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Investigation of the Histopathological and Histometric Changes in Rectum Tissue Biopsies of Hirschsprung and Non-Hirschsprung Disease in Neonate and Infant

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ABSTRACT

Hirschsprung disease is one of the most common and problematic infancy and childhood maladies. The most reliable method for diagnosis is the histopathological analysis of colorectal biopsies and the typical finding of Hirschsprung disease is the absence of ganglion cells. The study involved 36 cases of suspected Hirschsprung disease, in which 27 cases were males and 9 cases were females with an age range between (1 day - 1 year). All patients undergo complete history taking, physical examination, radiological investigations, and rectal biopsy. The tissue specimens were obtained from the rectum of neonate and infant patients, maintained in the fixative solution (formalin 10%) for histopathological analysis, and patients were divided into two groups (Hirschsprung disease and non-Hirschsprung disease group) according to histological findings. Statistical analysis was performed on the tabulated data by chi-square, and the automated computer-adopted image analysis program Image J® was utilized for the histometrical examination of rectum tissue. The Hirschsprung disease is more common in males than females. The delayed passage of meconium is the most common symptom of these diseases. We notice that there is a significant difference between the Hirschsprung disease group and the non-Hirschsprung disease group in clinical signs, empty rectum, tight anal sphincter on per rectal examination. Based on histopathology analysis of the rectal biopsy, out of the 36 cases, 27(75%) cases were diagnosed as Hirschsprung disease, and 9 (25%) cases as negative for Hirschsprung disease. In Histometric study of rectal biopsies tissues showed a significant difference in the mucosa, Submucosa, and muscularis externa between Hirschsprung disease and non-Hirschsprung disease patients.

KEYWORDS: Hirschsprung disease, rectum tissue, histopathology, histometric.

الخلاصة

يعد مرض هيرشسبرونج أحد أكثر أمراض حديثي ولادة والرضع شيوعًا. الطريقة الأكثر موثوقية للتشخيص هي التحليل النسيجي المرضي لخزعات القولون والمستقيم والنتيجة النموذجية لمرض هيرشسبرونج هي عدم وجود الخلايا العقدية. اشتملت الدراسة على ٣٦ حالة يشتبه اصابتها بمرض هيرشسبرونج ، منها ٢٧ حالة من الذكور و٩ حالات للإناث تتراوح أعمارهم بين (١ يوم-١ سنة). يخضع جميع المرضى لأخذ التاريخ الكامل والفحص البدني والفحوصات الإشعاعية وخزعة المستقيم. تم الحصول على عينات الأنسجة من المستقيم للمريض، وحفظها في محلول مثبت (فورمالين ١٠٪) لتحليل الأنسجة المرضية، وقسم المرضى إلى مجموعتين (المصابين بمرض هيرشسبرونج وغير المصابين بمرض هيرشسبرونج) حسب النتائج النسيجية. تم إجراء التحليل الإحصائي على البيانات المجدولة بواسطة مربع كاي، وتم استخدام برنامج تحليل الصور الآلي المعتمد بالكمبيوتر Image J® لفحص نسيج المستقيم. يعتبر مرض هيرشسبرونج أكثر شيوعًا عند الذكور أكثر من الإناث. ويعتبر تأخر مرور العقي أكثر أعراض هذا المرض شيوعًا. نلاحظ أن هناك فرقًا معنويًا بين مجموعة مرضى هيرشسبرونج ومجموعة غير المرضى هيرشسبرونج في العلامات السريرية، المستقيم الفارغ، العضلة العاصرة الشرجية الضيقة عند فحص المستقيم. على أساس تحليل التشريح المرضي لخزعة المستقيم من أصل ٣٦ حالة، تم تشخيص ٢٧ (٧٥٪) حالة على أنها مرض هيرشسبرونج ، ٩ (٢٥٪) حالات سلبية لمرض هيرشسبرونج. أظهرت دراسة قياس النسيج لخزعات المستقيم وجود فرق كبير في الغشاء المخاطي، وتحت المخاطية، والعضلات الخارجية بين مرضى هيرشسبرونج والذين لا يعانون من مرض هيرشسبرونج.

