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# *In- vitro* Contractility of Normal Human Vermiform Appendix involves 5-Hydroxytryptamine (5-$HT_3$) Pathways in addition to Muscarinic Transmission.

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**ABSTRACT**

Appendicitis is a cause of health concern in all age and sex worldwide. Alteration in appendicular peristalsis/contractility has been implicated as a precipitating factor in appendicitis. But, the neurotransmitters involved in the contractility of normal human vermiform appendix are not well understood. The present study was undertaken to investigate the neurotransmitter mediated pathways involved in mediating the contractility of longitudinal muscles of normal human vermiform appendix. Longitudinal muscle strips of human vermiform appendix were mounted in Dale's organ bath. *In vitro* contractility to agonist was recorded using Statham's isometric force displacement transducer. Experiments were performed to determine the dose-response of agonists (acetylcholine/5-HT/ histamine) and the dose that produced maximum contractions. In a separate set of experiments, the effect of antagonists (like atropine, ondansetron and chlorpheniramine maleate) on the agonist-induced contractions was evaluated. Acetylcholine (ACh) and 5-HT produced dose-dependent increase in contractions. ACh produced maximum contractions at 10 µM and it was partially (70%) but significantly blocked ($p < 0.05$) by atropine (100 µM) pretreatment. 5-HT produced maximum contractions at 1 µM and it was blocked ($p < 0.05$) by 5-$HT_3$ antagonist, ondansetron (10µM). The contractions produced by histamine were small, hence effect of histamine antagonist on histamine induced contractions were not analyzed. Thus, the present study suggests the involvement of 5-$HT_3$ pathway in addition to cholinergic-muscarinic transmission in mediating the contractility of normal human appendix. Histamine plays a minor role in overall contractility of normal appendix.

**Keywords**: Appendicitis, Appendicular contractions, Atropine, $H_1$-receptors, Histaminergic, Ondansetron.

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## INTRODUCTION

The appendix (6-9 cm in length) is a hollow outpouching generally located at the posteromedial wall of the caecum approximately 1.7 cm below the ileocecal valve in the abdomen [1]. Inflammation of the vermiform appendix (appendicitis) is a cause of health concern in human beings. Alteration in appendicular motility has been implicated in appendicitis [2, 3, 4]. Variety of key neurotransmitters of myenteric neuronal network have been reported to mediate contractions of gut muscles. They are acetylcholine (ACh), amines, 5-Hydroxytryptamine (5-HT) etc. These neurotransmitters/neuromodulators also affect the motility of the gut [5]. Since, vermiform appendix is developed from the midgut embryologically [6,7]. The neurotransmitters/neuromodulators/chemical mediators that affect the motility of gut, are expected to affect the motility/contractility of the appendix. There are only few reports regarding contractility of human vermiform appendix [8, 9]. Mansuri and Jindal [8] have reported contractions in the human isolated appendix by ACh and histamine. Ekblad *et al.,* [9] also reported that ACh played a role in the electrically evoked contractions, since atropine suppressed these contractions.

Further, 5-HT has been reported to mediate contractions in stomach and colon [10] but its effect on the contractility of appendix has not been reported till date. However, reports elsewhere mentioned that 5-HT was synthesized and stored in enterochromaffin cells and the myenteric plexus of the appendix [11]. Thus, in light of above observations, the role of 5-HT in the contractility of longitudinal muscles of normal human vermiform appendix was examined and compared with ACh and histamine. Further, the receptors involved were also examined.

## MATERIALS AND METHODS

The study was conducted as per the guidelines provided by the Ethical Clearance Committee of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

**Criteria for selection of samples:**

**Inclusion criteria:**

The normal human appendix specimens were collected from the patients of age between 15-50 years of both sexes whose gut were partially resected along with appendix through abdominal laparotomy for non-appendicular pathologies such as intussception, stricture and malrotation of gut, small bowel obstruction or from incidental appendicectomy. Longitudinal muscle strips showing no histopathological abnormalities were only selected for analysis.

