Intestinal muscularis propria increases in thickness with corrected gestational age and is focally attenuated in patients with isolated intestinal perforations

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ABSTRACT

Purpose: Intestinal perforations are common in premature infants, leading to a diagnostic dilemma between necrotizing enterocolitis and isolated intestinal perforation (IIP). IIP is thought to result from a congenital or acquired absence of the muscularis propria. However, developmental events leading to IIP are not well understood. This study examines the relationship between corrected gestational age (CGA) and intestinal muscle development in controls and patients with IIP.

Methods: Specimens from stillbirths and infants undergoing intestinal surgery from 8 to 48 weeks' CGA were collected from 2005 to 2012. Twelve patients with IIP were identified. Control specimens were collected during 25 fetal autopsies and 39 bowel resections. In each case, three sections of intestine were examined histologically for muscularis mucosa, circular and longitudinal muscle thickness. Comparisons of control and perforated specimens were performed via linear regression and ANOVA.

Results: Controls and adjacent normal segments in IIP showed a linear relationship between thickness of circular and longitudinal muscles with CGA. Circular and longitudinal muscles were thinner in perforated segments than in adjacent normals and CGA-matched controls ($p < 0.05$).

Conclusion: Intestinal muscularis propria increases in thickness with CGA. Muscle thickness is focally attenuated in patients with isolated intestinal perforations, while the remaining intestine is normal, suggesting that primary repair is an appropriate treatment.

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Paraffin sections of formalin-fixed tissues were cut (6 μm) and stained with hematoxylin and eosin, masson trichrome and α-smooth muscle actin using standard techniques [13,14]. In every case, three sections from each site, both normal and perforated, were examined histologically for muscularis mucosa (MM), circular muscle (CM) and longitudinal muscle (LM) thickness. A line was drawn perpendicular to the muscle plane and the number of cells passing through this line was counted to define the number of cell layers forming the specific muscle groups. If discrete spindle cell borders could not be identified in sections, then the number of nuclei passing through this line was counted as a surrogate marker for the number of cells. These measurements gave the thickness of each muscle layer (in μm), the cellularity (as the number of cell layers) and the approximate size of each individual smooth muscle cell (in μm, as approximated by the muscle thickness divided by the number of cell layers). Measurements and images were taken using an Axiovert S100TV microscope (Carl Zeiss, Thornwood, NY) and DFC490 digital imaging system (Leica Microsystems, Vertrieb, Germany). In order to detect myopathy, α-smooth muscle actin staining was performed for bowel segments from IIP patients and CGA-matched controls, as defined by IIP CGA range ± 0.5 weeks. ImageJ software v1.46r (National Institutes of Health, Bethesda, MD) was used to measure the density of α-smooth muscle actin staining of the smooth muscle fibers of the MM, CM, LM and blood vessel for each specimen, using the density of the blood vessel as an internal control to allow for objective comparisons between slides.

Comparisons of CGA, muscle thickness, cellularity, smooth muscle cell size, and α-smooth muscle actin stain density in control and perforated specimens were performed via linear regression analysis and ANOVA using GraphPad Prism version 6.0 software (GraphPad, San Diego, CA). Data are expressed as means ± SEM. A p value less than 0.05 was deemed statistically significant.

2. Results

Demographic data for control and perforated specimens are listed in Table 1. Of the total patients in the control group, five were CGA matched to the IIP group, and are listed in the CGA-matched control column for comparison. IIP was only found in the small bowel, with ileal lesions accounting for 64%. All affected patients were preterm in the CGA range of 25.4 to 33.7 weeks.

Histology slides were inspected thoroughly and no evidence of vasculitis or NEC was found in any of the control or IIP patients. IIP is characterized by preservation of the mucosal villus architecture and thinned muscularis propria in contrast to the coagulation necrosis, mucosal sloughing, hemorrhage, vascular thrombosis, pneumatosis and relatively thicker muscularis propria that are typical of NEC [15–17].

Control specimens showed a linear relationship between CGA and thickness of the small bowel muscle layers CM (R² = 0.5377) and LM (R² = 0.5660), but not the MM (R² = 0.0001) (Fig. 1). Similarly, there was a linear relationship between CGA and thickness of colon CM (R² = 0.5329) and LM (R² = 0.6119), but not the MM (R² = 0.0578). There was no evidence of muscle attenuation on thorough inspection of available sections from control specimens. Scatter plots of CGA and cellularity in each muscle group (data not shown) reveal similar relationships as the CGA and thickness of the muscle layer in both the small bowel and colon. Cell size remains relatively constant for each muscle layer when plotted against the CGA for both small bowel and colon with R² < 0.2. These findings are suggestive that the increases in muscle thickness of the normal small and large bowel muscularis propria (circular and longitudinal muscles) with increasing age are caused by a hyperplastic effect, rather than hypertrophy of individual cells.