INTRODUCTION

The submucosal (Meissner) plexus, the myenteric (Auerbach) plexus (between the longitudinal and circular muscle layers), and the smaller mucosal plexus are the three nerve plexuses that innervate the gut. All of these plexuses are intricately connected and play a role in regulating blood flow as well as absorption, secretion, motility, and other bowel functions [1]. The absence of these enteric neurons at the Meissner's plexus of the submucosa and Auerbach's plexus of the muscularis in the terminal rectum that extends in a variable distance proximally is the hallmark of Hirschsprung's disease (HD) [2][3]. leading to functional intestinal obstruction [4]. The HD is a complex genetic congenital condition. The Latin name for HD is megacolon congenitum [5]. According to reports, HD has a 4:1 male-to-female ratio and impacts 1 case out of every 5,000 live births globally [6]. The HD is often discovered throughout the first year of life in most individuals, but it can occasionally occur later [7], even in adulthood. Abdominal distension, feeding issues, failure to pass meconium, constipation, and frequently bilious emesis are common symptoms in infants [8]. The HD is an uncommon congenital condition [9], Parents need to be alert of any suspected HD signs, such as a delay in meconium transit lasting more than 48 hours during the neonatal period. Additionally, constipation, abdominal distention, nausea, vomiting, and attacks of explosive diarrhea are other non-specific symptoms that need to be taken seriously [10]. Because most specialist doctors find it difficult to diagnose HD and the different methods of diagnosis depending on clinical signs and radiological examinations, The study aims to establish scientific bases in diagnosing the disease based on the evaluation of histopathological changes that occur in the layers of the colon of a group of neonate and infants who suffer from chronic constipation since birth and are suspected of HD.

MATERIALS AND METHODS

Subjects

The study included the histopathological and histometrical examination of 36 children suspected of having HD. The children's ages ranged between (1 day-1 year) for both sexes, 9 females and 27 males. The samples were obtained from the Central Teaching Hospital for Children and teaching laboratories at the Medical City Hospital in Baghdad from July 2022 to December 2022.

Collection and Preparation of the Rectum Tissue Specimens

The tissue specimens were obtained from the rectum of 36 neonates and infants who clinically presented with symptoms suspicious of having HD after being diagnosed by specialized doctors. The cases were rectal punch biopsies as shown in Figure 1, and the tissue samples were maintained in the fixative solution (formalin 10%) for histopathological study.

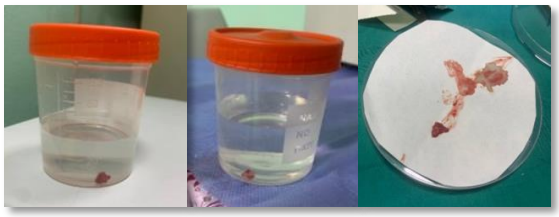

Figure 1. Rectal Biopsies sampling for different patients.

Tissue samples from the rectal biopsy were prepared for histological study to the method of Suvarna [11][12]. Each tissue sample was usually cut into small fragments about 2-3 cm long before fixation in a buffered isotonic solution of 10% formaldehyde for 24 hours. Each biopsy was processed for the dehydration process, which was done by passing them through progressive concentrations of ethanol alcohol and cleared by passing them through two steps of xylene. Then, tissues were infiltrated with paraffin wax and embedded in a metal template. After that, the paraffin blocks were sectioned by rotary microtome into sections 5µ in thickness. The slides were examined using a light microscope after staining with H&E for ganglion cell examination.

Histometric Analysis of Rectum Tissue

The automated computer-adopted image analysis program Image J® was utilized for the histometrical examination of rectum tissue. It is made by USA-based Sun Microsystems Inc.

Steps for isometric analysis:

a. From the program's (file) icon, the image of the inserted glass micrometer was opened. Next, a straight line with zero angles is drawn to indicate the distance between the first and last scales of the measurement that needs to be calibrated.

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b. To install this distance on the scale, the (analysis) icon was opened to calibrate the image to be measured.
c. The image to be measured opens from the (file) icon in the program and a straight line is then drawn to indicate the distance to be measured.
d. When the word "measuring" is clicked from the icon for "analysis," the micrometer measurement results will show up.

Statistical Analysis

Statistical Package for Social Sciences (SPSS) version 21 is used to interpret the data. The information is given in the form of a mean, standard deviation, and ranges. Frequencies and percentages are used to display categorical data. ANOVA was used to compare between tested mean Data expressed as mean±SD. The LSD test was used to calculate the significant differences between the tested mean. Values of $p>0.05$ were considered statistically non-significant while $p\leq0.05$ considered significant results.

RESULTS AND DISCUSSION

Patient and Control Distribution

Table 1 showed the age and gender comparison of both groups. The neonate ratio of the control group was (40%) and the infant ratio of the control group was (60%), the neonate and infant ratio of the HD patients group was (48,1%), (51,9%) respectively, and neonate and infant ratio of non-HD patients group was (44,4%), (55,5%). According to gender, the result showed the ratio of the control group (55% male and 45% female) and the HD patients' group (74% male and 26% female), and the non-HD patient group (77% male and 22% female. In this study, a predominance of males in patient groups can be observed, which means a little bit more common in males than females.

Table 1. The distribution of age and gender for the HD and non-HD patients and control group.

