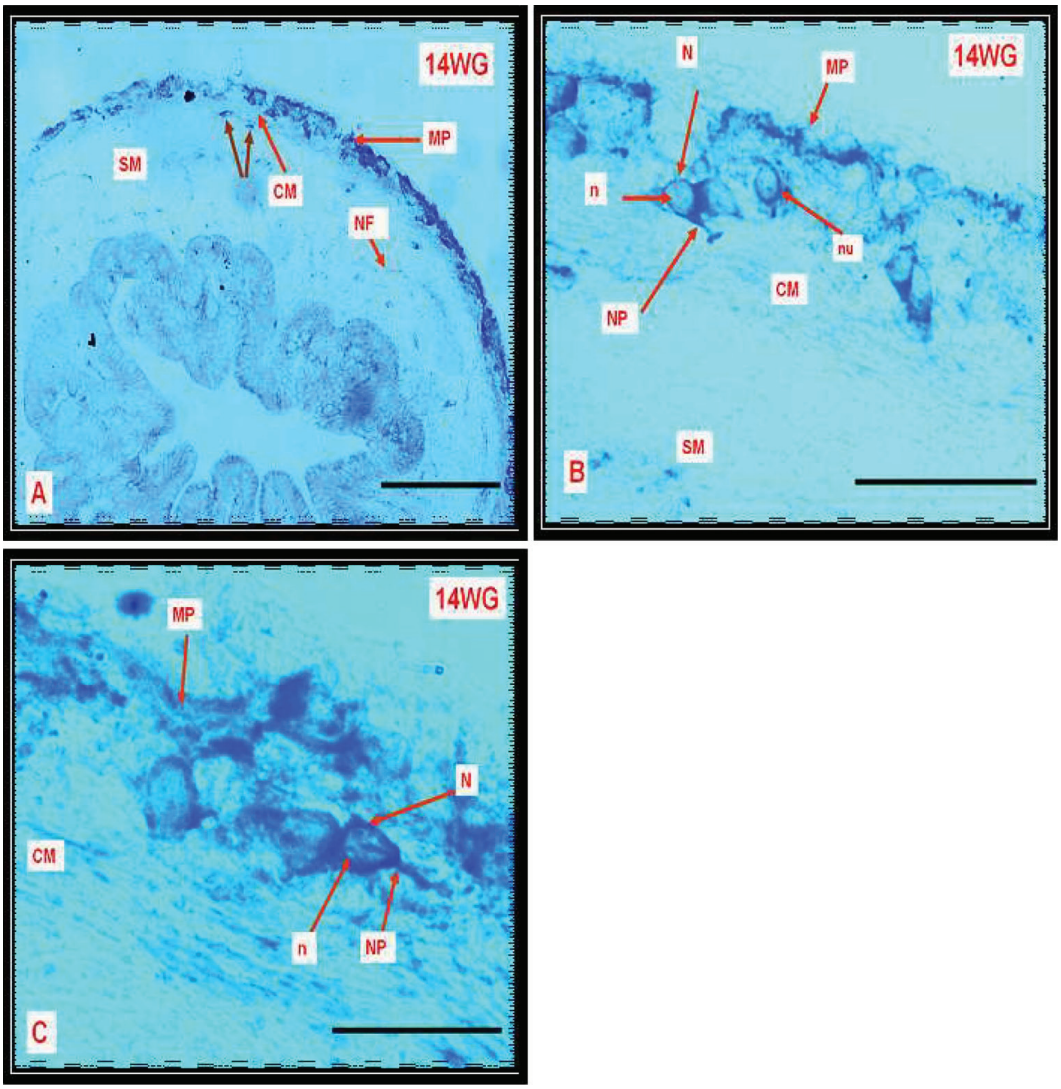

Figure 6. Cross-section of human foetal sigmoid colon in NADPH – diaphorase stain at 14WG. (A). Shows Myenteric plexus (MP) in the form of a continuous band.Scattered neurons are present in submucosa (SM) along inner border of CM. Very thin nerve fibres (NF) are present in the SM. (B). Higher magnification shows myenteric neurons (N) have round to oval nucleus (n) with scanty cytoplasm. Neuronal processes (NP) can be clearly demarcated. (C) Higher magnification shows round to oval nuclei (n). Neuropil is minimal. CM – circular muscle. Scale bar – 200µ(A), 50µ(B), 20µ(C).

