ORIGINAL RESEARCH PAPER
Volume - 12 | Issue - 02 | February - 2023 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

IMMUNOHISTOCHEMICAL EVALUATION OF NEUROMUSCULAR ARCHITECTURE IN ANORECTAL MALFORMATIONS IN CHILDREN

Histopathology

Dr. Naina* Consultant Pathologist *Corresponding Author
Dr. N. Neelaveni Professor, Dept. of Pathology, Osmania Medical College, Hyderabad
Dr. Ramya Sabbineni Post Graduate trainee, Dept. of Pathology, Osmania Medical College, Hyderabad.

ABSTRACT

Objective: The present study was conducted to evaluate neuromuscular architecture in distal rectal specimens of children with anorectal malformations(ARM) on the basis of histopathology and immunohistochemical markers. (Calretinin, S-100 and CD117) and to know whether the distal rectal pouch histology in ARMs is abnormal enough to explain the potential mechanism of the poor anorectal defecation function after anorectoplasty. **Materials and methods:** A total of 40 cases of anorectal malformations in children were included in the study. Routine processing for H&E was done, followed by immunohistochemistry using CD117, S-100 and calretinin immunostains. Masson's trichome special stain was also done. The statistical analysis for categorical variables was done using Pearson's Chi square test. The results were considered statistically significant if the p value is <0.05. **Results:** Most common histopathological finding was inflammation (95%) in mucosa, congestion (97.5%) and inflammation (92.5%) in submucosa, hypertrophy (60%), disorganized fibers in muscularis propria (50%) on Haematoxylin and eosin (H&E) sections and fibrosis on Masson trichrome stain (50%) in muscularis propria. CD117 expression was reduced in 89.5% cases implying a reduction in Interstitial cells of Cajal with a highly significant correlation (p- value < 0.001). S100 immunostaining showed presence of hypertrophic nerve bundles in 30% cases (p-value= 0.04). Calretinin expression was absent or reduced in 60% cases which shows decrease in number of ganglia. **Conclusion:** A number of specific and non-specific histopathological changes were observed in the malformed intestinal segments. Decreased density and distribution of Interstitial cells of Cajal (ICC) interpreted by decreased expression of CD 117 on immunohistochemistry may lead to failure of transmission of peristaltic waves across the distal rectal pouch. Presence of hypoganglionosis seen with decreased expression calretinin on IHC along with hypertrophic nerve bundles as interpreted by S100 immunostaining were indicative of a probable HD like neuronal dysfunction in these patients.

KEYWORDS

Anorectal malformations, CD117, ICC, Calretinin

INTRODUCTION

Anorectal malformations (ARM) are among the more frequent congenital anomalies encountered in pediatric surgery, with an estimated incidence ranging between 1 in 2000 and 1 in 5000 live births.[1] There is a wide spectrum of presentation ranging from low anomalies with perineal fistula having simple management to high anomalies with complex management. Although surgical techniques have improved significantly in the last decades, complete anatomic and functional restoration of congenital anorectal malformations (ARMs) cannot be achieved. Patients with ARMs often present with varying severity of defecation dysfunction (constipation, incontinence, and fecal soiling) following corrective operations. Constipation is one of the most frequent complications after correction of ARMs, occurring in 30% to 60% of patients.[2,3] However, the detailed mechanism remains unclear. Enteric nervous system (ENS), smooth muscle layer and Interstitial cells of Cajal are important for the normal functioning of GIT.[4] There are reports emphasizing that histology of the blind pouch has an important role in the post-operative bowel function apart from other factors like type of malformation and neuromuscular functioning of pelvic floor. There is a paucity of studies discussing the histopathological abnormalities of the distal rectum or pouch [5],[6]. Hence, we studied histomorphological changes and various immunohistochemical (IHC) markers (Calretinin, S-100, CD117) in intestinal wall specimens to assess neuromuscular dysfunction in a small cohort of children with ARM.