Study Groups	Neonat	infant	Gender	
			Male N (%)	Female N (%)
Control	8(40%)	12(60%)	11 (55%)	9 (45%)
HD	13(48,1%)	14(51,8%)	20 (74 %)	7(25,9%)
Non-HD	4(44,4%)	5(55,5%)	7(77,7%)	(2,22%)

The [13] reported that male: female ratio in the HD patients was 4:1 while it was 2:3 in the non-HD group which was almost the same result we get in our study as HD is more common in males than females, which was nearly the same outcome as that indicated by [14] in his study. Out of 107 hospitalized patients, 32 cases (or 3.5:1) of HD were found, with a male-to-female incidence ratio overall.

Clinical Features: Symptoms

Delayed passage of meconium, Abdominal distension, Constipation, Vomiting, failure to pass and fever are common symptoms in HD patients Table 2 shows the symptoms for HD and non-HD cases.

Table 2. the symptoms for HD and non- HD cases.

Symptoms	HD		Non-HD		P value
	+ve (%)	- ve%	+ve(%)	- ve (%)	
Delayed passage of meconium	26(96.3%)	1(3.7%)	3(33.3%)	6(66.6%)	***0.0001 SIG
Abdominal distension	25(92,6%)	2(7,4%)	7(77,7%)	2(22,2%)	0.025* SIG
Constipation	23(85,2%)	4(14,8%)	4(44.5%)	5(55.5%)	0.009 SIG
Vomiting	22(81.5%)	5(18.5%)	6(66.6%)	3(33.3%)	0.120 Non sig
Fever	10(37%)	17(62,9%)	3(33,3%)	6(66,6%)	0.682 Non sig

The delayed passage of meconium is the most common symptom of HD in our study which was almost the same result mentioned by [15] as the delayed passage of meconium in HD patients was reported to be about 90 % of patients in the HD group. While in [16] study reported the delayed passage of meconium in HD patients was to be about 81 %. Furthermore, [17] study reported in his study that only about 65% of HD patients presented with delayed passage of meconium and it is about 13% in non-HD group. These differences in the result might be due to the presence of a ganglionic segment of varying lengths of the distal colon [8] or maybe the early detection of HD group in the early age. In our study, there was just one instance of meconium passing without being delayed, and

this was because the patient had aganglionosis, which typically results in a delayed diagnosis, and only affected a small portion of the body [18]. According to the researchers, passing meconium on the first day of birth does not rule out the HD [19] reached in their study. This data is in stark contrast to those of healthy newborns, who pass meconium 95% of the time within 24 hours.

But recent research by Lorijn of 111 children suspected of having HD, showed the delayed meconium passing did not appear to be a good indicator of HD [20]. Abdominal distension did not clearly distinguish either group. In contrast to being modest in the non-HD group, the degree of distension was significantly more apparent (moderate to severe) in the HD group (figure 2), which was seen as visible abdominal vasculature and an everted umbilicus. This finding was nearly identical to that of [17] as abdominal distention in another study was 80% among HD patients while [21] mentioned that abdominal distention was present in 60.1% of the HD patient.

Most cases suffered from constipation were infants. [22] found that the most prevalent complaint was constipation, which accounted for 94.5% of all cases. This is because a sizable portion of individuals with HD continue to have constipation and soiling. Vomiting and fever are not significantly different between the HD group and the non-HD group.

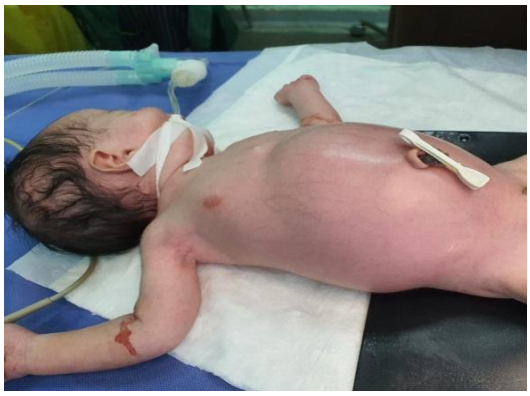

Figure 2. The abdominal distention in the HD patient.

This could be because these symptoms are common to many illnesses and are not specific to HD [23]. Approximately 77% of HD children came with vomiting, and fever was a symptom suggestive of enterocolitis and/or a substantial intestinal obstruction, according to the same study's findings [24]. According to our statistical analysis, a history of delayed meconium, abdominal distension, and constipation should alert a pediatric surgeon to the possibility of HD. Our results revealed a significant difference between the HD group and the non-HD group, which is consistent with the study of [25].

