

Toluidine Blue Stain and Crystal Violet Stain Versus H&E Stain in the Diagnosis of Hirschsprung's Disease : A Study in Sulaimani City in Kurdistan/Iraq

Hadeel A. Yasseen

Department of pathology, School of Medicine, University of Sulaimani. Iraq

Keywords: *Hirschsprung's Disease, Ganglion Cells, Cresyl Violet, Toluidine Blue, Mast Cells*

ABSTRACT

Background: Hirschsprung's Disease (HD) is a congenital disorder of the colon in which certain nerve cells, known as ganglion cells are absent.

Setting and Design: To demonstrate the efficacy of Cresyl Violet and Toluidine blue (Tb) special stains in the identification of ganglion cells in suspected Hirschsprung's disease and to find other adjuvant histological criteria for the diagnosis.

Method: In Sulaimani Teaching Hospital and Pediatric teaching hospital in Sulaimani Governorate/ Kurdistan-Iraq a total of fifty non selected cases biopsied for suspected HD were stained with hematoxylin and eosin (H&E) stain and divided into two groups: HD and-non-HD. All cases then should be stained with Tb special stain to identify ganglion cells and to count mast cells in the submucosa. Cases were stained with Cresyl Violet special stain to identify ganglion cells. H&E- and Tb-stained sections were examined for the presence or absence of hypertrophic nerve fibers in the submucosa.

Results: Both Cresyl violet and Tb stains were superior to H&E in the identification of ganglion cells with no statistically significant difference between the two stains. Mast cell count in the submucosa has no important effect on diagnosis while nerve bundle hypertrophy was found to be associated with absence of ganglion cells in Hirschsprung disease. **Conclusions:** Toluidine blue and/or Cresyl violet stains should to be used as the routine stain to highlight ganglion cells in suspected Hirschsprung's disease cases. Submucosal nerve bundle hypertrophy has to be assessed as an adjuvant histological criterion.

***Corresponding author:**

Dr Hadeel A. Yasseen, Lecturer at Department of Pathology, School of Medicine, University of Sulaimani/Sulaimani city, Kurdistan regional government, Iraq.

Phone: +91-7703591007

E-mail: hadeeladnanyas@gmail.com



Introduction

Hirschsprung's Disease (HD) is a congenital disorder of the colon in which certain nerve cells, known as ganglion cells are absent, causing chronic constipation¹. The first report of Hirschsprung disease dates back to 1691² however, the disease is named after Harald Hirschsprung, the Danish physician who first described two infants who died of this disorder in 1888³. The most accepted theory of the cause of Hirschsprung's disease is that there is a defect in the craniocaudal migration of neuroblasts originating from the neural crest that occurs during the first 12 weeks of gestation. Defects in the differentiation of neuroblasts into ganglion cells and accelerated ganglion cell destruction within the intestine may also contribute to the disorder⁴. Hirschsprung's Disease occurs in about one in 5000 births⁵. Most cases are diagnosed before the patient is 10 years of age⁶. The gold standard for definitive diagnosis of HD is rectal biopsy, looking for the absence of ganglion cells and the finding of hypertrophied nerve trunks. The biopsy is taken 2-3 cm above the dentate line on the posterior wall of the rectum. Going too distally may result in a false-positive diagnosis of HD because ganglion cells are normally absent in the anal canal. The most common technique used is **full thickness rectal wall biopsy**. A **suction rectal biopsy** can be used to obtain tissue for histologic examination. Rectal mucosa and submucosa are sucked into the suction device. In experienced centers, the accuracy is 99.7%⁷.

In 1884 Franz Nissl (1860-1919) a medical student in Munich, discovered darkly colored granules in neuronal perikarya in sections of brain stained with methylene blue, a cationic (basic) dye⁸. Advances in electron microscopy and biochemistry in the 1950s showed that the Nissl bodies represented aggregations of rough endoplasmic reticulum, containing numerous ribosomes. Here, mRNA is translated and proteins are synthesized⁹.