**Exclusion criteria:**

Gangrenous, perforated and tumourous appendices as well as appendix resected by laparoscopic surgery were not included in this study. Patients of younger age (<15 years) and older age (>50 years) were excluded. Histopathologically abnormal and inflammed appendices were excluded from the present study.

**Collection and transportation of appendix samples:**

Prior informed consent was taken in all the cases in a bilingual (Hindi / English) consent form. In the present study, 12 normal appendices were selected as per the inclusion criteria. Appendices were collected from General Surgery operation theatre of Sir Sunder Lal hospital, Banaras Hindu University. The appendix samples were cut in two parts, proximal half and distal half immediately after appendicectomy in the operation theater. The proximal half of the appendix was transferred to a wide mouthed bottle containing ice-cold, Krebs-Ringer solution already bubbled with 100% $O_2$. The bottle was then transported to the Department of Physiology, Institute of Medical Sciences, Banaras Hindu University for contractility study, whereas, the distal segment of appendix was sent to Department of Pathology, Institute of Medical Sciences, Banaras Hindu University for histopathological examination.

**Dissection and preparation of the appendix muscle strips:**

The full thickness specimen was placed in a petri dish containing ice-cold Kerbs Ringer solution with continuous 100% $O_2$ bubbling. After cleaning the serosal and adventitious layers from proximal segments, small longitudinal muscle strips (10-15 × 2-3 mm each) were isolated from each appendix. Three longitudinal muscle strips were prepared from proximal half of each normal appendix. Each of the strip in a given appendix was used to test only one agonist.

**Mounting and recording of contractile responses:**

The procedure for recording contractility in smooth muscle strips was followed as described elsewhere [10,12]. Briefly, one end of the muscle strip was fastened to a glass tube via a thread and the other end was also tied to a thread. Then, the tube with the muscle strip was transferred to Dale's organ bath (50 ml) containing Krebs-Ringer solution bubbled continuously with 100% $O_2$ and the temperature of the solution was maintained at 37 ± 0.5°C. Then the piece of thread tied with other end of muscle strip was fastened to a Statham's force displacement transducer. The tissue was given an initial tension of 0.5 g and allowed to stabilize for 30 min.

**Experimental protocol**

The experiments were divided into three groups and each group was divided into two subgroup (subgroup 1 and subgroup 2). In subgroup 1 series of experiments of each group, dose-response of agonist was performed to determine dose that produced maximum contraction. In subgroup 2 series of experiments of each group, the effect of appropriate antagonist on respective agonist (dose that produced maximum response) induced contractions in normal appendix was studied.

In group 1, subgroup 1 series of experiments, after stabilization of the muscle strip for 30 min, ACh was used in different doses in increasing order (0.1, 1, 10, 100 µM) and contractions were recorded for 5 min at each dose (n=6). In between two doses, the muscle strip was washed twice at an interval of 10 min with Krebs-Ringer solution and allowed to stabilize for another 10 min before recording contractile responses with next higher dose of ACh.

In group 1, subgroup 2 series of experiments, after stabilization, muscle contractions were recorded for 5 minute after exposure to ACh (10 µM). Then, after washing the tissue twice at an interval of 10 min with Krebs-Ringer solution and stabilization period of another 10 min, it was exposed to atropine (100 µM) for 5 min. Then, ACh (10 µM) response was obtained again.

In group 2, subgroup 1 series of experiments, after stabilization of the muscle strip for 30 min, 5-HT was used in different doses in increasing order (0.1, 1, 10 µM) and contractions were recorded for 5 min at each dose (n=6). In between two doses, the muscle strip was washed twice at an interval of 10 min with Krebs-Ringer solution and allowed to stabilize for another 10 min before recording contractile responses with next higher dose of 5-HT.

In group 2, subgroup 2 series of experiments, after stabilization, muscle contractions were recorded for 5 minute after exposure to 5-HT (1 µM). Then, after washing the tissue twice at an interval of 10 min with Krebs-Ringer solution and stabilization period of another 10 min, it was exposed to ondansetron (10 µM) for 5 min. Then, 5-HT (1 µM) response was obtained again.