All symptoms are similar between the two groups, and the reason is that there are many diseases whose symptoms are similar to the symptoms of HD. In one study [26], The Radiology Department received 21 consecutive infants (3 to 8 days old) with non-passage of meconium and aberrant features on a plain abdominal X-ray indicative of a low type intestinal obstruction likely caused by meconium ileus. Bowel atresia was the ultimate diagnosis in 10 patients, severe meconium ileus in 3, HD in 2, small left colon syndrome in 2, and simple meconium ileus in 4. Clinical signs of abdominal distention, bilious vomiting, and failure to pass meconium were seen in all patients.

Some of the cases in this study were diagnosed clinically as constipation in the first year of life, and the mother complained that her child had been constipated since birth. Because there is some misunderstanding about the passing of meconium, which is crucial and should occur within 24-48 hours in this area. Otherwise, the infant should be evaluated to rule out HD; this is crucial information in the past of individuals who are suspected of having HD [27].

Clinical Signs

Based on the symptoms above, A total of 36 patients were referred to pediatric surgeons for constipation, the cases had a rectal exam by the patient's pediatrician. It was noted that most children had an empty rectum, a tight anal sphincter per rectal examination followed by explosive passage of feces after removal of the finger. Our results showed in Table 3 a significant difference between the HD group and the non-HD group, where it turns out that the empty rectum was another representative sign of HD. However, it depends on the surgeon’s experience and is a subjective sign, and hypertonic anal sphincter or tightness or feeling of resistance on rectal examination also appears to make a significant difference. On the other hand, we found a non-significant difference between the HD group and the non-HD group in explosive diarrhea.

The HD can be differentiated from functional constipation if the child has these signs [28].

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Table 3. Per rectum exam between the HD group and the non-HD group.

Parameters			HD No. (27)	Non-HD No. (9)	Odd Ratio	95% CI	P value
Per rectum exam	Hypertonic anal sphincter	Yes	12	1	7.37	1.13 - 26.34	0.02 SIG
		No	15	8			
	Empty rectum	Yes	26	3	3.04	1.05 - 6.83	0.03 SIG
		No	1	6			
	Explosive diarrhea	Yes	3	1	1.08	0.11 - 8.44	0.08 NSIG
		No	24	8			

A rectal examination frequently results in the passage of meconium and the resolution of acute intestinal obstruction in HD cases. For a few days or weeks, these infants may have regular bowel movements before developing symptoms of intestinal blockage [16]. Children with HD frequently lack fecal incontinence, which is frequently present in children with functional constipation. Though it is treatable with dietary adjustments, laxatives, suppositories, or enemas, chronic constipation with or without abdominal distention may be a symptom of HD in a small percentage of individuals. Thus, it is evident that to avoid serious problems from HD, early identification between HD and functional constipation is required [29].

Due to the aberrant sphincter innervation caused by HD, the [30] findings demonstrated that non-relaxation of the anal sphincter complex is present in all HD patients. Functional gastrointestinal obstructive symptoms may persist if the residual bowel is unable to pass through this non-relaxing anal sphincter complex. Empty rectum is one of the symptoms of HD, and it can be used to distinguish HD from functional constipation according to the authors [31]. On the other hand, numerous researchers have demonstrated that explosive diarrhea is a sign that enterocolitis may be present [29]. And then 36 rectal biopsies were performed by a pediatric surgeon and sent to the laboratory to complete the diagnosis

Histopathological Study

The Hematoxylin and Eosin

Based on histopathology analysis of the rectal biopsy (H&E-stained sections), out of the 36 cases 27(75%) cases were diagnosed as HD, and 9 (25%) cases as negative for HD. In the normal segments, the myenteric plexus was observed between the inner and outer muscle layers, and the presence of ganglion cells was confirmed. Ganglion cells were identified in the submucosal (Meissner) plexus Figure 3.

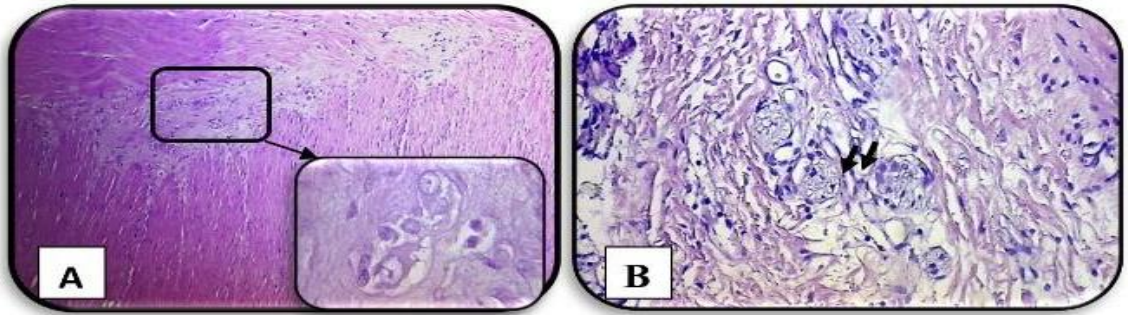

Figure 3. Cross section in rectal biopsy of the non-HD case showed (A): the presence of myenteric plexus (Auerbach's plexus) ganglion cells between the two layers of muscularis. (B): the presence of myenteric plexus (Meissner's plexus) ganglion cells in the submucosal layer (Hematoxylin and Eosin staining, large figure: X4, small figure: X40).