AIMS AND OBJECTIVES

- To evaluate neuromuscular architecture in distal rectal specimens of children with ARMs on the basis of histopathology and immunohistochemical markers. (Calretinin, S-100 and CD117).
- To know whether the distal rectal pouch histology in ARMs is abnormal enough to explain the potential mechanism of the poor anorectal defecation function after anorectoplasty.
- To assess whether the distal rectal pouch should be used for anorectoplasty with the treatment of ARMs or resected for better functional outcomes during radical outcomes during radical treatment.

MATERIALS AND METHODS

The present study was carried out in the Upgraded department of Pathology, Niloufer hospital for women and children from the year 2019 to 2021. Ethical clearance was granted by the institution. The study group consisted of 40 patients with clinical diagnosis of ARM who underwent surgery. In cases diagnosed as intermediate or high ARM, colostomy was done at birth and 6 months later a definitive procedure (PSARP/ASARP/AP pull through) along with colostomy closure was done. The colostomy specimen was sent at this stage. For low ARMs anoplasty or Primary ASARP was done and a biopsy from the rectum was sent for histopathology.

In cases of Congenital Pouch Colon, types III – V, the pouch is excised and end colostomy is done. For these cases, specimens for histopathological examination were sent at birth. For types I – II of CPC, a primary surgery of pouch colostomy is done followed by a definitive procedure,6 months later, of excision of the redundant pouch and tubularisation of the residual one. The type I and II CPC specimens were sent at this stage of surgery for histopathological evaluation.

The specimens were received in 10% formalin. After gross examination tissue bits from the distal rectal pouch and the fistula region were processed for one day and later embedded in paraffin which was cut at 5-micron thickness. Sections were stained with Hematoxylin and Eosin (H & E) as well as Masson Trichrome stains and thereafter reviewed by two experienced pathologists and diagnosis was made. All these 50 cases were subjected to immunohistochemical staining with markers CD117, S100 and Calretinin.

The control group consisted of 10 patients who presented to the hospital from 2019 to 2021 but did not show any clinical or radiological signs and symptoms of ARM. 7 of these cases underwent resection due to necrotizing enterocolitis and 3 others due to Idiopathic neonatal colonic perforation.

Following this application of IHC markers CD117, S-100 and calretinin was done by taking 3-micron thick sections on 3-Amino propyl tri-ethoxy silane [APES] coated slides followed by deparaffinization by xylene and hydration in descending grades of isopropyl alcohol. The slides were kept in jar containing antigen retrieval solution for 3 cycle (1st – 3 min, 2nd – 8 min, 3rd – 10 min) in microwave after setting the temperature at 95 degrees Celsius. After getting cooled for 30 minutes and wash by distilled water and Tris buffer ,the Endogenous peroxidase was blocked by application of 3%

Hydrogen peroxide solution for 15 minutes. Sections were then incubated with the primary monoclonal (Anti human mouse monoclonal antibodies, Biogenex) for 60 minutes. Super enhancer (Biogenex company) applied and incubated for 20 minutes followed by incubation with secondary antibody for 30 minutes. Substrate-chromogen solution (3,3'Diaminobenzidene) was applied for visualizing the reaction and then counterstaining with haematoxylin to visualize nuclei was done.

Positive and negative control were run with each batch of IHC stain. Positive controls for Calretinin and S100 were taken from normal colonic tissue and for CD117 sections from gastrointestinal stromal tissues were taken. Substitution of primary antibody by non-specific secondary antibody was done for achieving a negative control.

Scoring And Evaluation

The density and distribution pattern of ICC was determined by counting the cells in the muscular plane and the myenteric plexus. Strong and diffuse cytoplasmic staining with stronger accentuation along the cell membrane was considered positive.[7,8]

The semiquantitative estimation of CD117 was done and compared to the control group as shown in table [1].[9] Relatively reduced number of positive cells or total loss of CD117 staining was considered abnormal. S 100 cytoplasmic positivity in Schwann cells was used to assess presence or absence of nerve bundle hypertrophy. Calretinin shows strong nuclear and cytoplasmic positivity in ganglion cells which was used to check presence or absence of ganglion cells in the submucosal nerve plexus and myenteric plexus.