In group 3, subgroup 1 series of experiments, after stabilization of the muscle strip for 30 min, histamine was used in different doses in increasing order (0.01, 0.1, 1, 10 µM) and contractions were recorded for 5 min at each dose (n=6). In between two doses, the muscle strip was washed twice at an interval of 10 min with Krebs-Ringer solution and allowed to stabilize for another 10 min before recording contractile responses with next higher dose of histamine.

In group 3, subgroup 2 series of experiments, after stabilization, muscle contractions were recorded for 5 minute after exposure to histamine (1 µM). Then, after washing the tissue twice at an interval of 10 min

with Krebs-Ringer solution and stabilization period of another 10 min, it was exposed to chlorpheniramine maleate (CPM) 100 µM for 5 min. Then, histamine (1 µM) response was obtained again.

In above groups, at the completion of each experiment, muscle strip was removed from the glass tube and force transducer, blotted for 5-10 seconds and weighed on an electronic weighing balance to quantify the contractile responses per unit mass of muscle tissue (g/g).

**Drugs and solutions:**

Acetylcholine chloride, atropine sulphate, histamine dihydrochloride and serotonin creatinine sulphate monohydrate (5-HT) were obtained from Sigma chemicals Co., St. Louis, MO, U.S.A. Ondansetron hydrochloride was procured from Cipla Limited, Mumbai, India and Chlorpheniramine maleate was procured from Alkem Laboratories Ltd., Mumbai, India. The stock solutions of all agonists and antagonists were prepared in distilled water (10 mM concentration) and refrigerated. Subsequent dilutions were made with Krebs-Ringer solution at the time of experimentation. The composition of Krebs-Ringer solution was (in mM/L): 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; glucose, 11. All the chemicals used in the study were of analytical grade.

**Statistical analysis**

The data were presented as Mean ± SEM. One way analysis of variance (ANOVA) was used to test the significance of the dose-responses obtained at various doses of drugs. Statistical comparisons between two groups were performed using Students t-test for paired observations. A p-value < 0.05 was considered significant.

## RESULTS

**ACh elicited contractions in normal human vermiform appendix.**

ACh (0.1 µM-100 µM) produced muscle contractions in the longitudinal muscle strips of normal human vermiform appendix. ACh increased the magnitude of muscle contractions in a dose-dependent manner up to 10 µM. Maximum amplitude of contraction was obtained at 10 µM of ACh. Further increase in the dose of ACh did not increase the contractions (n=6 at each dose). The amplitude of contractions were significantly different at each dose ($p < 0.05$, One way ANOVA, Fig.-1A).

**Atropine partially blocked the ACh induced contractions**

In these experiments, 10 µM ACh produced contractions similar to those seen in dose-response experiment (Fig.-1A). The magnitude of ACh (10 µM) induced contractions (n=6) were partially and significantly blocked (about 70 % blockage) by pretreatment with 100 µM of atropine ($p < 0.05$, Student t- test for paired observations; Fig-1B and 1C).

**5-HT produced contractions in normal human vermiform appendix.**

5-HT (0.1 µM-10 µM) produced contractions of longitudinal muscle strips of normal appendix in dose-dependent manner. The amplitude of contractions was maximum at 1 µM (n=6; Fig.-2A). Thereafter, the magnitude of contractions was decreased with increased dose of 5-HT to 10 µM. Contractions were significantly different at each dose ($p < 0.05$, One way ANOVA, Fig.-2A).

**5-HT induced contractions were completely blocked by ondansetron**

5-HT (1 µM) induced contractions (n=6) were significantly blocked by prior exposure of the tissue to 10 µM of ondansetron ($p < 0.05$, Student t- test for paired observations; Fig-2B and 2C).