Hematoxylin and Eosin (H&E) staining, Acetylcholinesterase staining (AChE) are commonly used in the diagnosis of HD. However, diagnosis is not possible with H&E every times, because staining has limitations in the diagnosis of immature ganglion cells in neonates and the submucosal area in which the ganglion cells are small in number (three to five cells per ganglion) and irregularly distributed and so their identification is difficult and requires high expertise¹⁰. On the other hand although AChE activity is diagnostically the most useful set of enzyme-histochemical reactions, it is not sufficient; AChE stains the parasympathetic nerve fibers and trunks of fibers that increase dramatically in the lamina propria mucosa and submucous layer, but is not a specific marker for ganglion cell¹¹. AChE staining requires the experience of pathologists and in some instances

interpretation is difficult¹². There are reports of false positive and false negative results using this technique¹³.

The Cresyl Violet Stain shows cell bodies of neurons and processes by virtue of their abundant rough ER and ribosomes (rRNA) or Nissl substance¹⁴. Nissl substance is very basophilic and will be very sharply stained with basic aniline dyes like Cresyl violet¹⁵. DNA present in the nucleus stains a similar color. In all the neurons studied the most prominent cytoplasmic basophilia consists of a broad dense band lying near the cell periphery. This peripheral basophilic ring is somewhat granular and shows local variations in density. Additional smaller masses of Nissl substance are usually to be found lying in the more central cytoplasm. Frequently, small rounded basophilic masses are found arrayed against the nuclear membrane forming a nuclear cap¹⁶.

Toluidine blue stain (Tb): (also known as toloum chloride) is a synthetic, acidophilic metachromatic dye that has an affinity for nucleic acids, and therefore binds to nuclear material with a high DNA and RNA content, in chromatin or Nissl substance¹⁷ and selectively stains nucleus blue and cytoplasm light blue¹⁸.

Other acidic tissue components (sulfates, carboxylates, and phosphate radicals) are stained in shades of blue. Toluidine blue stains mast cells metachromatic violet (with histamine and heparin metachromatic granules)¹⁹. Metachromasia is attributed to stacking of dye cations at the sites of high density of anionic groups in the tissue. Stacking shortens the wavelength of maximum absorption, a hypsochromic shift, so that the maximum wavelength in the spectrum of the transmitted light is longer making the observed color red instead of blue²⁰.

The aim of this study was to demonstrate the efficacy of Cresyl Violet and Toluidine blue special stains in the identification of ganglion cells in suspected Hirschsprung's disease and to find other adjuvant histological criteria for the diagnosis.

Materials and Methods

This prospective cross-sectional study was carried out from February 2013 to February 2014, in Sulaimani Teaching Hospital and Pediatric teaching hospital in Sulaimani Governorate/Kurdistan-Iraq. A total 50 non selected cases biopsied for suspected HD were stained with H&E stain. Cases negative for ganglion cells were serially sectioned and at least 4 additional sections were examined to confirm the diagnosis and cases were divided accordingly into two groups: HD and non-HD. Mucosa and submucosa were available for review in all the cases. Muscularis propria was available in resection specimens only.

All 50 cases then were stained with:

- 1. Toluidine blue** (Santa Cruz Biotechnology) special stain. This stain was used for two purposes; *first* to identify ganglion cells. *Second* to count mast cells in five high power fields ($\times 400$) in the submucosa.^{21,22} Toluidine blue staining on paraffin sections was performed using a simple Tb method that required incubation of sections in 0.2% aqueous solution of Tb in 56°C for 30 minutes and mounting in a water based medium. (Mast cells in normal skin biopsies were used as positive control).
- 2. Cresyl Violet** (Santa Cruz Biotechnology) special stain. This stain was used to identify ganglion cells. Staining in 0.1% Cresyl Violet for 30 minutes was done at 56°C and mounting in water based medium. (Neurons in normal brain tissue were used as positive control, Figure 1a)

According to the two special stains all cases were graded as follows:

- 1 + Some what easy to identify
- 2 + Easy to identify
- 3 + Very easy to identify
- No ganglion cells seen²³

H&E- and Tb-stained sections were examined for the presence or absence of hypertrophic nerve fibers in the submucosa then the relations between ganglion cells, hypertrophied nerve fibers and mast cells were studied. The results of the three stains were evaluated together and compared with similar studies.

All data (mean, standard deviation, percentile and graphical presentation) were inferred and analyzed using the Statistical Package for Social Sciences SPSS version 21 software for windows 7. Chi square test was used to compare the differences between variables and other tests used where found applicable. Level of statistical significance was set at P value < 0.05 .

