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RESEARCH ARTICLE

A comparative study of *in vitro* contractility between gut tissues of Hirschsprung's disease and other gut malformations

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ABSTRACT

Background: Hirschsprung's disease and other gut malformations commonly present with obstructive features of gut in pediatric age group. Problem of obstruction persists even after resection and anastomosis operation. **Aims and Objectives:** This *in vitro* comparative study was performed to assess the gut contractility to chemical mediators such as Acetylcholine and Histamine between Hirschsprung's disease and other gut malformations. **Materials and Methods:** The longitudinal muscle strips of Hirschsprung's cases and other gut malformations (non-Hirschsprung's cases) were placed in Dales organ bath containing Krebs-Ringer solution, continuously bobbled with 100% O2 at 28°C. Gut contractions were recorded using Power Lab 4/ST system and was analyzed using software CHART-5 for windows. Control contractions were recorded against initial tension of 0.5 g. Subsequently, agonist (acetylcholine, histamine)-induced contractions were recorded before and after appropriate antagonists (atropine, pheniramine). Before values of agonist-induced contractions of Hirschsprung and non-Hirschsprung's cases were compared. Values of agonist-induced contractions obtained after pretreatment with antagonists were also compared between Hirschsprung and non-Hirschsprung's cases. **Results:** Acetylcholine enhanced contractions in non-Hirschsprung's cases and it caused small increase in amplitude of contractions in Hirschsprung's cases. Atropine pretreatment blocked acetylcholine-induced contractions significantly in non-Hirschsprung's cases, whereas it failed to block in the Hirschsprung's cases. Histamine augmented contractions in both Hirschsprung and non-Hirschsprung's cases. H1 antagonist, pheniramine failed to block the contractility in both the cases. **Conclusion:** Findings of this study suggested that acetylcholine increased gut contractility significantly in non-Hirschsprung's cases involving muscarinic-cholinergic pathways, whereas histamine increased gut contractility in both Hirschsprung's disease and non-Hirschsprung's cases and it is not mediated by H1 receptors.

KEY WORDS: Gut Contractility; Hirschsprung's Disease; Gut Malformations; Acetylcholine, Histamine

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INTRODUCTION

Congenital malformations of gut commonly present with features of partial/complete obstruction are commonly encountered by the pediatric surgeons. In Hirschsprung's disease, aganglionic bowel has been suggested as the cause of obstruction.[1] Intussusception is a gut disease

characterized by invagination of a portion of intestine into itself leading to intestinal obstruction.[1] Intestinal atresia is a common congenital gut disease[2] detected by prenatal ultrasonography in second to third trimester and confirmed at birth by intestinal obstruction.[3] Anorectal malformation is another congenital problem in neonates which may present with or without pouch. When it presents with pouch, the condition is commonly known as congenital pouch colon in which whole or a part of colon is replaced by pouch like dilation. The cases of pouch colon are mostly found in Asian countries especially in India with maximum number of cases reported from Northern India.[4]

However, the outcome of the surgery of these conditions often remains unsatisfactory.[5,6] This may partly be due to nonavailability of functional studies in terms of contractility of intestinal smooth muscles because most of the studies so far conducted on these disorders were focused on histology and immunohistochemistry.[7,8] Available histopathological studies[9] demonstrated abnormalities in colonic muscle and neuronal components in congenital pouch colon while another group of workers reported no structural deficiency. These histopathological studies are also inconclusive and contradictory in regard to the structural deficiencies in enteric nervous system and intestinal smooth muscles.[9-13] Thus, one of the important reasons for not achieving the desirable result in post-operative period may be very limited understanding of the functional derangement and mechanisms underlying the neonatal colonic motility in above-diseased conditions.