A descriptive study was done and the findings were statistically analyzed using Pearson's Chi square test for categorical variables. The results were considered statistically significant if the p value is <0.05.

OBSERVATIONS AND RESULTS

The age of cases ranged from 1 to 6 years. Mean age of cases of ARM was 1.12 years and that of controls was 1.9 years. Male to female sex ratio was 3:2. Most common presenting complaint was failure to pass meconium at birth (32%) followed by constipation (23%) and abdominal distension (20%). Majority of the cases were of congenital pouch colon (38%) followed by rectovesical fistula (33%).

Most common histopathological finding was inflammation (95%) in mucosa, congestion (97.5%) and inflammation (92.5%) in submucosa, hypertrophy (60%), disorganized fibers (50%) on H&E sections and fibrosis on MTS (50%) in muscularis propria.

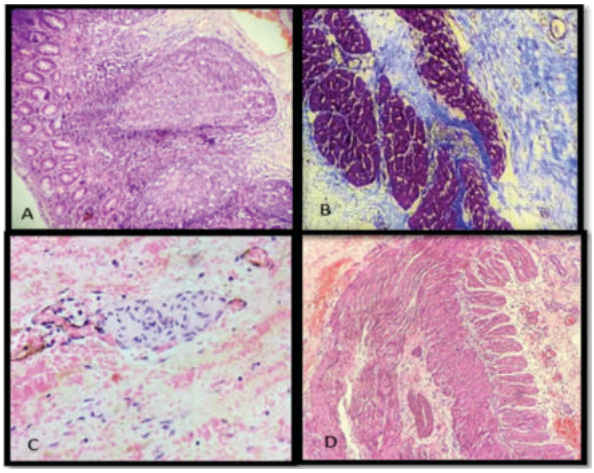

Fig 1 : A] Submucosal lymphoid follicles and inflammation (H&E,100X), B] Muscle fibrosis (M.T,400X),[C] Submucosal hypertrophic nerve bundles (H&E,400X), [D]Disorganised muscle fibres(H&E,100X)

Immunohistochemical examination showed that CD117 expression was reduced in 89.5% cases implying a reduction in Interstitial cells of Cajal (pacemaker cells of the gut) with a highly significant correlation. (p- value < 0.001). S100 immunostaining showed presence of hypertrophic nerve bundles in 30% with a significant correlation (p-value = 0.04). Calretinin expression was absent or reduced in 60% cases which shows decrease in number of ganglia (p value = 0.0006).

Table [1]: Grading of CD117 immunostaining among cases and controls for semiquantitative estimation of Interstitial cells of Cajal(ICC)

GRADE	MAGNIFICATION	OBSERVATION	CASES	CONTROLS
0	400X	No positive cells	2 (5%)	0
1+	400X	Occasional weakly positive cells	17 (42.5%)	0
2+	100X	Easily distinguishable	18 (45%)	0
3+	100X	Intense positive cells	3 (7.5%)	10

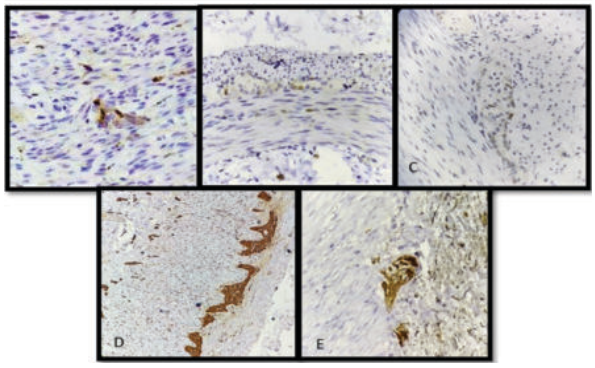

Fig [2] - Figure showing: [A,B,C]Grades of CD117 immunostaining patterns - A)GRADE -3 (CD117,100X), B)GRADE - 2(CD117,100X),C)GRADE – 1(CD117,400X), [D] Hypertrophic nerve bundles (S100,100X), [E] Ganglion cells in submucosa and myentric plexus (Calretinin,400X)

