In Vitro Evaluation of Carbachol and Endothelin on Contractility of Colonic Smooth Muscle in Hirschsprung’s Disease

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Abstract

Background: The hypomotility of colon observed in Hirschsprung’s disease (HD) has been attributed to congenital aganglionosis only. So far, it is not clear whether the contractility of colonic smooth muscle in this condition is altered or not. Therefore, the present study attempted to understand the contractile status of colonic segments of HD patients by examining carbachol and endothelin (ET-1) evoked colonic smooth muscle contractions in vitro.

Methods: Contractile responses were recorded from strips of colonic segments obtained from HD patients, using organ bath preparations. Cholinergic agonist carbachol and ET-1 along with their antagonists were used to evoke contractile responses. Thereafter, the samples were histopathologically confirmed for HD.

Results: Colonic strips of HD did not show any spontaneous contractions but responded to carbachol and ET-1 to a lesser extent. In HD, response of carbachol was blocked by atropine and hexamethonium by nearly 73% and 50% respectively. ET-1 induced contractile responses were blocked by ET-A and ET-B antagonist up to 40%, signifying the possible role of ET-A and ET-B receptors in HD colon contractility.

Conclusion: As evidenced by lack of spontaneous contractions and impaired carbachol and ET-1-induced contractile responses, it is concluded that, in addition to aganglionosis, decreased contractility of colonic smooth muscle may contribute to hypomotility observed in patients with HD.

Introduction

Hirschsprung’s disease is characterised by intestinal obstruction due to hypomotility of colon and congenital absence of ganglion cells in the enteric nervous system. The aganglionosis in HD is caused by the failure of the migration of neural crest cells during foetal development (1). Further, the pathogenesis of aganglionosis has been attributed to the genetically lack of the endothelin receptors (ET-A and ET-B) in some cases (2-4). So far, the colonic hypomotility observed in HD has been assigned to only aganglionosis or hypoganglionosis. However, even after complete excision of aganglionic...
segment of bowel, continued dysmotility of remaining bowel have been reported and patients present as post-operative recurrent enterocolitis and persistent constipation (5). On the other hand, there is lack of studies on the contractile status of colonic smooth muscles in HD patients. Therefore, the possibility of impaired contraction of colonic smooth muscle per se cannot be ruled out. Further, in studies with animal (rat) model of HD, it was shown that application of cholinergic agonist, carbachol caused enhanced contractile response in HD as compared to control (6). These experiments failed to provide concluding evidence about the contractile status of colonic smooth muscle in HD for two reasons. Firstly, the experiments were carried out in rat model and secondly, the HD model was produced by genetically knocking out endothelin ET-B receptors. Thus, the animal model seemed to be inadequate to represent the human HD, where there may not be total absence of ET-B receptors in various tissues. Consequently, it was felt that direct examination of the colonic tissue from HD patients is required to resolve the issue.

Therefore, the present study aimed for in vitro assessment of the contractile status of colonic smooth muscle in HD patients, by recording the contractions induced by cholinergic agonist carbachol and ET-1, with the help of organ bath preparations.

Methods

Specimens

The present study was carried out on the excised specimens (total 30 cases) of colon obtained from 19 patients of HD, 6 cases of ano-rectal malformation (ARM) and 5 cases of colonic atresia. These specimens were collected from the operation theatre in the Department of Paediatric Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. In absence of availability of normal colonic tissue, ARM and colonic atresia (i.e. 11 non-HD cases) were considered as working control to HD. In both of these cases, colonic strips were prepared from relatively healthy looking areas towards the end of dissected specimens. Grossly abnormal areas (e.g. atretic part of colonic atresia) were not considered for contractility studies. In case of HD, central areas were used for the study. Immediately after excision in the operation theatre, the specimens were collected in a wide mouth bottle containing ice-cold Krebs-Ringer solution bubbled with 100% oxygen. They were quickly transferred to the laboratory in the Department of Physiology for contractile studies. All the experiments were conducted as per the guidelines laid down by the ethical committee of the institute for handling human tissues.

Preparation of muscle strips

The excised specimens of HD and non-HD were transferred to a petri dish containing oxygenated ice-cold (4°C to 6°C) Krebs Ringer solution having the composition (in mM): NaCl, 119; KCl, 4.7; CaCl₂,2H₂O, 2.5; KH₂PO₄, 1.2; MgSO₄.7H₂O, 1.2; NaHCO₃, 5; and glucose, 11. Each specimen was cleaned with freshly prepared cold Krebs-Ringer solution to remove the faecal matter adhered to the tissue. Thereafter, the adventitious layer was removed and 2 to 3 mm wide and 15 to 20 mm long, rectangular colonic strips (1-2 strips) oriented along the longitudinal layer of smooth muscle were prepared from the HD and non-HD cases.