It is well known that the regulation of contractile mechanisms of intestinal smooth muscle involves neural control mechanisms through extrinsic (sympathetic and parasympathetic) and intrinsic (myenteric and submucosal plexus) pathways in addition to myogenic contractions. The major regulatory mechanisms of intestinal smooth muscle contractility involve cholinergic, adrenergic, and nonadrenergic-noncholinergic systems. The basic tools that may help in evaluating the functional status of smooth muscle in gastrointestinal tract is recording of spontaneous and chemically evoked contractions in *in vitro* preparations.[14,15] The spontaneous contractions involve pacemaker activity and the complex neuromuscular coordination in the intestinal tissues. Furthermore, the contraction evoked by cholinomimetic agents may help in assessment of the status of cholinergic contractile mechanisms which is known as major and important regulating mechanisms. Histamine-induced *in vitro* contractions have been used earlier in human gallbladder contractions.[14]

Till date, there are only a few studies that demonstrate the contractile mechanisms of human gut musculature of pediatric age group. Earlier observations reported that the contractile responses in anorectal malformations with pouch colon which failed to demonstrate spontaneous contractions[16] whereas another observation demonstrated spontaneous contractions in 66% of Hirschsprung's disease patients.[17] However, these studies failed to draw a comparative conclusion and relations between Hirschsprung's disease and anorectal malformations. Further, it is also not clear yet, how various neurohumoral agents regulating the contractions are ineffective in regulating the motility in various gut malformations. Thus, our question was whether such contractility regulatory mechanisms demonstrate same or different grade of activity in various obstructive gut malformations. Further, there are no comparative studies available that can differentiate in activity of various chemical agents between Hirschsprung's disease and other gut malformations.

In light of above observations, this study was conducted to compare the *in vitro* contractility in the gut tissues of Hirschsprung's disease and the other congenital gut malformations using acetylcholine and histamine as chemical mediators.

MATERIALS AND METHODS

This study was conducted after getting approval from Ethical Committee (Ref No. Dean/2008-09/452, dated 13/04/2009), Institute of Medical Sciences, Banaras Hindu University, Varanasi to understand the contractile differences between the longitudinal gut muscle strips of Hirschsprung's disease and non-Hirschsprung's cases (gut atresia, intussusception and anorectal malformations). This experimental *in vitro* study was performed from May 2009 to June 2010. Informed consent was taken in a bilingual (Hindi/English) form from the concerned patient party in all the cases.

Selection Criteria for Gut Samples from Patients

Human intestinal tissues (small/larges gut) obtained from the pediatric patients of age from neonate to 12 years, presenting with different types of intestinal malformations were the subject of the present investigation. The samples were divided into two broad groups. In one group, gut samples of Hirschsprung's disease patients were included while in other group, gut samples of other gut malformations excluding Hirschsprung's disease was included and it was represented as non-Hirschsprung's cases.

In the Hirschsprung's group, all histopatologically diagnosed cases of Hirschsprung's disease of less than the age of 12 years were included in the study. Normal specimens, gangrenous, tumorous and perforated gut samples of more than 12 years of age were excluded from the group. In the non-Hirschsprung's group, all other clinically diagnosed cases of gut malformations other than Hirschsprung's disease from neonate to 12 years of age were included in the study. Histopathologically normal samples were also excluded from non-Hirschsprung's group.

Collection of Samples and Sample Size

Surgically excised gut tissue samples were collected from Pediatric Surgery operation theater of Sir Sunder Lal Hospital, Banaras Hindu University, Varanasi, India. The samples were transferred to a bottle containing freshly prepared preoxygenated (100%) Krebs Ringer solution and bottle was kept in a container containing ice packs for transportation from the Department of Pediatric Surgery to Department of Physiology. A total of 12 samples were used out of which six samples were of Hirschsprung's disease and six were of non-Hirschsprung's group. On an average, two muscle strips were prepared from each sample. One was used for one type of agonist and another was used for another type of agonist.

Dissection and Preparation of Gut Muscle Strips

The sample was transferred to a Petri dish containing ice cold, freshly prepared Krebs Ringer solution with continuous 100% O_2 bubbling. The sample was thoroughly cleaned to remove the fecal matter adhered to the tissue. The serosal and mucosal layers were removed by gentle dissection and 2-3 mm wide and 15-20 mm long longitudinal muscle strips were prepared.

Recording of Contractile Responses

The recording of muscle contractions from gut strips was performed as described elsewhere.[16,17] In brief, the muscle strip was placed in Dale's organ bath (10 ml) containing Krebs Ringer at 28°C and continuously bubbled with 100% O_2. The strip was placed under an initial tension of 0.5 g and then allowed to equilibrate for 30 min. Isometric muscle contractions thus developed were amplified by Bridge amplifier (ML 110, AD Instrument, Australia), digitized via an analog/digital interface (Power Lab 4/ST system, AD Instrument, Australia, a computerized chart recorder) and were displayed on a personal computer. The contraction recordings were analyzed using software CHART-5 for Windows (AD Instruments, Australia). Before recording the contractile responses from each strip of the muscle, calibration for the tension was done with weights.