Small bowel samples of CGA-matched controls were compared to those with IIP (both adjacent normal and perforated sections) after stratification into two CGA ranges (25.1 to 29.0, and 29.1 to 34.0 weeks) for statistical analysis as summarized in Table 2. Representative masson trichrome stains of small bowel control, adjacent normal and perforated segments are shown in Fig. 2. No statistically significant differences were found in MM, CM or LM thickness and number of cell layers between control specimens and adjacent normal segments from patients with IIP within each CGA range. Small bowel musculature in controls and adjacent normal segments of IIP patients were significantly thicker with higher cell layer counts than perforated segments with respect to the CM and LM (p < 0.05), but not with MM. Average smooth muscle cell size of the MM, CM and LM did not differ significantly between the groups, suggesting that the decreases in thicknesses of the muscle layers for the CM and LM at the site of perforation are caused by a hypoplastic rather than a hypotrophic phenomenon. There was no difference in α-smooth muscle actin stain density between controls, adjacent normal and perforated segments in patients with IIP in each muscle group, suggesting the absence of a myopathy. Representative α-smooth muscle actin stains of small bowel control, adjacent normal and perforated segments are shown in Fig. 3.

Patient charts were reviewed to determine any differences in antenatal and postnatal treatment in CGA-matched controls and patients with IIP to determine potential risk factors (Table 3) and outcomes of operative management. Possible risk factors reviewed included the use of nonsteroidal anti-inflammatory agents (NSAIDs), steroids, enteral feeds, vasopressors and continuous positive airway pressure (CPAP). The sample size for this study was too small to perform odds ratios or a proper multivariate analysis to determine statistical significance. Of the 12 patients with IIP, 6 were treated with bowel resection and creation of an ostomy, while the other 6 were treated with bowel resection and primary anastomosis. There were no anastomotic leak complications in any of the patients who were reanastomosed primarily.

3. Discussion

This study’s observations are consistent with our hypothesis, showing a focal deficit and thinning of the muscularis propria in both the CM and LM at areas of perforation compared to adjacent normal segments and control patients. Based on histology and α-smooth muscle actin staining density, these areas of attenuation are most likely caused by smooth muscle cell hypoplasia and are not associated with NEC, vasculitis or myopathy. We have also confirmed that there is a linear relationship between CGA and thickness of normal small

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 64)</th>
<th>CGA-matched control (n = 5)</th>
<th>Isolated intestinal perforation (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>31</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Male</td>
<td>28</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Unknown</td>
<td>6</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Corrected gestational age (wks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (range)</td>
<td>31.8 (29.0–33.3)</td>
<td>30.4 (29.1–34.0)</td>
<td>28.7 (25.1–31.8)</td>
</tr>
<tr>
<td>Age at perforation (d), mean (μm)</td>
<td>N/A</td>
<td>N/A</td>
<td>7.1 (2–15)</td>
</tr>
<tr>
<td>Body weight (g), mean (range)</td>
<td>1895 (1129–2190)</td>
<td>1129 (856–1800)</td>
<td>945 (615–1873)</td>
</tr>
</tbody>
</table>

* n = 50 (body weights not available for the remaining 14).*
bowel and colon CM and LM, most likely related to smooth muscle cell hyperplasia. In patients with IIP, the muscularis propria of grossly normal adjacent segments of bowel follows the same linear growth with age. Previous studies have associated increases in thickness of the muscularis propria with peristalsis [8,18–20]. This description would be consistent with our findings, given that peristalsis has been noted to occur as early as 8 weeks of gestation [21–23]. The stage at which the CM and LM thicknesses plateau was not found in this study based on the upper CGA limit of our patient population.