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**Histamine failed to produce significant contractions in normal human vermiform appendix.**

Histamine (0.01 µM-10 µM) produced feeble contractions in longitudinal muscle strips of normal appendix. Amplitude of contractions were similar at each dose (n=6). However, it produced somewhat greater contractions at 1 µM (around 0.3 g/g). Contractions were not significantly different at each dose ($p > 0.05$, One way ANOVA, Fig.-3A). Further, the magnitude of contractions at 1 µM of histamine was nearly 1/10th of ACh (10 µM) or 5-HT (1 µM) induced contractions (Fig. 1, 2, 3).

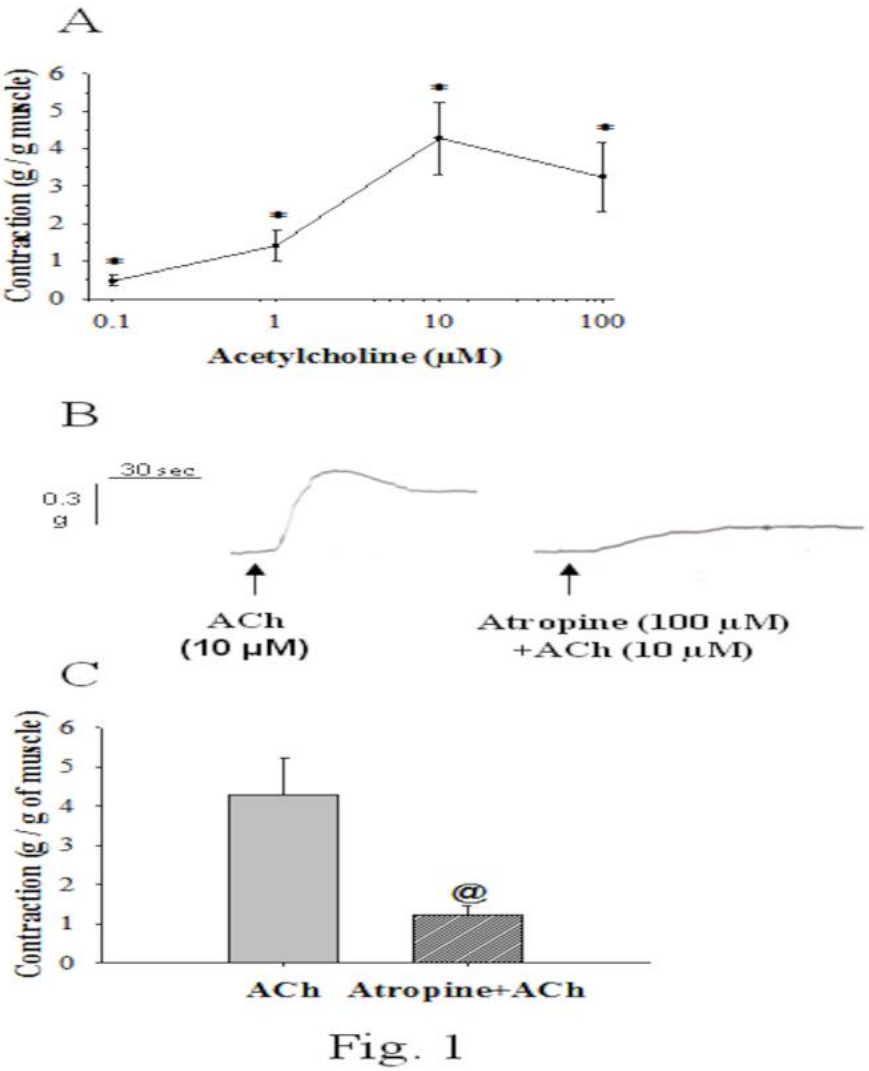


Fig. 1

**Fig. 1:** Upper panel (A) demonstrates dose-response of ACh (0.1-100 µM) induced muscle contractions (g/g) in normal appendix. Values are represented as Mean ± SEM (n=6) at each dose. The responses are significantly different from each other at different doses ($p < 0.05$, One way ANOVA). Asterisk (*) indicates significant difference of response from one dose to another. Middle panel (B) shows the actual recording of an experiment shows Ach (10 µM) induced contractions before and after exposure to atropine (100 µM). Scale for measuring the tension and time scale are given at the top left corner. Lower panel (C) shows Mean ± SEM value (n=6) of the ACh (10µM) induced muscle contractions (g/g) before and after the application of atropine (100 µM). Contractions elicited by ACh in muscle strips of normal appendix were partially but significantly blocked by atropine pre-treatment ($p < 0.05$, Student t- test for paired observations). Note after atropine pretreatment 30% of ACh-induced contractions were not blocked. @ indicates significant difference (Fig. 1A) from before values (Fig. 1C).