According to several accounts in the literature, identifying a ganglion cell in an H&E-stained section is not difficult for a skilled observer, especially if the tissue segment's histological quality is good [32]. Numerous cytoplasmic cells, an eccentric circular nucleus, a noticeable nucleolus, a perinuclear pallor, and peripheral chromatin are examples of classic cytological characteristics. Ganglion cells are typically, but not always, clustered in ganglia and/or in contact with the neuropil (neurites and glia) [33].

In a study conducted in 2020, the [34] explained that cytologically immature ganglion cells with less cytoplasm, stippled nuclear chromatin, and an unclear nucleolus can be problematic and may prevail in extremely young or premature newborns. However, with sufficient skill, even immature ganglion cells can be recognized. And [35] assert that, despite the limits discovered by some researchers, hematoxylin and eosin is still the diagnostic technique of choice for locating ganglion cells in submucosae in many locations. In contrast, in the ganglionic segments, ganglion cells were not observed between the two layers of muscularis, and the absence of myenteric plexus (Meissner's plexus) ganglion cells in the submucosa layer and the normal reticular arrangement of the myenteric nerves and ganglia were absent Figure 4.

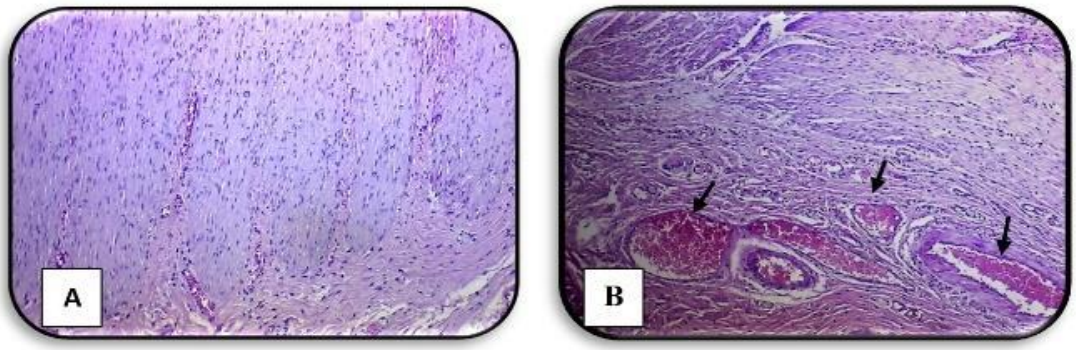

Figure 4. Cross section in rectal biopsy of HD case showed (A): the absence of myenteric plexus (Auerbach's plexus) ganglion cells between the two layers of muscularis, (B): showed the absence of myenteric plexus (Meissner's plexus) ganglion cells in submucosa layer with congestion of blood vessels (arrows) (Hematoxylin and Eosin staining, X4).

The [36] showed in the rectal biopsies' by using Hematoxylin and Eosin no ganglion cells were discovered in 13 of the 28 rectal samples that were used in this study, and these biopsies revealed nerve trunks of the submucosa that had hypertrophied. These results supported the diagnosis of aganglionosis, also 1 of the 28 instances had no submucosa in their and 14 of the 28 cases had ganglion cells visible. Rebiopsy was advised in this case since the biopsy was likely conducted in the HD transition zone, where there may have been a ganglion cell. Rectal mucosal biopsies are conducted in neonates 1 cm above the dentate line because they are technically straightforward procedures and the absence of ganglionic cells in the distal part of the anal canal is a normal variant [33]. But in the same study, the authors claimed that for the sections to be suitable for evaluation, they needed to have submucosa in the sections, to simplify the diagnosis, other studies have been conducted employing H&E staining in these remnants of rectal mucosa and submucosa [37]. The quick and easy way of detecting ganglion cells in the submucosal plexus and diagnosing HD with HE staining is still debatable. Numerous publications discuss how difficult it is to distinguish between the neurons in these plexuses because they are dispersed widely across the intestinal submucosa [38,39]. On the other hand, a study shows that H&E didn't provide a conclusive answer in 6 cases of full-thickness rectal biopsies (18% of the total), and 18 cases of split-thickness rectal biopsies (54.5%) of the total. In these cases, the interpretation of H&E sections was regarded as suspicious and inconclusive [40].