In the developing gut studied here, scattered neurons were present in submucosa on the inner surface of the CM at 14WG and then groups of neurons were present as age advanced. Similar neurons were observed in oesophagus by Hitchcock, Pemble, Bishop et al. (1992). In the present study it was observed that there was a gradual increase in the size of ganglions as foetal age advanced. Fu, Tam, Sham et al. (2004) also documented that as gut wall is formed, the ganglion plexus increased in size with more neurons and glia, and the formation of intra-plexus nerve fascicle, so our findings supports the available literature reports and shows normal growth pattern. It has been seen that the development of the enteric plexuses is different in mammals and avian large intestine because the submucosal region is colonised before the myenteric region in the large intestine of birds (BURNS and DOUARIN, 1998). This is likely to be due to early differentiation of CM and NCC found difficult environment than loose mesenchymal cells of submucosa. Many authors claimed that by E14.5, entire hindgut was colonized and the CM had started to differentiate in rats. From E14.5 to E16.5, the neurons were observed on the serosal side of the CM in the myenteric region, but not in the submucosal region. Scattered neurons were first observed in the submucosal region around E18.5 and groups of neurons forming ganglia were not observed until after birth (MURPHY and FOX, 2007; MCKEOWN, CHOW and YOUNG, 2001; ROTHMAN and GERSHON, 1982).

In present study we observed that at 14WG, the cells were of variable shape and size. Neuronal cells were small with rounded nuclei and many neuronal cells were having two nucleoli representing immature neuroblasts. Neuronal cells were arranged in a compact manner in the ganglia especially on the outer surface of the CM and these cells were