DISCUSSION

Anorectal malformations are one of the most commonly observed congenital anomalies of gastrointestinal system. They are a complex group of congenital anomalies which involve the distal anus and rectum, as well as the urinary and genital tracts in many cases. The prevalence is approximately one per 5000 live births, with a slight male preponderance.[10],[11]

Despite the various advancements in diagnosis and management of ARMs surgical procedures available for ARMs, patients suffer from variable degrees of post operative bowel incontinence. It has been observed that most cases of low ARM have good bowel function and better quality of life. A third of patients with high or intermediate type of ARM suffer from fecal soiling. An impaired urinary and sexual function has also been noted in some of these patients.[12] Most of these post operative complications have been attributed to the dysfunctional neuronal function and histopathological alterations in the malformed segments in ARMs.

In the present study, histopathological findings were seen in the mucosa and submucosa similar to other studies. Aganglionosis was observed in 60% of cases with ARM with the presence of giant ganglion cells in 4 (10%) cases. However, presence of ganglion cells was seen in all cases in the study by Bhatia Y. et al (2017) [9]. Constriction bands and additional muscular coat which observed in studies by Tiwari A. et al [13] and Udawat H. et al [14] was not noticed in present study. The various histopathology findings in various studies have been compared with the present one in table 2.

Table [2]: Comparison of histopathological features in ARM with various studies

FINDINGS IN MUCOSA	PRESENT	Bhatia Y. et al	Udawat H. et al	Tiwari et al
Mucosal erosion	42.50%	57%	6.20%	
Inflammation	95%	87%	40%	60%
Haemorrhage	37.50%	40%	20.40%	40%
MM disruption	40%	60%	7.50%	53.30%
FINDINGS IN SUBMUCOSA				
Widening	42.5%	47%	77.50%	66.60%
Congestion	97.5%	80%	96%	
Haemorrhage	77.5%	35%	76%	60%
Inflammation	92.5%	28.50%	35%	80%
Lymphoid follicles	30%		28.50%	

Fibrosis	65%	67%	27%	
Absent ganglion cells	60%	0%	63.20%	
Edema	25%		27%	
FINDINGS IN MUSCULAR LAYER				
Hypertrophy	60%	50%	37%	86.60%
Thinning	25%			
Disorganised	50%		96%	20%
Fibrosis	50%		49%	

Complete absence of ICC was seen in 5% cases and mild or markedly reduced expression in 87.5% cases with a p-value of 0.0000001 in this study. Hypertrophic nerve bundles present in 30% of cases (p-value =0.04) and absent ganglion cells in 60% of cases (p value =0.00068) showing a Hirschsprung like picture. Most of the findings in present study are consistent with that of the previous studies done by Bhatia et al (2017) [9], Udawat et al (2017) [14], Xiao et al (2018) [15], Lombardi L. et al (2013) [16] and Agarwal et al [17]. Contrary to the present study, Anatol et al [18] found no abnormality in ICC. Absence of ganglion cells was not seen in any case by Bhatia et al whereas it was seen in 60% cases in our study with giant ganglion cells seen in 4 cases (10%).

The present study corroborates the histomorphological changes like focal erosion, denudation, haemorrhage, congestion and lymphoid follicle in mucosa and submucosa along with transmural inflammation, thickening and disorganised arrangement of fibres in the muscularis propria. In addition, the intrinsic abnormalities of myenteric plexus and marked decrease in density and distribution of ICC was substantiated. The study suggests that that the severe anomalies like fibrosis of the muscle layer which is a sign of irreversible damage, abnormalities in myenteric plexus and reduction in the number of ICCs and ganglion cells may be responsible for the post operative soiling, constipation and incontinence in ARMs.