There are many signs that HE staining has only mediocre accuracy for the diagnosis of HD, according to the data [41]. Despite the significance of employing H&E and rectal biopsy in the diagnosis of HD, the pathologist may have

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difficulty identifying ganglion cells in H&E sections [42]. The rectal biopsy may be performed to distinguish HD from other causes of intestinal pseudo-obstruction, in which case ganglion cells will be present on histologic examination. Even though abdominal plain X-ray documented bowel obstructions in all patients, it had low specificity in the diagnosis of the cause of obstruction [43]. This is evident in nine cases in our study where HD was excluded, the biopsy results showed that 9 cases do not suffer from HD, but suspicion led us toward it. This is because of similar symptoms to HD; the most common symptom is failure to pass meconium and other symptoms include abdominal distention and bilious emesis.

In our result, we found that one of them had a small left colon syndrome. Infants born to diabetic mothers are more likely to experience neonatal small left colon syndrome, an unusual functional illness of the lower colon that causes signs and symptoms of intestinal blockage [44]. In particular, the sigmoid and descending colon are involved. Although the cause of NSLCS is unknown, many theories including functional immaturity of the ganglion cells, aberrant autonomic nervous system, drug use by the mother, immaturity of bowel innervations and motility in term newborns, are thought to be the major contributing factors [45]. Clinically, NSLCS newborns have a history of vomiting, growing abdominal distention, and non-meconium passing in the first 24 hours after birth. Nevertheless, a pathological examination revealed that the sigmoid colon had few ganglion cells, therefore we excluded HD [46]. And in another, newborns with Megacystis Hypoperistalsis showed signs of bowel and bladder blockage, abdominal distention, and delayed meconium passing leading to the suspicion of HD. This syndrome's genetic origin is due to mutations [47]. One of nine cases had meconium ileus, meconium retention and the absence of ganglion cells in the distal intestine tract distinguish meconium ileus from HD, which shows similarly [44]. According to the [26] study, 13 cases of small intestinal atresia, a difficult form of meconium ileus, HD, duplication cysts, and one case of Gastrografin enema nonresponse required surgical intervention. According to clinical and X-ray findings, patients who had delayed meconium passage and likely low bowel obstruction had final diagnoses of 9, 6, 3, 1, and 3 cases of small bowel atresia, simple form of meconium ileus, complicated form of meconium ileus, small left colon syndrome, bowel atresia, and duplication, HD, respectively. Meconium obstruction affected half of the examined cases overall, and the other half had different types of obstruction. Also, one of the mothers took a drug (pethidine) in the last month of pregnancy, which is believed to affect the health of the fetus and cause lethargy in its intestines. Since they are the most frequently prescribed medication to relieve severe pain in the Western world, opioids serve as the foundation of pain management. Opioids are being used more and more to manage pain that is not cancerous. However, opioids are linked to gastrointestinal (GI)-related side effects, which are collectively known as opioid-induced bowel dysfunction (OIBD). GI-related side effects include constipation, nausea, vomiting, dry mouth, gastroesophageal reflux, abdominal cramping, spasms, and bloating. Constipation caused by opioids is the most frequently reported and long-lasting side effect in people using opioids for pain [48].

Histometric Study of Rectal Biopsies Tissues

Based in histometric of rectal biopsies tissues (mucosa, submucosa, muscular layer) in HD and non-HD cases, the data in Table (4) exhibited a significant difference ($p < 0.05$) in Mean ± SD of rectal biopsies mucosa between HD patients (439.51±17.25) and non-HD patients (336.38±71.61). Also, the results showed a significant difference ($p < 0.05$) in the Mean ± SD of Submucosa between HD patients (458.83±13.25) and non-HD patients (165.03±18.19). Likewise, there was a significant difference ($p < 0.05$) in the Mean ± SD of Muscularis inner circular in HD patients (574.88±32.38) and non-HD patients (326.42±9.24), and Muscularis outer longitudinal HD patients (260.30±30.55) and non-HD patients (153.54±13.57). Microscopic examinations for rectal biopsies tissues. Figure 5 showed the thickness of mucosa, the thickness of the submucosal layer, and the thickness of Muscularis (inner circular and outer longitudinal). On the other hand, Figure 6 showed the thickness of the mucosal**,** submucosal**,** muscularis circular layer**,** muscularis longitudinal layer in the rectal biopsy

tissues of the HD case**.** Mechanically, the large intestine is anisotropic (e.g., stiffer in the longitudinal direction than in the circumferential direction), viscoelastic, and contractile [49]. The positions and morphology of PN afferent endings in various levels of the colorectum were delicately disclosed by sparse neural-tracing experiments, which revealed a concentration of afferent endings in the submucosal and muscle layers [50][51]. Two studies that examined the large intestine as a biological structure with layers found that the submucosa and muscular layers were primarily responsible for the tissue's mechanical strength and that the serosa and mucosa did not exhibit any appreciable stiffness [52][53]. The layered structure of the intestinal wall suggests variable biomechanical capabilities along the thickness of the wall [53].