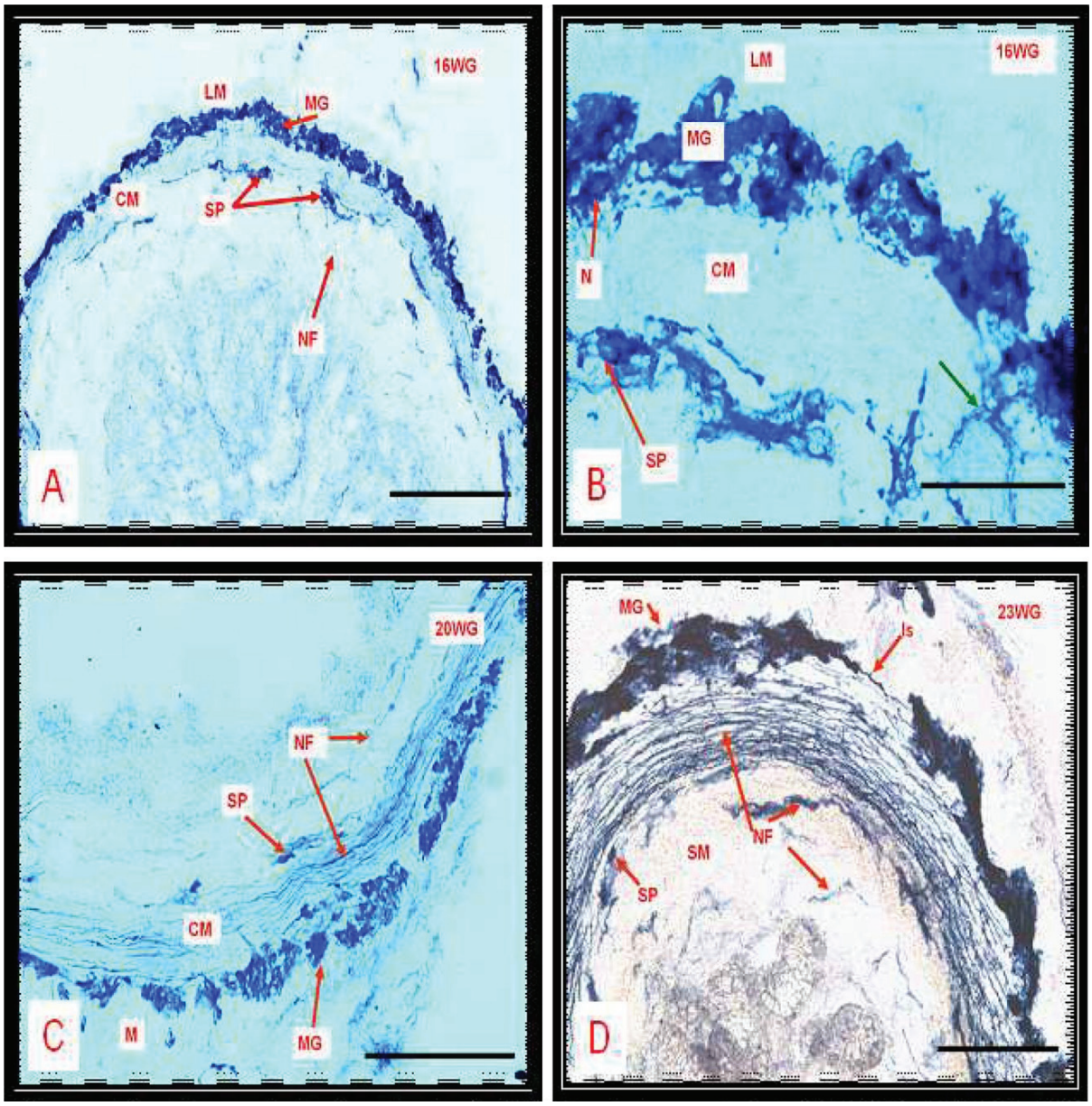

Figure 7. Cross-section of human foetal sigmoid colon in NADPH – diaphorase stain at 16WG(A, B), 20WG(C), 23WG(D). (A, C, D) shows progressive increase in the sizes of Myenteric ganglia (MG), nerve fibres (NF) present in CM (inner circular muscle) and neuropil from 16WG to 23WG. Submucosal plexus (SP) are present in the submucosa (SM). (B) Shows the connection between MP and SP (green arrow). Neurons in MG and submucosa (SM) having round to oval nuclei. (D) Internodal strand is seen between two MG. LM – outer longitudinal muscle, M – Mesentery, Scale bar - 200µ (A, C, D) and 50µ (B).

appearing larger than others. Undifferentiated mesenchymal cell were present in the mesentery. A similar feature of the neurons in human small intestines and oesophagus during foetal life were described earlier (RESCH, FEKETE and BAGYÁNSZKI, 2000; HITCHCOCK, PEMBLE, BISHOP et al., 1992). Nissl substance was scanty as the neuronal cells had very little cytoplasm. Nissl substance represents accumulation of new RNA in the neurons during development(HUGHES, 1955).

In present study, smaller cell profiles of immature glial cells formed by 14WG. As described earlier, these cells are non neuronal cells which appear along with the neurons around 10WG. These cells had ovoid nuclei and rich heterochromatin. It can be concluded that glial cells appear within the MP at the very beginning of ganglionic morphogenesis. They appear together with the primordial ganglion cells, long before the formation of the LM (FEKETE, RESCH and BENEDECZKY, 1995). Thus, these cells provide the first morphological elements of the ganglionic microenvironment, necessary for the ganglionic morphogenesis. They might be involved in providing the mechanical support, isolation and nutrition for the developing ganglia as described previously by many authors (FERRI, PROBERT, COCCHIA et al., 1982; MIRSKY and JESSEN, 1983; SMITH, KOBAYASHI and FURNESS, 1989). This needs to be confirmed by demonstration of early glial cell markers like GFAP and