The initial recording was made after period of 30 min against tension of 0.5 g and amplitude of contraction was evaluated. Thereafter, chemicals were administered in the organ bath, and subsequently, contractions were recorded. Agonists (acetylcholine, histamine) and their appropriate antagonists (atropine, H1 blocker-pheniramine maleate) were used to evaluate the cholinergic and histaminergic mechanisms. Different chemicals were used in different gut strips. The strip was removed from the force transducer and glass tube after the completion of recording. The strip was soaked on bloating paper for 5 s. The tissue was then weighed in the balance to express the contractile response per unit weight of tissue for the purpose of normalization.

Drugs and Solutions

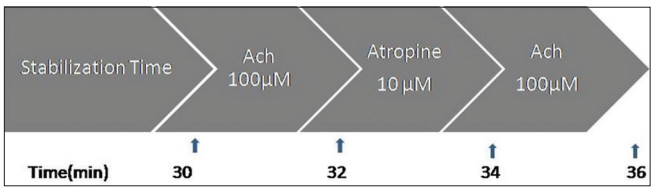
Acetylcholine, cholinergic antagonist-atropine, histamine, and H1 antagonist-pheniramine maleate were procured from SD-Fine Chemicals Pvt. Ltd., Mumbai, India. Stock solutions were prepared with 10 mM concentration in distilled water and refrigerated. Subsequent dilutions were made with Krebs Ringer solution at the time of experimentations. The composition of Krebs Ringer solution in mM was – NaCl - 119, KCl - 4.7, $CaCl_2.2H_2O$ - 2.5, KH_2PO_4 - 1.2, $MgSO_4.7H_2O$ - 1.2, $NaHCO_3$ - 5 and glucose - 11.

Experimental Protocol

Longitudinal gut muscle strips from the patients of Hirschsprung (n = 6) and non-Hirschsprung's cases (n = 6) were used in this study. The tissue was allowed to stabilize against initial tension of 0.5 g for 30 min at 28°C. Then, spontaneous contractions were recorded for 2 min, calculated and expressed in g/g of tissue and was considered as initial response. Subsequently, the tissue was exposed to acetylcholine (100 µM) and recordings were obtained at 0.5, 1.0, 1.5 and 2.0 min. These recordings were calculated in g/g of tissue and were expressed as % of initial response.

Then, tissue was exposed to atropine (10 µM) for 2 min, and subsequently, recordings were obtained after exposure to acetylcholine (100 µM) again at 0.5, 1.0, 1.5 and 2.0 min. Contractions thus obtained were calculated in g/g and were expressed as % of maximum responses obtained before pretreatment with atropine. Above mentioned experimental protocol was also followed for longitudinal muscle strips obtained from non-Hirschsprung's cases (n = 6).

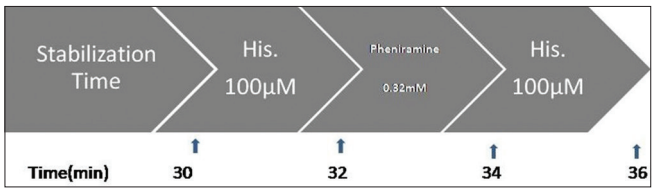
Finally, the time matched acetylcholine (100 µM)-induced amplitude of contractions before and after exposure to atropine (10 µM) of Hirschsprung's disease (H) were statistically compared with their corresponding values of non-Hirschsprung's cases (before values of H were compared with before values of NH and after values of H were compared with after values of NH).



Similar experimental protocol was also followed with histamine (100 µM) in Hirschsprung's cases (n = 6) and non-Hirschsprung's cases (n = 6) before and after pheniramine (320 µM). The contractions between Hirschsprung and non-Hirschsprung's cases were statistically compared as mentioned above.

Data were expressed as mean ± standard error of mean values. Statistical comparisons were made between Hirschsprung

and non-Hirschsprung's cases using Mann–Whitney U-test. A $P < 0.05$ was considered as significant.