Groups, treatment and recording of contractile responses

Specimens were divided into two groups, HD and non-HD groups. HD group was further subjected to treatment with a) Carbachol, in presence or absence of atropine and hexamethonium and b) ET-1, with or without ET-A / ET-B antagonist. The non-HD group had same treatment excepting the use of ET-A / ET-B antagonists.

The procedure for recording of contractile response has been described earlier (7). Briefly, the prepared strips were mounted in Krebs-Ringer filled organ bath (12 mL) maintained at 37°C±1°C and continuously bubbled with 100% oxygen. One end of the muscle strip was fastened to a glass tube support, and the other end was fixed to an isometric force transducer (MLT 0210). The strip was placed under an initial
tension of 0.5 g and then left to equilibrate for 30 minutes, with replacement of Krebs-Ringer solution every 15 minutes. The output signals from the transducer were amplified by bridge amplifier and digitized by A/D converter (Power Lab 4/ST system) and the recording of isometric contractions was stored in a personal computer. The recording was displayed and analysed with the help of software Chart-5 for windows. The transducer, amplifier, digitizer system, and software were procured from AD Instruments, Sydney, Australia. Before, as well as after recording the contractile responses, calibration for the tension (0-10 g) was performed. After stabilization, the initial recording was made for 30 minutes without any external chemical interventions, so as to assess the presence of any spontaneous contraction. Subsequently, the tissue was exposed to different concentrations of carbachol (0.1, 1, 10, and 100 µM) and endothelin-1 (1, 10 and 100 nM). The contractions were recorded for a minimum period of 15 minutes for each concentration. In one set of experiments, carbachol (100 µM)-induced contractions were recorded in presence of atropine (100 µM), and in another set same was recorded after pre-treatment with hexamethonium (100 µM). After the recording of contractions, the strips were removed from the bath and placed on blotting paper for lightly soaking the extra water from the tissue. Two ends of the strip beyond their attachments were cut and discarded because these parts of tissue did not participate in the recorded contractions. The wet tissue was then weighed in a fine balance to express the contractile response per unit weight of tissue (g/g wet tissue).

Drugs and solutions

Aqueous solutions of carbachol, ET-1, hexamethonium, (Sigma Aldrich, New Delhi, India) and atropine sulphate (Sd-Fine Chemicals, Mumbai, India), were used in this study. The stock solutions of these chemicals were prepared with distilled water and refrigerated. Required dilutions were made in Krebs-Ringer solution just before the experimentation.

Histopathological examination

Fresh tissue from spastic site of all clinically suspected cases of HD were processed for preparing frozen section and subsequently stained with haematoxylin and eosin (H&E) and acetylcholinesterase (AChE) stains for confirmation of HD.

Statistical analysis

The amplitude of contractions was noted as tension (g weight) after the calibration procedure. The tensions were then expressed per unit mass of colonic tissue (g/g of wet tissue). The initial tension was normalised as 100% and the change in tension following chemical intervention was expressed as % of initial. The values were pooled to calculate the mean±SEM. The statistical significance of differences in mean values was examined with the help of Student’s t-test and 2-way analysis of variance (ANOVA) as and when applicable. P value of less than 0.05 was considered as significant. The software ‘GraphPad Prism’, version 5.0 was used for statistical analysis.

Results

Contractile study was carried out with a total of 29 colonic strips obtained from 19 cases of HD and 18 strips obtained from 11 cases of non-HD to evaluate spontaneous as well as chemically (ET-1 and carbachol) evoked contractions. Most of the strips responded well to carbachol and ET-1.

Absence of spontaneous contractions in Hirschsprung’s disease

Only two (6.9%) of 29 HD strips demonstrated spontaneous contractions and another two strips from two cases of HD did not respond to any concentration of carbachol and ET-1. In contrast, most of the strips from the non-HD cases (78%) i.e. 14 out of 18 showed spontaneous contractions. The spontaneous contractions observed in non-HD specimens were characterized with tonic contractions and
contractions as compared to non-HD samples. ET-1 produced quicker response with lower duration of contraction in HD as compared to non-HD. The data for carbachol and ET-1 responses in HD and non-HD samples are presented in the Table I.

### Dose-responses to carbachol

A concentration dependent increase in the amplitude of contractions was observed with 4 different concentrations (0.1-100 µM) of carbachol in both HD and the non-HD specimens. There was a significant (P<0.05, 2-way ANOVA) lower response evoked by carbachol on HD as compared to non-HD specimens (Figure 2 upper right panel). At 100 µM bath concentration of carbachol, the response in HD was increased by 5.5 times (554.52±104.58% of initial; n=5) as compared to nearly 8 (795.63±291.71% of initial; n=5) times in non-HD samples. EC-50 of carbachol for non-HD was approximately 2 µM and that in HD was 7 µM.