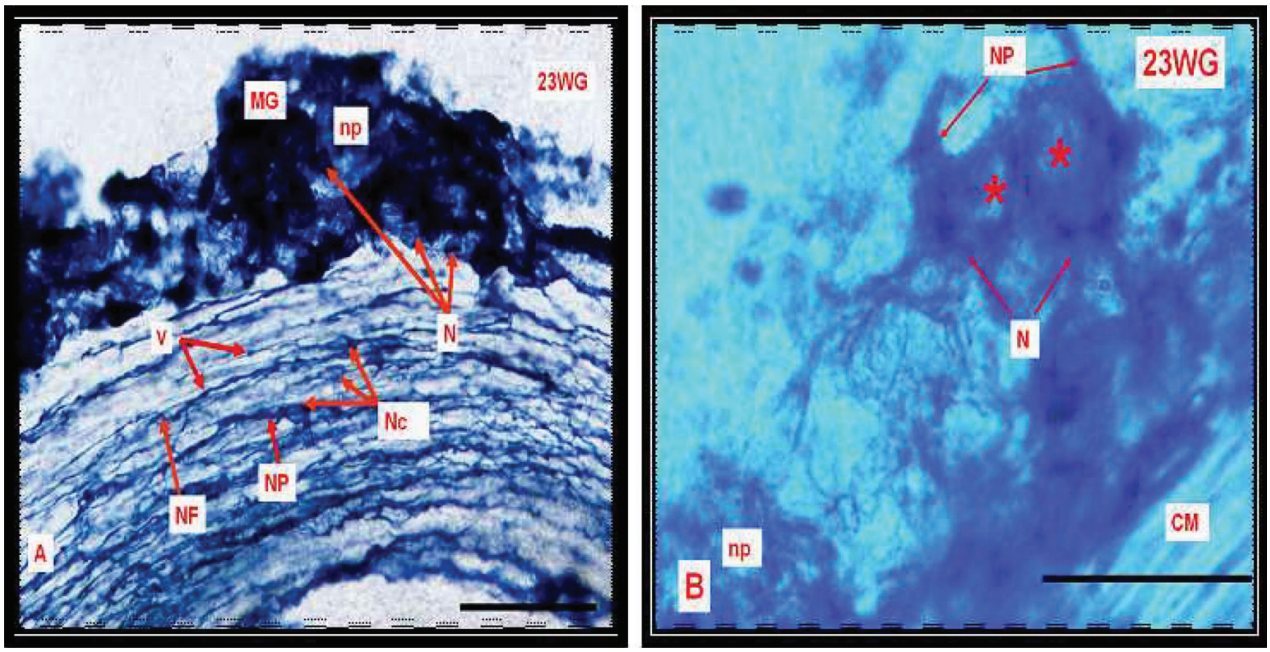

Figure 8. Photomicrograph of NADPH – diaphorase stained sigmoid colon at 23WG. (A) Shows increase in sizes of myenteric ganglia (MG) and nerve fibres (NF) in CM. Neurons (N) with nucleoli are seen in MG. Remarkable increase in neuropil (np) is there. Few neurons (Nc) are present in the CM, oriented along the axis of CM fibre. These neurons are having oval nuclei and long neuronal processes (NP). Varicosities are visible in the NF. (B) Higher magnification shows large neurons with nucleus (asterix) and extensive neuropil are visible. Thickened NP are visible clearly. CM – inner circular muscle. Scale bar - 100µ (A), 20µ (B).

S100 protein using immunocytochemical methods (FERRI, PROBERT, COCCHIA et al, 1982).