According to the study of [54], the colon and rectum of individuals with lower GI illnesses or in animal disease models were also subjected to macroscopic mechanical characterization. With a few notable exceptions, such as active ulcerative colitis, increased colon stiffness has been observed in the majority of inflammatory bowel disease situations [55]. In addition to having a constricted lumen, colons from patients with diverticular disorders had decreased mechanical strength, reduced distensibility, and premature relaxation to distension [56]. The submucosa and muscular layers make up the majority of the intestine wall's mechanical strength, while the serosa and mucosa have no discernible strength, according to research done by the [52] on layer-separated large intestinal tissues taken from human cadavers. In recent biaxial tensile experiments, the [53] observed greater compliance in the mouse rectum than in the distal colon. However, according to a study by [57], the biomechanical characteristics of the human rectum and sigmoid colon *in vivo* are comparable. This most likely reflects the existence of smooth muscle tone, which under physiological circumstances causes an increase in rectal stiffness. The [50] explained that although the circular muscle layer is substantially thicker, it has a little higher density of sensory nerve terminals (25%). In contrast, the longitudinal muscle layers have very few extrinsic sensory innervations, according to [58].

Table 4. Histometric of rectal biopsies tissues (mucosa, submucosa, muscularis layer) in HD and non-HD case.

Layers thickness	Non-HD cases (Mean µm ± SD) (No.= 9)	HD cases (Mean µm ± SD) (No.=27)	P-value between tested groups
Mucosa	336.38±71.61	439.51±17.25	0.001
Submucosa	165.03±18.19	458.83±13.25	0.001
Muscularis (inner circular)	326.42±9.24	574.88±32.38	0.001
Muscularis (outer longitudinal)	153.54±13.57	260.30±30.55	0.001
SD: standard deviation, *Significant difference (p <0.05)			

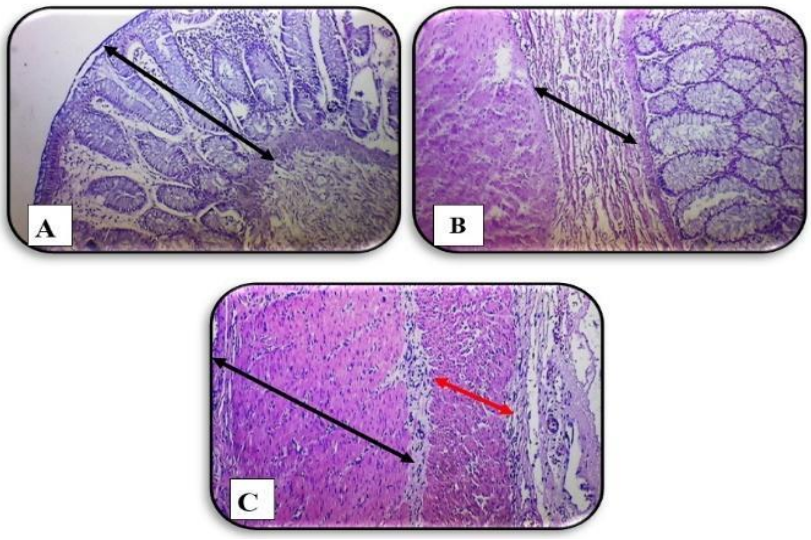

Figure 5. Cross-section in the rectal biopsies tissues of the non-HD case showed (A): mucosal thickness (double arrow), (B): submucosal thickness (double arrow), and (C): muscularis circular layer

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thickness (black double arrow) and muscularis longitudinal layer thickness (red double arrow) (Hematoxylin and Eosin staining, X10).