Carbachol-induced contractions were blocked by atropine

In HD, after treatment with atropine (100 µM), carbachol (100 µM) produced approximately 27% (n=7) of its initial contraction. Thus, there was nearly 73% blockade of carbachol-induced response after atropine pre-treatment. Whereas, in non-HD samples the blockade was 53%, i.e. after atropinisation carbachol produced 47% (n=6) of initial response (Table II).

Carbachol-induced contractions were blocked by hexamethonium

In HD as well as in non-HD cases, after pre-

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**TABLE I :** Showing mean±SEM values of latent period, contraction period/duration in minutes from various experimental groups after carbachol and ET-1 treatments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Experimental group</th>
<th>Latent period (minutes)</th>
<th>Contraction durations (minutes)</th>
<th>Time to reach peak (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol (100 µM)</td>
<td>HD</td>
<td>0.39±0.01*</td>
<td>7.89±0.07*</td>
<td>1.84±0.03*</td>
</tr>
<tr>
<td></td>
<td>Non-HD</td>
<td>0.29±0.01*</td>
<td>5.84±0.13</td>
<td>1.12±0.03</td>
</tr>
<tr>
<td>ET-1 (100 nM)</td>
<td>HD</td>
<td>0.18±0.003*</td>
<td>2.54±0.231*</td>
<td>0.99±0.1*</td>
</tr>
<tr>
<td></td>
<td>Non-HD</td>
<td>0.39±0.03</td>
<td>8.12±0.29</td>
<td>4.72±0.29</td>
</tr>
</tbody>
</table>

*p<0.05 (Student’s t-test, paired) as compared to Non-HD group. µM=Micromole, nM=Nano-mole, HD=Hirschsprung’s disease, Non-HD=Non-Hirschsprung’s disease.
administration of hexamethonium (100 µM), carbachol (100 µM) produced nearly 50% (HD, n=17; Non-HD, n=7) of its initial contraction, indicating 50% blockade in both HD and non-HD cases (Table II).

Dose response to ET-1

A concentration dependent increase in the amplitude of contractions was observed with 3 different concentrations of ET-1 in both HD and the non-HD specimens. There was significantly reduced response in HD as compared to non-HD group. At 100 nM bath concentration of ET-1, the response of HD

<table>
<thead>
<tr>
<th>Drugs used in pre-treatment</th>
<th>% of initial response in HD samples</th>
<th>% of initial response in Non-HD samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine (100 µM)</td>
<td>26.59±9.93*</td>
<td>46.74±9.51*</td>
</tr>
<tr>
<td>Hexamethonium (100 µM)</td>
<td>50.63±6.57*</td>
<td>50.74±5.93*</td>
</tr>
<tr>
<td>ET-A antagonists (100 nM)</td>
<td>61.72±1.59</td>
<td>—</td>
</tr>
<tr>
<td>ET-B antagonists (100 nM)</td>
<td>60.34±13.53</td>
<td>—</td>
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</tbody>
</table>

*p<0.05 (Student’s t- test, paired) as compared to the response without antagonist. Data for ET-A/ET-B antagonists on Non-HD group was not available.

Fig. 2: Left panel: Showing actual recordings of contractions induced by carbachol in non-HD (A) and HD (B) and ET-1 in HD (C) non-HD (D) samples. Vertical and horizontal bars represent contractile tension (g) and time (min) respectively. Arrows indicate point of application of drugs. Right panel: The upper and lower right panel shows dose-response curve for carbachol and ET-1 respectively. Data points indicate mean±SEM. An asterisk indicates P<0.05 (Two-way ANOVA).
(352.76±99.72 % of initial tension, n=5) was nearly 3 times lesser than that of non-HD (866.78±318.30% of initial tension, n=4) (Figure 2, lower right panel).

**ET-1-induced contractions were blocked by ET-A and ET-B antagonists**

On pre-treatment with either ET-A or ET-B antagonists (100 nM each), ET-1 produced nearly 60% of initial contractile tension in both cases. In other words, these antagonists caused around 40% blockade of ET-1 induced response (Table II).

**Confirmation of HD by histopathological examination**

All the sections from the spastic segment of clinically suspected cases of HD showed absence of ganglion cells. In addition, some of the cases also showed presence of hypertrophied nerve bundle. These cases also demonstrated positive staining for AChE, and therefore confirming the diagnosis of HD. Section from the Non-HD cases showed presence of ganglion cells.