Result

A total of 50 cases of chronic constipation in children below 7 years were studied. The age of the patients ranged from 0.6 month (2 days) to 84 months (7years) (mean \pm 2 SD = 19.425 ± 20.098 months). 27 cases (54%) were rectal punch biopsies while 23cases (46%) were resection specimens. Based on the findings of H&E stained sections of rectal biopsies, they were divided into two groups: HD included 20 cases (40%) and non-HD included 30 cases (60%). In HD group a male to female ratio = 1.5. Biological demography of all cases is shown in both Table-1and 2.

By using Toluidine blue special stain the ganglion cells were identified in 34 (68%) out of 50 cases and the ganglion cells were very easily identified in 36% cases. While only in 16 cases no ganglion cells were identified i.e. HD group. By using Cresyl violet special stain (figure1,b) the ganglion cells were identified in 36 (72%) out of 50 cases i.e. non-HD group while only in 14 cases no ganglion cells were identified i.e. HD group as shown in Table 3.

So the HD group consists of 20 cases by H&E stain while it consists of 16 cases by Tb and only 14 cases by Cresyl violet stain.

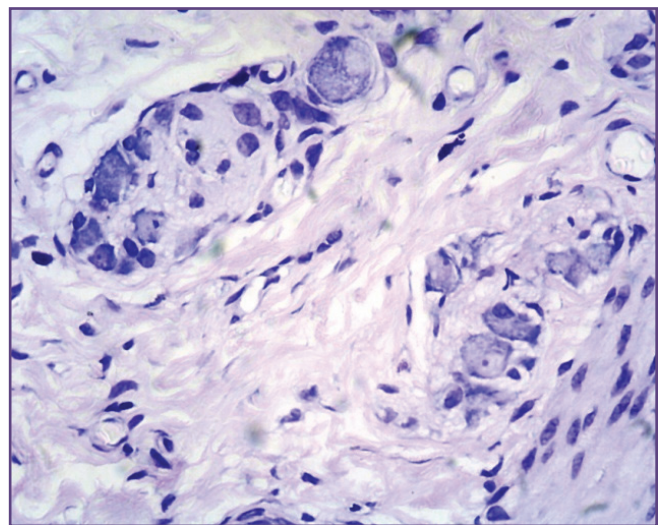
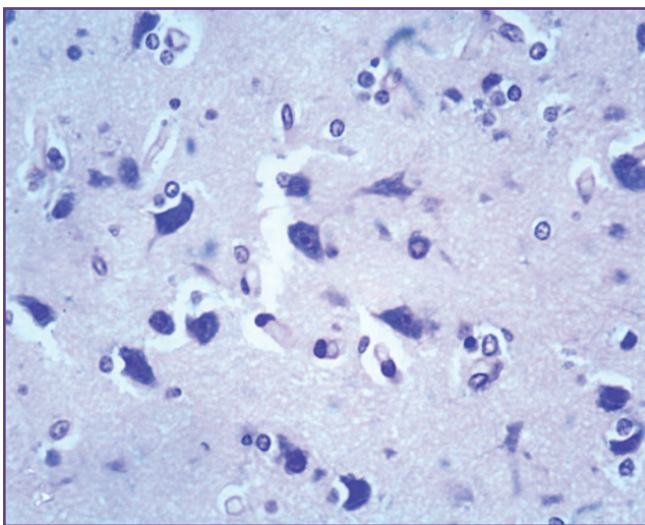


Fig. 1: (a) Normal brain tissue as positive control. Note the distribution of the stain in the nucleolus, perikaryon and dendrites. (b): Different size ganglion cells in non-HD case. (Cresyl Violet, $\times 400$)

Table 1: Relation between gender and age groups in months in HD and non-HD patients.

Age group of patients in months	Gender of patients			
	male		female	
	ganglionic cells presence in H&E stain		ganglionic cells presence in H&E stain	
	HD	Non-HD	HD	Non- HD
< 1	1	1	0	1
1- 11.9	7	11	2	3
12- 23.9	0	2	1	2
24- 35.9	0	3	5	2
36- 47.9	2	1	0	0
48- 59.9	2	0	0	0
60+	0	3	0	1
Total	12	21	8	9
	33		17	

Table 2: Descriptive Statistics in all cases.