In the present study, it was noted that the thickness of the CM increased along with that of the LM from 14WG onwards. It may be due to hypertrophy and hyperplasia of the CM occurring during developmental morphogenesis. In the oesophageal development, it was observed that the CM and the LM thickness becomes approximately equal during third trimester. The LM develops relatively late (13WG). Swallowing does not commence until the LM is well developed (HITCHCOCK, PEMBLE, BISHOP et al., 1992). It has been also reported that the LM layer develops in 10-12WG in small intestine and the oroanal peristalsis begins in 27-30WG in humans (RESCH, FEKETE and BAGYÁNSZKI, 2000). The development of taeniae coli occurs in the 11-12WG when haustra appear described earlier during gut morphogenesis (STANDRING, 2008).

In the present study it was also noted that the neurons were more numerous in the mesenteric zone than at the free edge of the gut in all foetuses investigated. As NCC migrates through mesentery, it is expected that the developing neurons will be more towards mesenteric border. Nemeth, Fourcade, Puri et al. (2000) observed that in the premature infants the neuron density of MP was significantly higher in the mesenteric border of the small bowel compared with antimesenteric border. He found that differences in the neuronal density in the mesenteric and antimesenteric border of the small bowel gradually became less striking as the gestation progressed with no differences evident at 32WG. The marked morphological differences observed in neuron density at two sites in the small bowel of premature infants may contribute to immature small bowel activity (NEMETH, FOURCADE and PURI 2000; McLELLAND and ALI, 1979).

It was noted that neurons were oriented on the outer surface of the CM from 14-20WG. At 23WG, the neurons were uniformly arranged within the MG. One EM study reported that at 10WG, neurons can be distinguished on the outer surface of the CM layer (RESCH, FEKETE and BAGYÁNSZKI, 2000). Throughout 18WG, the CM provides the mechanical surface for the developing MP, which is attached firmly to the CM. Around 18WG, the mechanical points of attachment shift from the CM to the LM layer. This relocation may be accompanied by the appearance of specific surface molecules recognized by developing neurons (OKI, DOMOTO and BISHOP, 1990). Both the CM and the MP appear by 10WG but cannot be recognized as separate entities. Neurons, muscle cells, nerve plexuses and nerve terminals are in close contact with each other without an intervening basal lamina. A similar arrangement was described by many authors. (BOROS and FEKETE, 1992). There is no evidence that neurons are dependent on neurotrophic support for their survival during development (TORIHASI, WARD and BURNS, 1994). But, there is evidence that the number of neurons, the density of the MP, and the average neuronal size are greater in areas where the smooth muscle are thicker (GABELLA, 1989).

4.1 Nitrergic neurons

In the present study, we have also focused our attention on a specific population of neurons, the NADPH diaphorase positive neurons in the MP in the human sigmoid colon. There is growing evidence of the abnormalities in nitrergic neurons in the several pathological conditions like

Hirschprung's disease (HSCR), pyloric stenosis, achlasia cardia and intestinal neuronal dysplasia.

In the present study, from 14-23 WG, a continuous band of cells dividing the elongated compact ganglia into several parts was noted. The well developed ganglia were noted at 20WG in the present study. Roman, Bagyanszki, Krecsmarik et al. (2004) demonstrated that the nitrergic neurons form aggregated groups within each ganglion due to an intrinsic pattern-forming force during the development of MP in humans. The distribution pattern of the nitrergic neurons changed markedly between 14 and 22 WG. The nitrergic neurons were randomly distributed at 14WG but were aggregated in the plexus and within the individual ganglia at 19WG (ROMAN, BAGYANSZKI, KRECSMARIK et al., 2004). This is in agreement with our observations. A similar pattern of development of ganglia in MP was also observed in chicks (BOROS and FEKETE, 1992; MURATA, YAGI, KUBOTA et al., 2003).

Brandt, Tam and Gould (1996) noted that by 12WG, nitrergic neurons appeared in the MG in the gut and the SP became evident after 14WG. Innervation became richer and more organized from 12-23WG. They observed an increasing numbers of nerve fibres in the CM similar to the present study. By 23WG, nitrergic innervation has matured to the pattern observed in the postnatal gut (BRANDT, TAM and GOULD, 1996). These results support the observations that the nitrergic neurons mature at the end of mid gestation.