RESULTS

Comparison of Acetylcholine-induced Gut Contractility between Hirschsprung's and non-Hirschsprung's Cases

In Hirschsprung's disease after administration of 100 µM of acetylcholine, contractility at 0.5, 1.0, 1.5 and 2.0 min was 108.34 ± 2.36, 111.80 ± 2.18, 111.24 ± 2.66 and 107.97 ± 3.46% of initial, respectively (Figure 1). In non-Hirschsprung's disease after administration of 100 µM of acetylcholine, contractility at 0.5, 1.0, 1.5 and 2.0 min was 155.80 ± 20.16, 187.42 ± 34.70, 162.22 ± 14.43 and 161.56 ± 9.57% of initial, respectively (Figure 1). In Hirschsprung's cases, effect of acetylcholine was almost negligible but in non-Hirschsprung's cases, contractility was found to be increased by 1.5-1.8 times of initial value ($P < 0.05$, Mann–Whitney U-test).

Comparison of Acetylcholine-induced Contractility after Atropine Pretreatment between Hirschsprung's and non-Hirschsprung's Cases

In Hirschsprung's disease, acetylcholine (in atropine pretreated group)-induced contractility at 0.5, 1.0, 1.5, and 2.0 min was 92.56 ± 3.85, 93.59 ± 1.60, 92.78 ± 1.90, and 91.00 ± 1.67% of maximum, respectively (Figure 2). In non-Hirschsprung's cases, acetylcholine (in atropine pretreated group)-induced contractility at 0.5, 1.0, 1.5, and 2.0 min was 69.20 ± 5.38, 54.66 ± 5.98, 52.64 ± 6.35, and 50.85 ± 7.15% of maximum, respectively (Figure 2). Result shows that acetylcholine-induced contractility was blocked in non-Hirschsprung's cases than in Hirschsprung's cases in atropine pretreated group ($P < 0.05$, Mann–Whitney U-test).

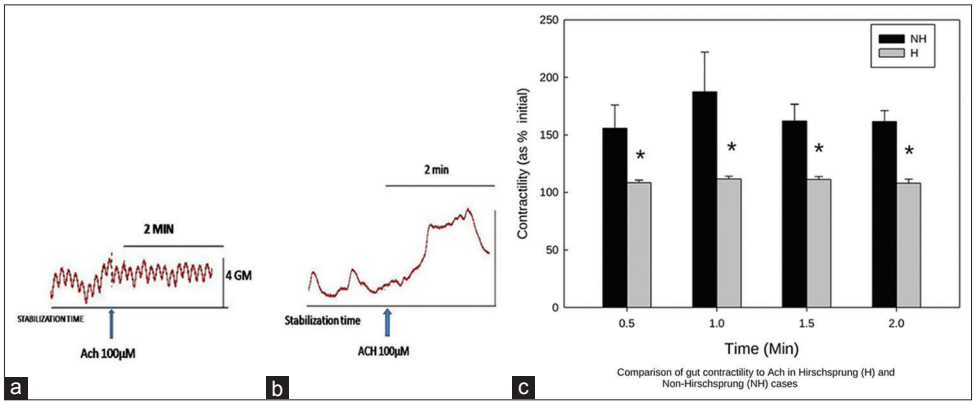


Figure 1: Original recordings from individual experiments showing effect of acetylcholine on longitudinal muscle strips obtained from Hirschsprung's cases (a) and non-Hirschsprung's cases (b). Arrow indicates point of administration of drug. Scale for measuring amplitude of contractions and time is given at the right upper corner of the both tracings. (c) The comparison of contractility effect of acetylcholine between Hirschsprung's cases (n = 6) and non-Hirschsprung's cases (n = 6). An asterisk indicates a significant difference between Hirschsprung and non-Hirschsprung's cases ($P < 0.05$ for Mann–Whitney U-test)