Age of patients in months	Number	Minimum	Maximum	Mean	Std. Deviation
All cases	50	0.6	84.00	19.4258	20.09820
HD	20	0.13	48.00	19.0565	16.41888
Non-HD	30	0.6	84.00	19.6720	22.48861

Table 3: Identification of ganglion cells by Toluidine blue and Cresyl violet stains

Grading	Toluidine blue		Cresyl violet	
	Frequency	Percent	frequency	percent
No ganglion cells seen	16	32%	14	28%
Some what easy to identify	8	16%	10	20%
Easy to identify	8	16%	12	24%
Very easy to identify	18	36%	14	28%
Total	50	100%	50	100%

No statistically significant difference between Cresyl violet and Toluidine blue in the identification of ganglion cells is found as shown in table 4 (p-value=0.96).

The number of mast cells in the submucosa in 5hpf was counted. The relation between mean number of mast cells in the submucosa in Toluidine blue stained sections between HD (18.10 ± 10.06) and non-HD (18.0 ± 9.94) groups in H&E stain was done and no statistically significant correlation between the two variables was found (P-value=0.971). In HD group the mast cells were characteristically distributed around the nerves and blood vessels in addition to being randomly scattered. Mast cells were also present in large numbers in some non-HD cases (figure 2 a, b) mainly near submucosal blood vessels.

Kobayashi et al²², Demirbilek et al²⁴ and Amit et al²¹ in their studies found there is statistically significant difference between the number of mast cells in the submucosa of HD and non-HD groups but with no clear cut base line value. The mean number of mast cells in the submucosa of HD group in this study was compared with mean number of mast cells in the previous studies and a no statistically significant difference is found between this study and Amit et al study (p. value 0.159) as shown in Table 5.

The mean number of mast cells in the submucosa of non-HD group in this study was compared with mean number of mast cells in other similar studies and no statistically significant difference is found between this study and **Demirbilek** et al²⁴ study as shown in Table 6.

Table 4: Relation between Identification of ganglionic cells by Cresyl violet stain & Toluidine blue stain.

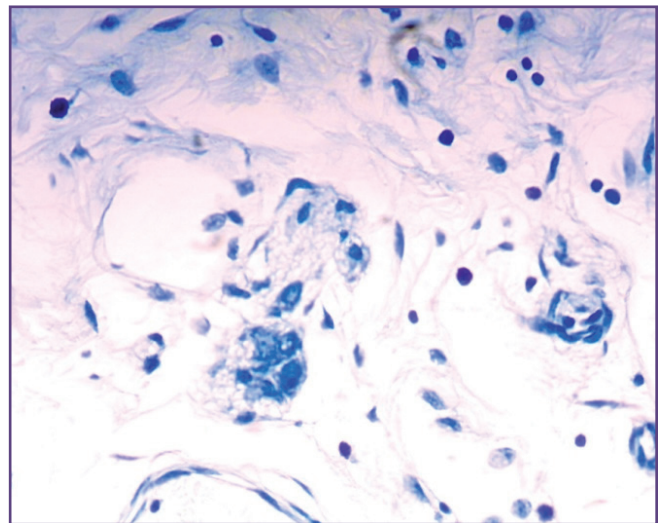
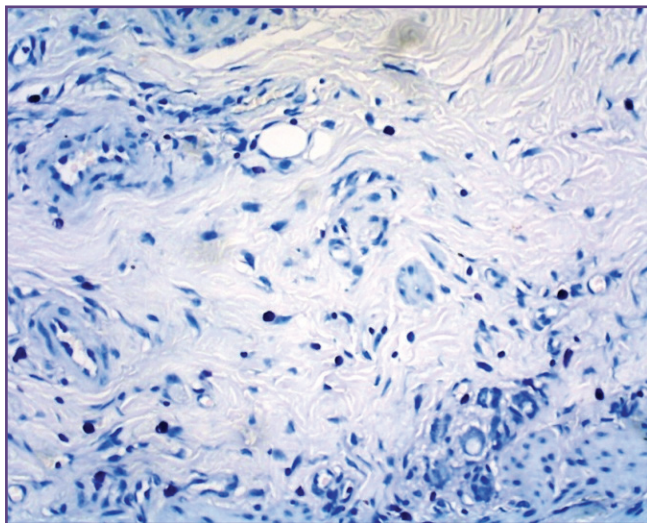
		Toluidine blue stain		Total	P- value
		No ganglion cells	ganglion cells		
Cresyl violet stain	No ganglion cells	14	0	14	0.96
	ganglion cells	2	34	36	
Total		16	34	50	

Table 5: Comparison of mean number of mast cells/5 hpf in submucosa of HD cases with previous studies.