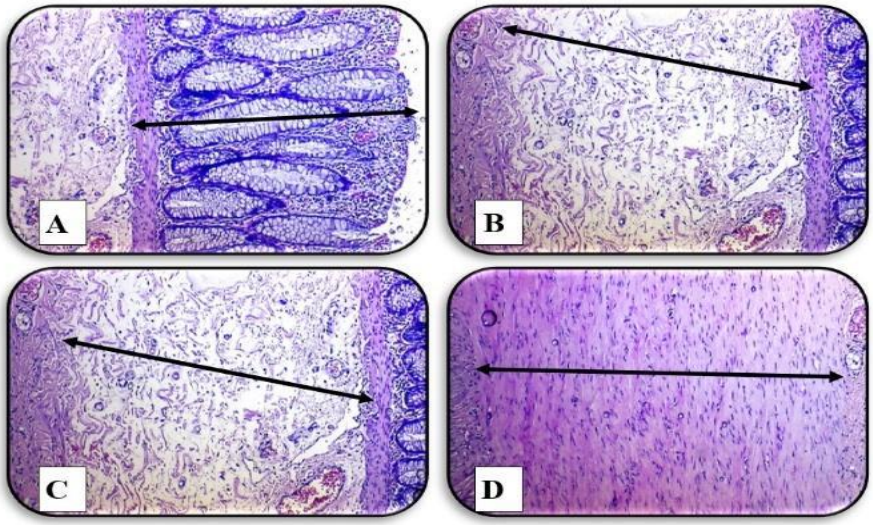

Figure 6. Cross-section in the rectal biopsies tissues of HD case showed (A): mucosal thickness, (B): submucosal thickness, (C) muscularis inner circular layer thickness, (D): muscularis outer longitudinal layer thickness (double arrow) (Hematoxylin and Eosin staining, X10).

Given that the rectum is substantially thicker than the colon, particularly at the circular muscular layers, this suggests that PN sensory endings are more negatively impacted by smooth muscle activities during normal GI functions than their LSN counterparts [56]. There have also been reports of primary myopathic diseases of the colon's longitudinal muscle coat, in which the circular muscle is comparatively unaffected but the "sick" longitudinal muscle coat contains vacuolated smooth muscle cells [59].

It is believed that smooth muscle cells are easily repairable and can adapt to increased or decreased demands in the gut, as well as in other tissues by hypertrophy or atrophy. However, there isn't enough information available about this shift in the colorectum. Free nerve terminals in the rectum and colon are likely parasympathetic nervous system-derived sensory fibers [60]

In HD, there is an absence of myenteric plexus and myenteric ganglia in the atonic segment and similar changes are found in patients with megacolon secondary to hollow visceral neuropathy, where the study conducted by [61] displays HD characteristics, which are comparable with the results previously reported, including loss of ganglionic neurons in the colon and rectum, increased circular muscle thickness, and increased intraluminal pressure during colonic expansion. In contrast, patients with irritable bowel syndrome did not appear to affect their colonic or rectal biomechanics. In conclusion, the majority of lower gastrointestinal illnesses, except for functional disorders like irritable bowel syndrome, are accompanied by changes in the large intestine's microscopic biomechanics [53]. As a result, we conclude that muscle thickness in intestinal wall thickening is a common feature in HD patients and unrelated to numerous clinical variables and supplements [62]. Obstruction can alter the morphological and mechanical properties of the tissue by altering the intestinal structure and morphology [63].

According to studies, a chronic partial intestinal obstruction can cause the muscle layer to thicken, increasing the size of the lumen's outer wall relative to its inner wall, lowering the inner wall's residual strain and expansion angle [64]. According to the research by [65], correlation analysis revealed a substantial positive link between the submucosal area, circular muscle area, and longitudinal muscle area and the circumferential residual stretch rate of the mucosa. The thickness of the submucosa considerably positively is linked with the axial residual stretch rate of the mucosa.

Additionally, there is a noticeable increase in collagen concentration close to the blockage location. While the wall stiffness primarily depends

on the thickness of the submucosa layer, the opening angle and residual strain primarily depend on the thickness of the muscle layer [66].
Numerous contents may build up in the proximal intestinal lumen as a result of intestinal blockage. It may result in an increase in intestinal burden and intestinal wall distortion. The growth of the obstructed proximal intestine, muscularis hyperplasia, intestinal wall neuronal hypertrophy, and an increase in collagen content are all possible side effects of long-term obstruction [65].
Additionally, the blockage may alter the morphological and mechanical characteristics of the tissue by altering the intestinal structure and morphology [66].

CONCLUSIONS

The HD should be suspected in all neonates with late meconium passage. The most reliable method for diagnosis is the histopathological analysis of colorectal biopsies and the typical finding of HD is the absence of ganglion cells. Hematoxylin & Eosin stain is considered an indicator for HD detection, where patients are divided into two groups (HD and non-HD group) according to the absence of ganglion cells. The histometric study showed the thickness of the rectum layer in HD patients in comparison with non-HD, accordingly our study concluded that thickness in bowel wall thickening is a common feature in patients with HD.

Informed Consent and Ethical Approval

All participants agreed to provide the investigator with the specimens. The ethics committee of the College of Science, Mustansiriyah University approved this work (Ref. No.: BCSMU/0622/0012Z).

Disclosure and Conflict of Interest: The authors declare that they have no conflicts of interest.

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