REFERENCES

1. Levitt MA, Pena A. Imperforate anus and cloacal malformations. In: Holcomb GW III,Murphy JP, editors. *Ashcraft's Pediatric Surgery.* 5th ed. Philadelphia, PA: Saunders Elsevier; 2010. pp. 468–90
2. Rintala RJ. Fecal incontinence in anorectal malformations, neuropathy, and miscellaneous conditions. Seminar Pediatr Surg 2002;11:75–82.
3. DeVries PA, Peña A. Posterior sagittal anorectoplasty. J Pediatr Surg 1982;17:638–43. PMID: 7175658.
4. Young B, O'Dowd G, Woodford P. Gastrointestinal Tract. In: Young B, O'Dowd G, Woodford P, editors. Wheater's Functional Histology: A Text and Colour Atlas. 6th ed. Philadelphia: Churchill Livingstone; 2104.p.251-75.
5. Holschneider AM, Pfrommer W, Gerresheim B. Results in the treatment of anorectal malformation with special regard to the histology of the rectal pouch. Eur J Pediatr Surg 1994;4:303–9.
6. Holschneider AM, Ure BM, Pfrommer W, et al. Innervation patterns of the rectal pouch and fistula in anorectal malformations: a preliminary report. J Pediatr Surg 1996;31:357–62.
7. Blair PJ, Rhee PL, Sanders KM, Ward SM. The significance of interstitial cells in neurogastroenterology. J Neurogastroenterol Motil. 2014;20:294-317.
8. Horisawa M, Watanabe Y, Torihashi S. Distribution of c-kit immunopositive cells in normal human colon and in Hirschspring's disease. J Pediatr Surg. 1998;33:1209-14.
9. Bhatia Y, Singh S, Rattan KN, Parmar P, Sahni D, Sen R. Anorectal malformations: histomorphological and Immunohistochemical evaluation of neuronal dysfunction. Journal of neonatal surgery. 2017 Apr;6(2).
10. Levitt MA, Peña A. Outcomes from the correction of anorectal malformations. Current opinion in pediatrics. 2005 Jun 1;17(3):394-401.
11. Levitt MA, Peña A. Anorectal malformations. Fundamentals of pediatric surgery. 2011:499-512.
12. Gangopadhyay AN, Pandey V. Anorectal malformations. Journal of Indian Association Pediatric Surgeons. 2015 Jan;20(1):10.
13. Tiwari A, Naik DC, Khanwalkar PG, Sutrakar SK. Histological Study of Neonatal Bowel In Anorectal Malformations.Int J Anat.2014, 2(2):318-24.
14. Udawat H, Nunia V, Mathur P, Udawat HP, Gaur KL, Saxena AK, Mohan MK. Histopathological and Immunohistochemical Findings in Congenital Pouch Colon: A Prospective Study. Pathobiology. 2017;84(4):202-209.
15. Xiao H, Huang R, Cui DX, Xiao P, Diao M, Li L. Histopathologic and immunohistochemical findings in congenital anorectal malformations. Medicine (Baltimore). 2018 Aug;97(31)
16. Lombardi L, Bruder E, Caravaggi F, Del Rossi C, Martucciello G. Abnormalities in "low" anorectal malformations (ARMs) and functional results resecting the distal 3 cm. J Pediatr Surg. 2013 Jun 1;48(6):1294-300.
17. Agarwal K, Chadha R, Ahluwalia C, Debnath PR, Sharma A, Choudhury SR. The histopathology of congenital pouch colon associated with anorectal agenesis. European journal of pediatric surgery. 2005 Apr;15(02):102-6.
18. Anatol T, Mohammed W, Rao C. Interstitial cells of Cajal and intestinal function in Trinidadian children. West Indian Medical Journal. 2008 Sep 1;57(4).