	Mean NO. of Mast in Other Studies	Mean NO. of Mast in this study	P value
Kobayashi et al22	7.2 ± 3.4	18.10 ± 10.06	0.000
Demirbilek et al 24	5.4 ± 1.2	18.10 ± 10.06	0.000
Amit et al21	14.8 ± 5.3	18.10 ± 10.06	0.159

Table 6: Comparison of mast cells/5 hpf in submucosa of non-HD cases with previous studies.

	Mean NO. of Mast in Other Studies	Mean NO. of Mast in this study	P value
Kobayashi et al 22	23.9 ± 6.6	18.0 ± 9.94	0.003
Demirbilek et al 24	18.2 ± 3.3	18.0 ± 9.94	0.913
Amit et al 21	36.36 ± 39.58	18.0 ± 9.94	0.000

**Fig. 2: (a) Note the presence of many mast cells in a non-HD case (Tb x200). (b) Mast cells around submucosal ganglia in another non-HD case. (arrows to submucosal ganglia), (Tb, x400)**

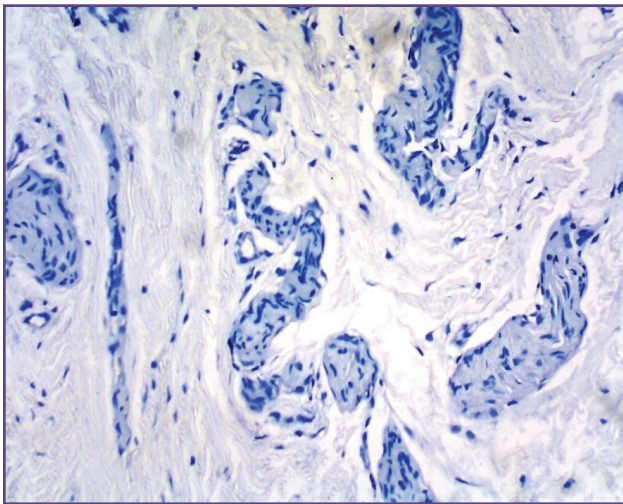
The correlation between the hypertrophied nerve fibers presence and ganglion cells presence by H&E stain in all fifty cases were studied and statistically a significant difference is found between HD and non HD groups as shown in Table7. All the seven cases of hypertrophied nerve bundles were found in HD group confirmed by Toluidine blue (figure 3) and Cresyl violet.

Discussion

Evaluation of suction rectal biopsies for the presence of ganglion cells remains very challenging, especially in negative or equivocal cases. The majority of these biopsies are performed on newborn infants, in which the immature ganglion cells can be confused with endothelial cells or fibroblasts. As a result of these difficulties, a large number

Table 7: Relation between HD and non-HD groups and the presence hypertrophied nerve bundles.

Presence of ganglion cells in H&E stain	presence of hypertrophied nerve bundles		Total	P-value
	absent	present		
HD	13	7	20	
Non-HD	29	1	30	0.048
Total	42	8	50	

**Fig. 3: Hypertrophied nerve bundles with no associated mast cells in HD case. (Tb, x200)**

of histochemical and immunohistochemical stains have been proposed to assist in the identification of ganglion cells, or to delineate the nature of the nerve fibers in suction rectal biopsies²⁵.

Cresyl violet or Toluidine blue with or without H&E were used in many studies as the main stains to highlight the ganglion cells in suspected Hirschsprung's disease by the principle of metachromasia²⁵⁻²⁸.

This study was performed to find a stain superior to H&E for identification of GC spatially when immunohistochemistry is not available. It showed that both Toluidine blue and Cresyl violet stains are superior to H&E for identification of ganglion cells in rectal specimens with no statistically significant difference between the two stains. This is partially in agreement with HM. Canil et al who used Tb stain and found that Toluidine blue method is a reproducible and reliable way of demonstrating ganglion cells in frozen rectal biopsies. This method provides faster and easier identification of ganglion cells than with H&E staining²³. Both Tb and Cresyl violet stains are fast, easy, cheap, do not need a counter stain and can be performed

in any lab. as a routine stain instead of H&E in suspected HD cases. Grading of specimens according to the two stains was somewhat identical; the minor differences seen were related to the scantiness of remaining material for processing for Cresyl violet and obviously encountered in punch biopsy only.