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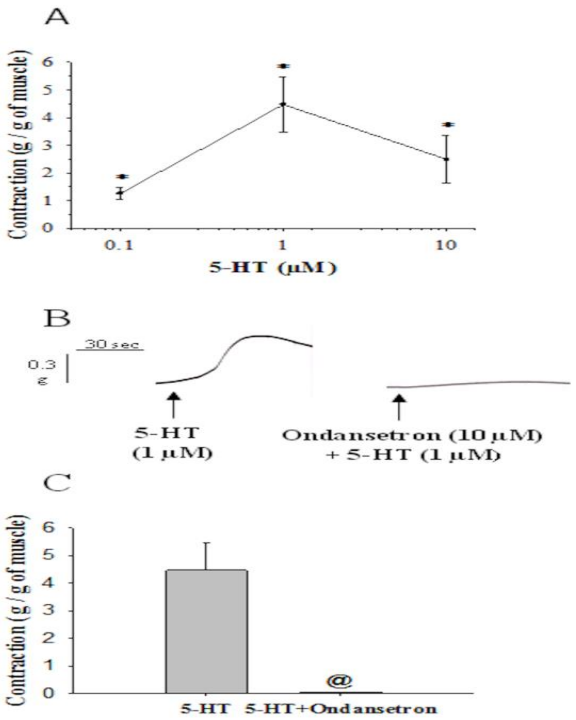


Fig. 2

**Fig. 2:** Upper panel (A) demonstrates dose-response of 5-HT induced muscle contractions (g/g) in normal appendix. Values are represented as Mean ± SEM (n=6 at each dose). The responses are significantly different from each other at different doses. ($p < 0.05$, One way ANOVA). Asterisk (*) indicates significant difference of response from one dose to another. Middle panel (B) shows the actual recordings of muscle contraction from individual experiment induced by 5-HT (1 µM) before and after exposure to ondansetron (10 µM). Scale for measuring the tension and time scale are given at the top left corner. Lower panel (C) showing Mean ± SEM values (n=6) of the 5-HT (1µM) induced muscle contractions (g/g) before and after the application of ondansetron (10 µM). Contractions induced by 5-HT were completely and significantly blocked by ondansetron pre-treatment ($p < 0.05$, Student t- test for paired observations). @ indicates significant difference (Fig. 2A) from before values (Fig. 2C).

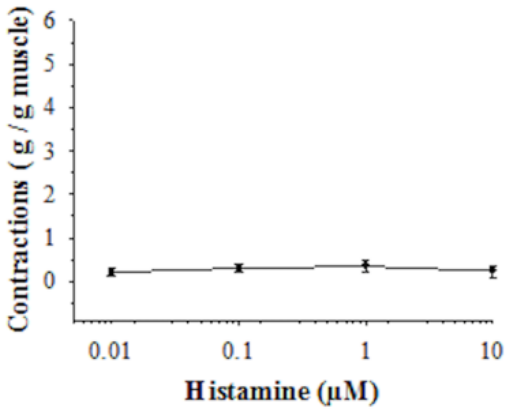


**Fig. 3**

**Fig. 3** demonstrates dose-response of histamine (0.01-10 µM) induced muscle contractions (g/g) in normal appendix. Values are represented as Mean ± SEM (n=6 at each dose). The responses are not significantly different from each other at different doses. ($p > 0.05$, One way ANOVA).