Shao and Xin (2001) also studied development of nitrergic neuron development in large intestine in human foetus. He observed varicosities equivalent to the bead like structures in the CM increasing remarkably at 23WG as described in our study. The nerve fibres with neurons in CM form circular muscle plexus (FURNESS and COSTA, 1987). This plexus was well formed at 23WG in the present study. At this age neurons in the CM and submucosa were observed in our present study. Few thin nerve fibres were observed in all the layers at 14WG. But in his study he observed similar features at 32-40 WG. This difference may be due to different in the ethnic group of population studied. He also observed an increase in intensity between 20WG and 40WG which was not in agreement with our study. This could be due to various reasons like delay in sample collection or fixation or processing of tissues which can be resolved by using large number of samples and taking care of all factors responsible for variations in results.

In the present study, it was noted that there was increase in thickness of nerve fibres and varicosities. These varicosities signify increase in the amount of neurotransmitters. Puri, Paran and Rolle (2008) concluded that the axonal thickness in the MP undergoes striking changes during the first 12 weeks in the MP in piglets. It has also been described that these varicosities on the glial cell surfaces might function as communication sites between glial cells and nitrergic neurons. Although the nature of this communication is not clear, the glial cells closely related to nitrergic nerves might directly benefit from the trophic effect of NO, which has recently been reported (FUJISAWA, OGURA and NAKAYAMA, 1996).

Nitrergic nerve density in intestinal smooth muscle decreases during foetal development (12-23WG) as a result of increased interspacing between MG and a disproportionately larger increase in smooth muscle area than neuronal area described by Brandt, Tam and Gould (1996) (quantified using a computerized image analyzing system). But, another author claimed that the density of nerve fibres (protein gene product-PGP 9.5) within CM of oesophagus increased in early second trimester (16-20WG), then reduced in late second trimester which continues throughout the gestation and into the infancy (HITCHCOCK, PEMBLE, BISHOP et al., 1992). In the present study, there was remarkable increase in the neuropil and nerve fibres in the CM with the increase in the thickness of CM from 14-23WG (qualitative). Thus it may be concluded that extensive innervation is established in late second trimester. This discrepancy might be due to rostrocaudal gradient of maturation in gut. However, other authors claimed that nerve fibres gradually colonize the muscle layer from 17WG to birth with maturation of neurons (GABELLA, 1971). It was also noted earlier that there was a significant regional difference in the density of nitrergic neurons observed between the small intestine and the colon in guinea pig (SHUTTLEWORTH, YOUNG and FURNESS, 1992).

In the present study, it was observed that in CM nerve fibres were more in numbers as compared to LM. This signifies that, circular muscle plexus is well developed than the other smooth muscle plexuses. Timmermans, Barbiers and Scheuermann et al. (1994) also described morphological differences between nitrergic neurons within the developing human gut and noted that in the colon, nerve cells and fibres were numerous in the MP and SP. The CM layer had a much denser NOS- immunoreactive innervation than the longitudinally oriented taenia. Hence it is in agreement with the results of the present study. We have also observed the majority of the nitrergic neurons and the nerve fibres were in the MP and CM respectively

The limitations of the present study was less number of samples due to non-availability of foetus and autolysis of tissue.

5 Conclusions

This study represents a detailed qualitative morphometric analysis of the development of human sigmoid colonic innervation during second trimester. During prenatal life there is an increase in the neuron cell size from 14-23WG signifying maturational process and extensive innervation is established at 23WG. The present study also revealed that there is correlation between the development of the circular muscle layer and the myenteric plexus. This study supports previous suggestions that nitrergic neurons are the subpopulation of neurons present in myenteric plexus. Such studies will answer important questions regarding the normal and pathologic development of the ENS. Hence this study may promote the link between theory and clinical practice.

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Received October 27, 2012
Accepted May 22, 2013