This study was also designed to find adjuvant histological criteria that assist in the diagnosis of HD cases. Mast cells secrete a great variety of biological active substances, MC synthesize, store and release nerve growth factor (NGF) responsible for the growth and repair of nerve fibers^{22,29-32}. MC are observed in great amount in digestive tract and may exert an important effect on differentiation and regeneration of intestinal nervous system.³³⁻³⁵. The exact role of MC in HD is still not known^{22,24,33,34}. Toluidine blue stain serves to highlight mast cells. Recently there has been a lot of interest in the role of mast cells in HD. Kobayashi et al described an increased number of mast cells in the aganglionic segment of the colon in patients with HD²². Similar findings were reported by Demirbilek et al²⁴ and Amit et al²¹. They mentioned a transmural distribution of these cells in HD cases and notably around nerve fibers and perivascularly. This is highly in disagreement with this study which shows statistically no significant difference in mast cell number in the submucosa between HD and non-HD groups. In this study mast cells were characteristically distributed around the nerves and blood vessels in addition to being randomly scattered HD group. However, in one study done by Hermanowicz et al³³, it was mentioned that MCs were increased in the mucosa and lamina propria of HD but the increase in mast cells in the submucosa, muscularis propria and serosa were not statistically significantly changed, which is in agreement to our finding.

In this study mast cells were also seen in the ganglionic segment. A similar finding was mentioned by Amit et al²¹. This may be due to the response of the mast cells to infectious agents or allergens rather than their association with aganglionosis. An increased number of mast cells is also reported in various other gastrointestinal disorders such as acute appendicitis, ulcerative colitis, celiac disease and gluten enteropathy^{36,37}. This creates a dilemma in the interpretation of a rectal biopsy in suspected HD and may lead to some bios.

Cholinergic nerve hyperplasia is a consistent finding in the aganglionic segment of bowel. whole-mount studies have suggested that the hypertrophic nerve trunks are blind ending, bulbous terminations of extrinsic enteric serosal nerves and not from intrinsic submucosal and muscle nerves³⁸. Clearly NGF and its receptor play a role in this stimulus, possibly from NGF produced by the hypo-

innervated colonic muscle or from the distorted intrinsic nerve plexuses³⁹. In this study Nerve bundle hypertrophy in the submucosa was another histological criterion to be noted in suspected HD cases. M. K. Babu et al²⁶ used H&E- and Tb and AChE-stained sections in their study to measure the thickness of nerve trunks in the submucosa using the Leitz oculometer. Kakita et al in their study used 2 nerve markers-erythrocyte-type glucose transporter (GLUT-1), and nerve growth factor receptor and they conclude that measurements greater than 50 microm in diameter (a figure which may be used as a threshold for hypertrophic nerves), are suggestive of Hirschsprung disease⁴⁰. While Monforte-Muñoz H et al conclude that submucosal nerve trunks that are 40 microm or greater in diameter strongly correlate with abnormal innervation/aganglionosis⁴¹. Since no clear cut line for nerve bundle hypertrophy, in this study simple histological criteria in H&E and Toluidine blue stained sections were used; the hypertrophic nerve bundles, unlike the random arrangement of the round nuclei of Schwann cells in normal neuronal plexuses, had a parallel, longitudinal pattern of elongated Schwann cell nuclei in the nerve trunks. Collagen and a distinct perineurium were clearly seen in the hypertrophic nerve bundles but not in normal plexuses. A significant correlation between the presence of submucosal nerve bundle hypertrophy and HD cases is found in this study and confirmed by Toluidine blue and Cresyl violet. One case in which hypertrophied nerve bundles were seen in non-HD case could be a case of intestinal neuronal dysplasia which is out of the scope this study.

Conclusion

Toluidine blue and/or Cresyl violet stains should to be used as the routine stain to highlight the ganglion cells in suspected Hirschsprung's disease cases. Submucosal nerve bundle hypertrophy has to be assessed as an adjuvant histological criterion. While mast cell count in the submucosa is a non significant criterion in HD.

Acknowledgements

I would like to thank Dr. Nabil Salmo the head of the department for his technical advises also special thanks to Dr. Ali Ibrahim in helping to fix the article statistics.