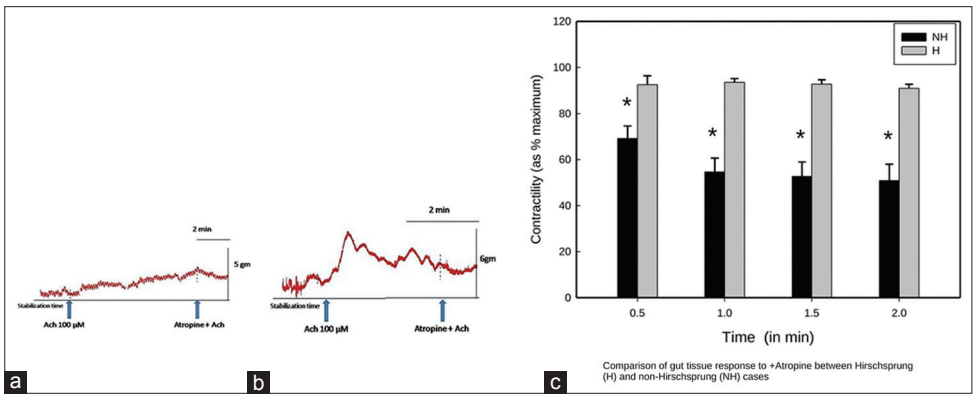


Figure 2: Original recordings from individual experiments showing effect of atropine pretreatment on acetylcholine-induced contractility on longitudinal muscle strips obtained from Hirschsprung's cases (a) and non-Hirschsprung's cases (b). Arrow indicates point of administration of drug. Scale for measuring amplitude of contractions and time are given at the right upper corner of the both tracings. (c) The comparison of contractility effect of acetylcholine between Hirschsprung's cases (n = 6) and non-Hirschsprung's cases (n = 6) after atropine pretreatment. An asterisk indicates significant difference between Hirschsprung and non-Hirschsprung's cases ($P < 0.05$ for Mann–Whitney U-test)

Comparison of Histamine-induced Gut Contractility between Hirschsprung and non-Hirschsprung's Cases

In Hirschsprung's disease, histamine (100 μM)-induced contractility at 0.5, 1.0, 1.5 and 2.0 min was 134.85 ± 11.78, 144.47 ± 7.45, 151.23 ± 7.26, and 141.76 ± 9.33% of initial, respectively (Figure 3). In non-Hirschsprung's disease, histamine (100 μM)-induced contractility at 0.5, 1.0, 1.5, and 2.0 min was 133.69 ± 6.22, 147.41 ± 7.63, 151.88 ± 10.07, and 139.94 ± 8.80 % of initial, respectively (Figure 3). In Hirschsprung and non-Hirschsprung's cases, effect of histamine is almost similar and contractility was found to be increased by 50% of the initial ($P > 0.05$, Mann–Whitney U-test).

Comparison of Histamine-induced Contractility after Pheniramine Pretreatment between Hirschsprung's and non-Hirschsprung's Cases

In Hirschsprung's disease, histamine (in pheniramine pretreated group)-induced contractility at 0.5, 1.0, 1.5 and 2.0 min was 92.79 ± 1.71, 84.20 ± 3.62, 81.71 ± 4.00 and 79.76 ± 4.15% of maximum, respectively (Figure 4). In non-Hirschsprung's disease, histamine (in pheniramine pretreated group)-induced contractility at 0.5, 1.0, 1.5 and 2.0 min was 93.02 ± 2.04, 86.59 ± 2.57, 84.68 ± 3.40, and 82.59 ± 2.97% of maximum, respectively (Figure 4). Result shows that histamine-induced contractility was not blocked in both non-Hirschsprung and Hirschsprung's cases by pheniramine pretreatment ($P > 0.05$, Mann–Whitney U-test).

DISCUSSIONS

In this *in vitro* study, acetylcholine enhanced amplitude of contractions in longitudinal muscle strips of non-Hirschsprung's cases, whereas enhancement in amplitude of contractions was small in Hirschsprung's cases. On comparison between non-Hirschsprung and Hirschsprung's cases, acetylcholine-induced contractility was significantly augmented in non-Hirschsprung's cases. While comparing the effect of cholinergic blocker, atropine (a muscarinic blocker) on acetylcholine-induced contractility between non-Hirschsprung and Hirschsprung's cases, it was observed that gut contractility induced by acetylcholine was blocked significantly in non-Hirschsprung's cases whereas in Hirschsprung's cases atropine failed to block the responses. Histamine increased amplitude