![Fig. 3: A. HD samples showing positive AChE stained thin nerve fibres (arrow) in between the crypts in lamina propria and muscularis mucosa in a case of HD, (100X). B. Negative stain with AChE in the lamina propria, in a case of non-HD (100X). C. Non-HD sample stained with H&E stain, showing several neural units (arrow) and presence of ganglion cells (200X). D. No ganglion cell was seen with H&E stain. Arrow shows hypertrophied nerve bundles in myenteric plexus in a case of HD (100X).](image-url)
Discussion

The present study was carried out to understand the functional status of colonic smooth muscles in HD, by studying in vitro contractility of colonic strips. In this study, except two strips, none of the HD specimens showed spontaneous contractions. In contrast, in earlier in vitro studies with normal colon, spontaneous contractions from almost all of the colonic strips were observed (8-9). Similar observations in non-HD colon were also made in our study. The origin of spontaneous contraction is related to the activity of interstitial cells of Cajal (10). These contractions have been found reduced in other pathologic conditions of colon including ulcerative colitis (9, 11). The nonappearance of spontaneous contractions in HD samples could be correlated to absence or very sparse ganglion cells as observed in histopathological examinations. However, the present functional study demonstrated that the colonic strips from HD responded to both ET-1 and carbachol in vitro.

Carbachol dose-response curve indicated that the colonic smooth muscle responded feebly in HD as compared to non-HD cases (EC-50 for HD was around 7 µM, against 2 µM in non-HD samples). Thus it may be presumed that the cholinergic contractions are preserved in HD, although to a lesser extent. Further, it was seen that pre-administration of atropine could abolish 73% of carbachol response in HD. This observation indicated that the carbachol-induced response was largely mediated through muscarinic receptors. Interestingly, carbachol-induced response was also reduced by 50% after pre-treatment with ganglion blocker hexamethonium. At present, it is difficult to explain, how hexamethonium could interfere with muscarinic action of carbachol in colonic muscle. However, there is evidence that hexamethonium can antagonise the carbachol induced contractions in canine vascular smooth muscle (12). The same may be true here also, since hexamethonium produced 50% blockade of carbachol-induced contractile responses in both HD and non-HD cases.

The HD specimens also responded to application of ET-1. However, the contractile response to ET-1 was lesser as compared to non-HD samples, as evidenced by EC-50, which was 5 times more in HD (EC-50, HD=10 nM; non-HD=2 nM). In mouse colon, it was shown that ET-1-induced contractions are mediated via ET-A and ET-B receptors (13). On the other hand, in rat model of HD (produced by knocking out ET-B receptors), demonstrated that the ET-1-induced contractions were not mediated through ET-B receptors (14). In our observations with human HD, it was revealed that, at least 40% of ET-1-induced contraction was mediated through ET-B receptors. This difference in responses for ET-B receptors may be due to variations in species. Therefore, it is suggested that the colonic contractions in human HD samples were partially mediated via ET-A and ET-B receptors.

The ET-B receptors are implicated in the pathogenesis of some but not all cases of short-segment HD. The expression of this receptor is required for neural crest cell migration and development of ganglion in enteric nervous system. Failure or absence of these receptors may lead to development of HD (15-17). However, our results on contractions with ET-1 and its blockers indicated the presence of ET-B receptors in colonic smooth muscles of HD.

It may be noted that we, for the first time, used human colonic tissue from HD patients and attempted to resolve the contractility issues with the help of in vitro techniques. In earlier experiments in rat model (6), it was shown that carbachol increased the contractile response in colonic smooth muscle. On the other hand, we did not observe any substantial histological change in muscle layer. Further, the ET-B receptors were present in colonic tissue, as evidenced by ET-1-induced contractile response and its antagonism by ET-B antagonist. Therefore, it is reasonable to say that, the rat model of HD does not represent a perfect model of human HD. Histologically, though there was absence of ganglion cells in myenteric plexus and submucosa and also presence of hypertrophied nerve fibre in the submucosa but no gross abnormality in muscle layer, attributable to the altered contractile responses, was seen.

The major limitation of this study was non-availability
of age matched normal human colonic tissue. Difficulty in obtaining the normal colon was obviously due to ethical reasons. In the present study, the non-HD specimens obtained from the paediatric patients with ARM and colonic atresia were considered as working control. These non-HD specimens were histologically normal but may not be functionally healthy tissue. Although, at this stage of the study it is hard to clearly correlate the histological findings with contractile responses of colonic smooth muscle, nevertheless, this study provides evidence for altered contractile function of the colonic muscle in HD. Thus, the observations of this study may help in the formulation of better clinical management strategies in future.

In conclusion, it may be said that, in addition to aganglionosis, impaired contractility of colonic smooth muscle may be responsible for colonic hypomotility in Hirschsprung’s disease as evidenced by lack of spontaneous contractions and reduced carbachol/ET-1 induced contractions.

References