Funding

None

Competing Interests

None declared

Reference

1. Worman S, Ganiats TG. Hirschsprung's disease: a cause of chronic constipation in children. *Am Fam Physician*.1995; 51: 487-94.
2. Hirschsprung's Disease and Allied Disorders. Berlin: Springer. 2007. ISBN 3-540-33934-5.
3. Hirschsprung, H. "Stuhlträchtigkeit Neugeborener in Folge von Dilatation und Hypertrophie des Colons". *Jahrbuch für Kinderheilkunde und physische Erziehung*(Berlin).1888; 27: 1-7.
4. Kays DW. Surgical conditions of the neonatal intestinal tract. *Clinics in Perinatology*. 1996; 23 (2): 353-75.
5. Suita S, Taguchi T, Ieiri S, Nakatsuji T. Hirschsprung's disease in Japan: analysis of 3852 patients based on a nationwide survey in 30 years. *Journal of Pediatric Surgery*. 2005; 40 (1): 197-201; discussion 201-2.
6. Goldman, Lee. *Goldman's Cecil Medicine* (24th ed.). Philadelphia: Elsevier Saunders. pp. 867. ISBN 1437727883.
7. Lukman O. Abdur-Rahman, Brian H. Cameron. Hirschsprung's Disease in Africa in the 21st Century. *Surgery in Africa Monthly Review*. Hamilton, Canada. January. 2011 <http://ptolemy.library.utoronto.ca/sites/default/files/reviews/2011/January%20-%20Hirschsprung's%20Disease.pdf>
8. Kiernan JA. Nissl: the man, the stain and the substance. *Microscopy Today*. 2005;13(6):50.
9. Bear MF, Connors BW, Paradiso MA. *Neuroscience: Exploring the Brain*, 3rd ed. Philadelphia: Lippincott, Williams & Wilkins, 2007, pp.31-34;94-98.
10. Rosai J. Large Bowel Disease. In: *Ackerman's Surgical Pathology*. Philadelphia: Mosby; 2004. p. 777-9.
11. Martucciello G. Hirschsprung's disease, one of the most difficult diagnoses in pediatric surgery: a review of the problems from clinical practice to the bench. *Eur J Pediatr Surg*. 2008;18:140-9.
12. Chen F, Winston JH 3rd, Jain SK, Frankel WL. Hirschsprung's Disease in a young adult: report of a case and review of the literature. *Ann Diagn Pathol*. 2006;10:347-51.
13. Petras R. Hirschsprung's disease. In: *Sternberg, SS. Diagnostic surgical pathology*, Williams and Wilkins; Philadelphia: 2004. p. 1390-1.
14. STAINING MANUAL - NERVE TISSUE <http://library.med.utah.edu/WebPath/HISTHTML/MANUALS/NISSL.PDF>
15. Tanisha N. Neely, Diana Harrington. MediaLab course, Histology Special Stains: Nervous Tissue. http://www.medialabinc.net/spg538507/luxol_fast_blue_lfb
16. Arline d. Deitch, Montrose J Moses. The Nissl substance of living and fixed spinal ganglion cells. *J. Biophysic. and Biochem. Cytol.*, 1957;3: 449-455
17. Gokul Sridharan, Akhil A Shankar. Toluidine blue: A review of its chemistry and clinical utility. *J Oral Maxillofac Pathol*. 2012;16: 251-255.