In this study, IIPs were only reported to occur in the small bowel, with ileal lesions accounting for 64%. All affected patients were preterm in the CGA range of 25.4 to 33.7 weeks. In the literature, IIPs have been reported to occur in infants 25 weeks’ gestational age to full term, and in the stomach, small bowel and colon, but most commonly at the terminal ileum [6,16,24–27]. According to the classification developed by Attridge et al. [9], the majority of our perforated patients (11/12) were late presenters, indicating likely associations with postnatal treatment risk factors. Although this study has limited power to determine the significance of risk factors for perforation, we observed that a higher proportion of babies with IIP had received antenatal NSAIDs and steroids, postnatal steroids and vasopressors, and initiation of enteral feeds and CPAP, when

![Fig. 1. Muscle thickness vs. corrected gestational age in small bowel control specimens. Muscle thicknesses (in μm) for the (A) muscularis mucosa, (B) circular muscle and (C) longitudinal muscle are plotted against corrected gestational age (in weeks). Results of linear regression analysis: muscularis mucosa $R^2 = 0.0001$, circular muscle $R^2 = 0.5377$ and longitudinal muscle $R^2 = 0.5660$.](image)

Table 2
Muscle layer measurements compared to corrected gestational age (CGA) stratification of CGA-matched controls, adjacent normal and perforated segments from patients with isolated intestinal perforation.

<table>
<thead>
<tr>
<th>Muscle layer measurement</th>
<th>Corrected gestational age stratification</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25.1–29.0 wks</td>
<td>29.1–34.0 wks</td>
</tr>
<tr>
<td></td>
<td>CGA-matched control (n = 2)</td>
<td>Isolated intestinal perforation (n = 8)</td>
</tr>
<tr>
<td></td>
<td>Adjacent normal</td>
<td>Perforated segment</td>
</tr>
<tr>
<td>Muscularis mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness (μm)</td>
<td>47.6 ± 5.6</td>
<td>38.1 ± 3.5</td>
</tr>
<tr>
<td>Cellularity (no. of cell layers)</td>
<td>10.3 ± 0.3</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>5.5 ± 0.4</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>Alpha-smooth muscle actin density</td>
<td>1.02 ± 0.05</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>Circular muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness (μm)</td>
<td>135.4 ± 20.8</td>
<td>169.5 ± 9.0</td>
</tr>
<tr>
<td>Cellularity (no. of cell layers)</td>
<td>24.2 ± 1.5</td>
<td>27.6 ± 2.0</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>6.8 ± 0.1</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>Alpha-smooth muscle actin density</td>
<td>0.76 ± 0.04</td>
<td>0.88 ± 0.02</td>
</tr>
<tr>
<td>Longitudinal muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness (μm)</td>
<td>95.4 ± 8.6</td>
<td>126.9 ± 6.3</td>
</tr>
<tr>
<td>Cellularity (no. of cell layers)</td>
<td>21.0 ± 1.3</td>
<td>20.8 ± 1.5</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>4.6 ± 0.5</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>Alpha-smooth muscle actin density</td>
<td>0.84 ± 0.06</td>
<td>0.89 ± 0.02</td>
</tr>
</tbody>
</table>

Data expressed as means ± SEM.

* Relative to density of blood vessel wall as internal control.
* Compared to CGA-matched control in 25.1– to 29.0-week range.
** Compared to adjacent normal in 25.1– to 29.0-week range.
*** Compared to CGA-matched control in 29.1– to 34.0-week range.
**** Adjacent normal in 29.1– to 34.0-week range.
compared to controls. Previous studies, including a large data set from the Pediatrix Medical Group and several case series, have suggested possible antenatal risk factors including anatomic abnormalities (low birth weight, congenital diverticula, omphalomesenteric remnants, intussusceptions, compression from intraluminal meconium), physiologic deficiencies (hypoxia, ischemia), infections (chorioamnionitis), and medications (NSAIDs, steroids) [10,11,16,18,25,28–34]. Antenatal NSAIDs are commonly used as first-line agents in tocolysis (indomethacin), as well as for analgesia (ketorolac, ibuprofen), while steroids are used to accelerate fetal lung maturity and reduce mortality from respiratory distress syndrome [35,36]. With the increased use of these interventions, several data sets have been developed to investigate adverse associations with IIP, however, no consensus has been established [34,37,38]. Postnatal risk factors for IIP have also been described, most notably the early use of indomethacin and steroids, especially when used in combination [8,10,16,18,30–32,34,38–45]. Indomethacin and ibuprofen have been shown to be equally effective in the closure of patent ductus arteriosus (PDA), and are still widely used in preference to surgical ligation owing to operative risks, despite the association with IIP [46–48]. Fortunately, widespread use of postnatal steroids has decreased with current guidelines no longer recommending its use in the treatment of neonatal chronic lung disease because of associations with neurodevelopmental impairments [49]. Vasopressors have also been implicated in the development of IIP, but are still used given its necessity in hemodynamic support [10]. Although several authors have postulated that early enteral feeding and administration of CPAP may lead to IIP, this has not been supported by other sources [50,51]. CPAP has been used increasingly as an alternative to or transition from invasive mechanical ventilation, while early trophic feeds have been advocated to improve intestinal function and feeding tolerance, and decrease bacterial translocation and sepsis; with these trends, we may expect to see an increase in the incidence of IIP [52–63].