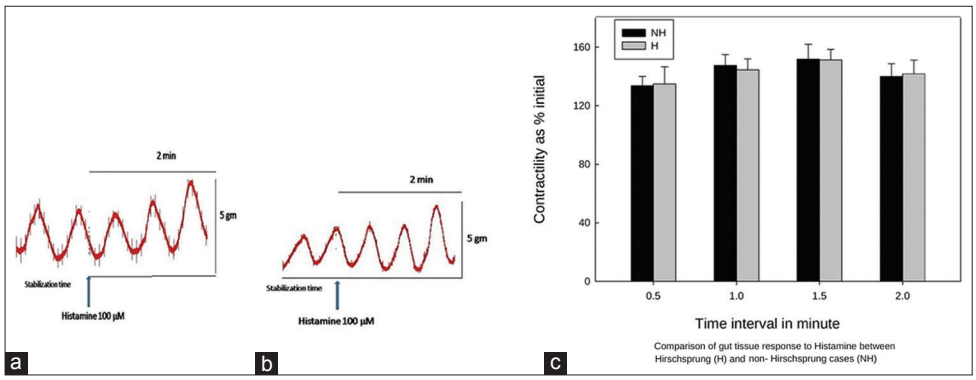


Figure 3: Original recordings from individual experiments showing effect of Histamine on longitudinal muscle strips obtained from Hirschsprung's cases (a) and non-Hirschsprung's cases (b). Arrow indicates point of administration of drug. Scale for measuring amplitude of contractions and time is given at the right upper corner of the both tracings. (c) The comparison of contractility effect of histamine between Hirschsprung's cases ($n = 6$) and non-Hirschsprung's cases ($n = 6$). There was no significant difference between the two groups ($P > 0.05$ for Mann-Whitney U–test)

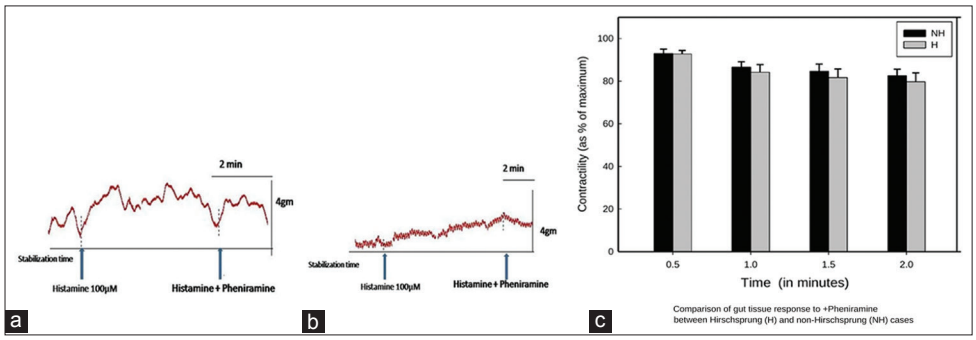


Figure 4: Original recordings from individual experiments showing effect of pheniramine pretreatment on histamine-induced contractility on longitudinal muscle strips obtained from Hirschsprung's cases (a) and non-Hirschsprung's cases (b). Arrow indicates point of administration of drug. Scale for measuring amplitude of contractions and time is given at the right upper corner of the both tracings. (c) The comparison of contractility effect of histamine between Hirschsprung's cases ($n = 6$) and non-Hirschsprung's cases ($n = 6$) after pheniramine pretreatment. There was no significant difference between the two groups ($P > 0.05$ for Mann–Whitney U-test)

of gut contractility in longitudinal muscle strips of both non-Hirschsprung as well as Hirschsprung's cases and contractility responses were almost equal and similar in magnitude. There was no significant difference between non-Hirschsprung as well as Hirschsprung's cases. Pheniramine (H1 blocker) failed to block the histamine-induced gut muscle contractility in both non-Hirschsprung and Hirschsprung's cases.