18. John A. Kiernan. *Staining Sections of the Central Nervous System*. Canada. 2012: 62
19. Toluidin blue stain for histology. http://reactiveshops.com/index.php?route=product/product&product_id=97
20. Kumar GL, Kiernan JA. 2nd ed. Dako North America, California: 2010. Education guide: Special stains and H & E.
21. Yadav AK, Mishra K, Mohta A, Agarwal S. Hirschsprung's disease: Is there a relationship between mast cells and nerve fibers? *World J Gastroenterol*. 2009 March 28; 15(12): 1493–8.
22. Kobayashi H, Yamataka A, Fujimoto T, Lane GJ, Miyano T. Mast cells and gut nerve development: implications for Hirschsprung's disease and intestinal neuronal dysplasia. *J Pediatr Surg*. 1999;34(4):543-8.
23. M. Canil, K. Meir, G. Jevon, T. Sturby, S. Moerike, A. Gomez. Toluidine Blue Staining Is Superior to H&E Staining for the Identification of Ganglion Cells in Frozen Rectal Biopsies. Technical bulletin for histotechnology. 2007; Vol. XL, No. 1:1-3
24. Demirbilek S, Ozardali HI, Aydm G. Mast-cells distribution and colonic mucin composition in Hirschsprung's disease and intestinal neuronal dysplasia. *Pediatr Surg Int*. 2001;17:136–139.
25. Johan Widenfalk, Christopher Nosrat, Andreas Tomac, Heiner Westphal, Barry Hoffer, Lars Olson. Neurturin and Glial Cell Line-Derived Neurotrophic Factor Receptor- β (GDNFR- β), Novel Proteins Related to GDNF and GDNFR- α with Specific Cellular Patterns of Expression Suggesting Roles in the Developing and Adult Nervous System and in Peripheral Organs *The Journal of Neuroscience*, 1997, 17(21): 8506-19
26. M. K. Babu, Usha Kini, Kanishka Das, Anand Alladi, Ashley j. D'cruz. A modified technique for the diagnosis of Hirschsprung disease from rectal biopsies. *The National Medical Journal of India* 2003;16:245-248.
27. Robertas Bagdzevičius, Sigita Gelman, Ligita Gukauskienė, Vytautas Vaičekauskas. Application of Acetylcholinesterase Histochemistry for the Diagnosis of Hirschsprung's Disease in Neonates and Infants: a Twenty-year Experience. *Medicina (Kaunas)* 2011;47(7):374-9
28. Singh S, Shariff et al. Prenatal development of the myenteric plexus in human sigmoid colon. *J. Morphol. Sci.*, 2013;30:156-166
29. Barczyk M, Debek W, Chyczewski L. Mast cells in the gastrointestinal tract. *Rocz Akad Med Białymst* 1995;40: 36-57.
30. Bonini S, Rasi G, Bracci-Laudiero ML, Procoli A, Aloe L. Nerve growth factor: neurotrophin or cytokine? *Int Arch Allergy Immunol* 2003;131: 80-84.
31. Dvorak AM. New aspects of mast cell biology. *Int Arch Allergy Immunol* 1997;114: 1-9.
32. Leon A, Buriani A, Dal Toso R, Fabris M, Romanello S, et al. Mast cells synthesize, store and release nerve growth factor. *Proc Natl Acad Sci USA* 1994;91: 3739-3743.
33. Hermanowicz A, Debek W, Dzienis-Koronkiewicz E, Chyczewski L. Topography and morphometry of intestinal mast cells in children with Hirschsprung's disease. *Folia Histochem Cytobiol* 2008;46:65–68.
34. Dines KC, Powell HC. Mast cell interactions with the nervous system: relationship to mechanisms of disease. *J Neuropathol Exp Neurol* 1997; 56: 627-640
35. Yadav AK, Mishra K, Mohta A, Agarwal S. Hirschsprung's disease: is there a relationship between mast cells and nerve fibers? *World J Gastroenterol*. 2009;15: 1493-8.
36. Stead RH, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J. Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. *Gastroenterology*. 1989;97:575–585.
37. Krishnaswamy G, Kelley J, Johnson D, Youngberg G, Stone W, Huang SK et al. The human mast cell: functions in physiology and disease. *Front Biosci*. 2001;6:D1109–D1127.
38. Tam PKH, Boyd GP. Origin, course and endings of abnormal enteric nerve fibers in Hirschsprung's disease defined by whole mount immunohistochemistry. *J Pediatr Surg* 1990; 25: 457-61.
40. H Kobayashi, D S O'Briain, P Puri. Nerve growth factor receptor immunostaining suggests an extrinsic origin for hypertrophic nerves in Hirschsprung's disease. *Gut* 1994; 35: 1605-1607
41. Kakita Y, Oshiro K, O'Briain DS, Puri P. Selective demonstration of mural nerves in ganglionic and aganglionic colon by immunohistochemistry for glucose transporter-1: prominent extrinsic nerve pattern staining in Hirschsprung disease. *Arch Pathol Lab Med*. 2000;124:1314-9.
42. Monforte-Munoz H, Gonzalez-Gomez I, Rowland JM, Landing BH. Increased submucosal nerve trunk caliber in aganglionosis: a "positive" and objective finding in suction biopsies and segmental resections in Hirschsprung's disease. *Arch Pathol Lab Med*. 1998;122:721–725.