Further, the effect of CPM on histamine induced contractions were not assessed as the induced contractions were feeble and insignificant.

## DISCUSSION

In the present study, ACh and 5-HT elicited contractions in longitudinal muscle strips of normal human vermiform appendix. ACh induced contractions were partially but significantly blocked by atropine pretreatment, whereas 5-HT induced contractions were completely blocked by ondansetron.

In the present observations, ACh and 5-HT demonstrated dose-dependent increase in the magnitude of muscle contractions. However, the muscle contractions were decreased when the dose of ACh and 5-HT were increased further to 100 µM and 10 µM respectively (Fig. 1A and 2A). Histamine did not demonstrate any dose-dependent changes in the muscle contractions and contractions were almost similar at each dose (Fig. 3A). Dose-dependent increase in the muscle contractions may be associated with the increased sensitivity of the tissue to ACh or 5-HT. This may be attributed to the involvement of more and more receptors and muscle fibres [13]. The decrease in the contraction at higher dose may be due to down-regulation of the receptors as has been reported earlier [14, 15].

ACh induced contractions were reported in other parts of gut tissues especially in stomach, small intestine and colon [16]. Appendix contains a well defined enteric nervous system as seen in the other parts of intestine. The myenteric Auerbach's plexus is present between the outer longitudinal and middle circular muscle layers. They are also innervated by parasympathetic fibres of vagus nerve [13]. Further, neurotransmitter ACh released from the nerve endings mediates contractions of gut muscle involving muscarinic receptors [17]. In a study elsewhere [9], electrically evoked contractions were blocked by atropine suggesting cholinergic involvement. The present study substantiate the cholinergic involvement as ACh produced contractions of longitudinal muscle strips of the normal appendix. Further, ACh induced contractions were significantly attenuated by atropine pretreatment indicating muscarinic involvement (Fig. 1C). However, 30% of the contractions still persisted. Thus, ACh may mediate its contractile responses in longitudinal muscle of normal appendix involving cholinergic-muscarinic pathways and by additional mechanisms.

Further, the partial blockade of ACh induced contractile response by atropine (70%) observed in the present study indicates the involvement of mechanisms other than muscarinic pathways. There are reports for the existence of different types of neurotransmitter secreting interneurons in the enteric nervous system of the gut which are innervated by parasympathetic fibers [17, 18]. Thus, it is speculated that the cholinergic activation of interneurons releases different type of neurotransmitters like amines, 5-HT etc. which in turn contract the smooth muscles. Hence, there is a possibility that non-muscarinic pathways mediate ACh induced contractions.

5-HT has been reported to mediate contractions in other part of gut tissues directly or via myenteric network of neurons as reported elsewhere [5, 10]. Further, 5-HT is endogenously synthesized and stored in enterochromaffin cells and the myenteric plexus of the appendix [11]. In the present study, we have shown the contractions induced by 5-HT demonstrating the involvement of 5-HT. Further, ondansetron pretreatment blocked 5-HT induced contractions of normal appendix (Fig.2B and 2C). Ondansetron is a known $5\text{-HT}_3$ blocker [6], thus 5-HT induced contractions in the normal appendix involve $5\text{-HT}_3$ receptor pathways. Interestingly, maximum contractions produced by 5-HT were almost similar to ACh. Thus, the results also suggest the major role for 5-HT in mediating the contractility of the normal human appendix in addition to ACh.

In the present study, histamine produced very feeble contractions of normal appendix (Fig. 3A). The maximum contractile response produced by histamine was about 1/10th of the maximum responses produced by ACh or 5-HT (Fig. 1, 2 and 3). Therefore, present observation suggests that histamine has a minor contribution in mediating the contractility of the normal appendix.

## CONCLUSIONS

The observations of the present study suggest the involvement of $5\text{-HT}_3$ pathways in mediating the contractility of longitudinal muscle strips of the normal human vermiform appendix in addition to ACh-

muscarinic pathways. However, histamine plays a minor role in mediating the contractility of normal appendix in comparison to ACh or 5-HT.

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