In this study, acetylcholine (100 μM) increased the gut contractility significantly in non-Hirschsprung's cases in comparison to Hirschsprung's cases (Figure 1). Acetylcholine has been reported to mediate its actions via muscarinic receptors (M1, M2, M3, M4, and M5). M1 receptors also play a role in the coordination of intestinal muscle contraction and relaxation.[18] Studies in the guinea pig ileum demonstrated that M1 receptors modulate acetylcholine release from neurons.[19-21] M2 receptors are found in the heart. M3 and M4 receptors are reported to be genetically expressed in the smooth muscle, thus facilitating contraction of smooth muscle.[22,23] Thus, M1, M3, and M4 receptors may mediate the gut contractility actions of acetylcholine. Acetylcholine is also known to shift the unstable membrane potential of smooth muscle to spike potential; thus, it has been suggested that it activates smooth muscle contraction by depolarization of muscle cell membrane.[22] Further, in this study atropine, a nonspecific muscarinic receptor blocker, significantly blocked the acetylcholine-induced contractility in non-Hirschsprung's cases (Figure 2). It confirms the role of muscarinic receptors in mediating the gut contractility as well as adequacy of muscarinic receptors in myenteric and Meissner's plexus of enteric nervous system in non-Hirschsprung's cases. In Hirschsprung's cases, reports are available that it was caused by failure of development/migration of ganglion cells in both myenteric and meissner's plexus of enteric nervous system.[24,25] Further, muscarinic receptors are suggested to be present on myenteric and meissner's plexus of the enteric nervous system.[22] The meager response observed with acetylcholine in Hirschsprung's cases may be attributed to either lack of receptors or absence of neurons containing muscarinic receptors. Further, atropine failed to block acetylcholine-induced gut contractility in Hirschsprung's cases (Figure 2), also confirms non-availability or absence of neurons or receptors in intrinsic nervous system of gut. However, feeble contractile responses seen in Hirschsprung's cases may be due to the involvement of such a receptor which is partially activated by acetylcholine but poorly blocked by atropine. Therefore, evidence suggests existence and involvement of another type of receptor (possibly cholinergic-dependent - nonmuscarinic) causing feeble contractions in Hirschsprung's cases.

In this study, contractility responses of gut muscle tissue to histamine were also assessed. Histamine increased gut contractility in both non-Hirschsprung as well as Hirschsprung's cases. Responses were equal in amplitude and magnitude of contractions and were not significantly different between non-Hirschsprung and Hirschsprung's cases (Figure 3). Four types of histamine receptor (H1 to H4) are reported. H1 receptor activates phospholipase C and causes smooth muscle contraction especially in intestine. H2 receptor increases cyclic adenosine monophosphate concentration in smooth muscle and causes their relaxation. H3 receptors are presynaptic and mediate the inhibition of release of histamine involving G-protein.[15,22,26] In our observation, pheniramine (H1 blocker) failed to block histamine-induced gut muscle contraction *in vitro* in both the groups (Figure 4). Therefore, our observations do not support the involvement of H1 receptor in mediating the gut smooth muscle contraction. On the contrary, our observations contradict reports of other workers.[15,26] It is reported elsewhere[15] on the rabbit colon that histamine caused increased tone in muscularis mucosa involving H1 receptor. Further, it was also reported that neural effect of histamine was mediated by H1 receptors.[27] It was demonstrated in the pig proximal colon that H2 blocker famotidine antagonized histamine-induced short circuit current.[28] In an observation, it was concluded that H1, H2, H4 receptors are expressed in human gastrointestinal tract while H3 receptors were absent.[29] Thus, above evidence and findings of the present observation suggest the operation of a non-H1, non-H2 and non-H3 histaminergic population of receptors responsible for producing gut contractility in this study. Therefore, involvement of H4 receptor or any other receptor may be speculated to be present in human gastrointestinal tract causing smooth muscle contraction. Further, it is also confirmed that gut muscles are functionally active in both the cases.

This *in vitro* study fails to identify the cholinergic-dependent non-muscarinic pathways in Hirschsprung's and non-Hirschsprung's cases. In this study, we have not used frequency of contraction as a parameter; hence, its use might have provided additional information. However, positive and strong information added to the pool of knowledge by this study was that non-Hirschsprung's cases of pediatric age group may be benefitted by the use of cholinomimetic and histaminergic drugs, especially after resection and anastomosis operation.

CONCLUSION

Findings of this study suggested that acetylcholine increased gut contractility significantly in non-Hirschsprung cases (gut atresia, intussusception anorectal malformations) involving muscarinic-cholinergic pathways, whereas this muscarinic-cholinergic pathway was less developed or absent in Hirschsprung's disease. Histamine increased gut contractility in both Hirschsprung and non-Hirschsprung's cases. Histamine receptors mediating histamine-induced gut contractility are well expressed in both non-Hirschsprung and Hirschsprung's cases, but these are not mediated by H1 receptors. Further, gut contractility increased by histamine also points that gut muscle is functional in both cases. However, further investigations are required to identify other histaminergic receptors mediating the contractility in gut.

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