In our series, it was interesting to note that one patient from each group (control 1/5 vs. IIP 1/12) did not have any of the aforementioned treatment risk factors. Major described risk factors, including postnatal steroids, NSAIDs and vasopressors, were absent in half of patients studied (control 2/4 vs. IIP 6/12). This would suggest that these risk factors are not an absolute requirement for the development of IIP, but rather act as a “second hit,” increasing the likelihood of perforation at an area already weakened by congenital muscular deficiency. Physiologic insults, infections and medications may have a common pathway in their effects on muscular development. Although enteral feeding and CPAP are unlikely to cause muscular thinning, they may instead apply mechanical stress on the area of muscle attenuation and increase the risk of perforation. Fortunately, the risk of IIP decreases with age, likely because of replacement of deficient muscular tissue with hyperplastic muscle fibers or with fibrotic tissue [64].

All IIP patients in this study were treated with a laparotomy and bowel resection, with half proceeding to a primary anastomosis and the other half receiving an ostomy. None of the patients who were reanastomosed primarily suffered complications of anastomotic leakage. This suggests that primary anastomosis is an appropriate treatment option in suspected cases of IIP. This would decrease the morbidity of an ostomy and requirement for a second surgery. Unfortunately, the majority of operative reports in this series did not comment on the gross appearance of bowel wall thickness, and as such, we are unable to determine any correlation with microscopic muscle thickness. However, the adjacent normal bowel at the resection margins was normal in appearance grossly and microscopically compared to controls. This suggests that a minimal resection or excision of the perforation with primary anastomosis is sufficient and that areas of gross thinning without perforation may be oversewn with adjacent thicker muscle.

There were several limitations to this study, including its retrospective nature and small sample size from a single institution. There were a limited number of patients in the control group who fell within the same CGA range as the IIP group for comparison. Also, although 36% of the perforations were in the jejunum, we were unable
to analyse small bowel separately for jejunum and ileum because of too few jejunal control samples. In addition, there was an inherent unreliability of using a morphometric histological approach to determine muscle thickness given the artifact created by variable amounts of intraluminal content, stretching of the tissue during mounting, timing and method of storage, which could not be controlled in this retrospective study. It was also difficult to distinguish if the area of attenuation was truly congenital or secondary to intense muscle contraction as a reaction to the perforation itself, given that there were no specimens revealing muscle deficits without perforation. However, if this effect was a result of muscle contraction, we would expect the cellularity of the muscle layer to be unchanged despite the decrease in muscle thickness. The hypothesis that muscle thinness is caused by intense muscle contraction could be tested by performing the same analysis on perforations caused by NEC or using an animal model with an iatrogenic perforation to determine changes in muscle thicknesses in those cases. Future directions of study include determining the muscle thicknesses, cellularity, cell size, and α-smooth muscle actin stain density of early IIP patients given that this group is a unique variant of perforations caused by NEC or in iatrogenic perforations in animal models may shed light on the effect of muscle contraction as secondary event after perforation. Deviating from the subjectivity of a morphometric study, a more quantitative approach with Western analysis to determine the smooth muscle content after microdissection of the MM, CM and LM may be considered. In addition, a larger database of patients would be able to determine any age-related decreases in IIP risks and identify specific risk factors. With advances in diagnostic imaging resolution, there may come a time when bedside ultrasounds will be able to discern the thickness of bowel musculature and assist in decision making for whether postnatal treatment risks factor for IIP should be used.

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References

Table 3
Antenatal and postnatal treatment in corrected gestational age (CGA)-matched controls and patients with isolated intestinal perforation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CGA-matched control (n = 5)</th>
<th>Isolated intestinal perforation (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antenatal</td>
<td>1/4 (25%)</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>3/5 (60%)</td>
<td>10/12 (83%)</td>
</tr>
<tr>
<td>Steroids</td>
<td>0/4 (0%)</td>
<td>1/12 (8%)</td>
</tr>
<tr>
<td>Postnatal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enteral feeds</td>
<td>1/4 (25%)</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>Vasopressors</td>
<td>1/4 (25%)</td>
<td>6/12 (50%)</td>
</tr>
<tr>
<td>CPAP</td>
<td>1/4 (25%)</td>
<td>6/12 (50%)</td>
</tr>
</tbody>
</table>

NSAIDs, nonsteroidal anti-inflammatory agents; CPAP, continuous positive airway pressure.


