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Modulation of acetylcholine induced smooth muscle contractile activity of rat ileum by histamine

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Abstract
Histamine is a short-acting endogenous amine found abundant in the gastrointestinal tract. It is playing an important role in the inflammatory conditions of the intestine. Even though, many literature have described the complexity of its control on gastrointestinal motility, a clear interpretation of its involvement is lacking. Hence, the present study was undertaken to assess the role of histaminergic drugs in the normal propulsive motion of the intestine and modulation of acetylcholine induced contraction in rat ileum. The contractile responses to the agonist alone and in presence of antagonists were recorded using a Digital Dale's Mono bath with isometric transducer connected to a recorder. The cumulative dose response of rat ileum to muscarinic and histaminergic drugs was determined. The median effective and inhibitory concentrations were calculated and the dose response curve was plotted. Histamine did not elicit any response in the rat ileal tissue even at the highest concentration added. The histamine receptor antagonists, chlorpheneramine maleate and ranitidine dihydrochloride didnot any effect on the rat ileum with prior exposure to histamine. However, pre-treatment with submaximal doses of atropine and histamine attenuated the contractile response to acetylcholine. On the other hand, the acetylcholine induced contraction was not altered by adding graded doses of histamine. The lack of response may be due to the absence of histaminergic receptors in the rat ileum. The modulation of response to acetylcholine indicates an involvement of H_3 receptor mediated pathway.

Keywords: Enteric nervous system, small intestine, histamine H_3 receptor, relaxation, atropine

1. Introduction
Intestinal smooth muscle contractility is a complex process coordinated by the enteric nervous system and the neurohormonal components. This involves various neurotransmitters such as acetylcholine, opioid, 5-hydroxytryptamine, epinephrine, gamma-aminobutyric acid (GABA), histamine, etc. The role of each neurotransmitter is different and depends on the part of the intestine it acts upon and the subtype of receptor present there. They bind to specific receptors to activate contraction in smooth muscle. Consequent to binding, there is an increase in the phospholipase C activity *via* coupling through G protein Gq/G_{11}. The activation of the phospholipase C pathway results in the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is hydrophilic and results in mobilization of Ca^{+2} leading to contraction of smooth muscle and secretion. DAG is hydrophobic and activates protein kinase C leading to phosphorylation of intracellular proteins (Blumenthal, 2018) [9].
Histamine was identified as an autocoid in the body and later found to have plenty of function in the body. The cellular sources of histamine include the gastric enterochromaffin-like cells, histaminergic neurons as well as mast cells and basophils. The actions of histamine are mainly seen in the respiratory system, gastrointestinal system and nervous system. Histaminergic receptors are also G–protein-coupled receptors classified into H_1, H_2, H_3, and H_4. The contraction of the intestinal smooth muscle is mainly mediated through the H_1 receptor and secretory activity is through the H_2 receptor (Ash and Schild, 1966 [4]; Black *et al.*, 1972 [7]. The H_3 receptor, which was initially identified in the brain and thought to be an autoreceptor was later found to have action on the gastrointestinal system as well (Bertaccini *et al.*, 2000) [5]. It was observed that the H_3 receptor produced a lesser effect on the propulsive activity of the intestine (Poli *et al.*, 2001) [19]. Besides, being potential immunomodulatory, histamine played a major role in inflammatory conditions of the intestine. A thorough understanding of the involvement of histamine in the regulation of gastrointestinal motility is deficient in the literature.

However, there is evidence indicating that excess production of histamine by mast cells may be responsible for diarrhoea caused by increased neuronal secretomotor function (Fabisiak *et al.*, 2017) [13].

Hence, the present study was undertaken with the primary objective of describing the role of histaminergic drugs in the normal propulsive motion of the intestine. The secondary objective was to access the modulation of acetylcholine induced contraction by histamine.

2. Materials and Method

2.1 Drugs

All the chemicals used in preparation of Tyrode solution were of analytical grade and procured from M/s Merck India Ltd., Mumbai, India and M/s Sigma Aldrich India Pvt. Ltd., Bengaluru, India. The drugs such as acetylcholine, atropine sulphate, histamine hydrochloride, ranitidine hydrochloride and chlorpheneramine maleate were procured from the M/s. Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru, India.

2.2 Animal

The study was conducted on eight healthy Sprague dawley rats of five to six months'age with an average body weight of 150-200 g. The rats were procured from Small Animal Breeding Station (SABS), College of Veterinary and Animal Sciences, Mannuthy and maintained for three weeks for acclimatization under standard uniform management conditions. All the experiments involving rats and rat tissue were reviewed and approved by the Institutional Animal Ethics Committee (IAEC/COVAS/PKD/20/2019) and conformed to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. They were housed in polypropylene rat cages in an environmentally controlled facility at temperature of 24ºC and relative humidity (RH) of at 50-60%. The rats were fed with standard feed diet as per Bureau of Indian Standards (BIS) and had access to water *ad libitum*.

2.3 Instrument

The study was done using thermostatically controlled Dale's Digital Mono bath (M/s. INCO Pvt. Ltd, Ambala, India) comprising of a temperature regulation board, water stirrer, glass tissue bath, oxygen tube and a force transducer (INCO T-305; ft 1173). The force transducer is connected to a three channel digital polygraph machine which converts the mechanical stimulus into electrical signals. The system is operated using INCO-Niviqure Digital Data Acquisition System software ver. 60.1.1.

2.4 Preparation of stock solution and physiological saline solution (Tyrode solution)

The drugs were administered as aqueous solution. Aqueous master stock solutions of all the drugs (10^{-2}M) were prepared using endotoxin free MilliQ®water. The master stock solution of each drug was then serially diluted using endotoxin free MilliQ® water to obtain working test solutions of concentration 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} 10^{-6}, 10^{-7}, 10^{-8}. The working test solutions were freshly prepared on the day of experimentation and administered in volumes not exceeding 0.5% of the bath volume. Further, the drugs were stored under refrigeration/ deep freezer (-20 ºC) as specified in the label. The prepared master stock solutions were stored at -4 ˚C.

The Tyrode solution was prepared as per the standard protocol described by Ghosh (2011) [14]. The composition of Tyrode solution used was sodium chloride (NaCl) 137mM, potassium chloride (KCl) 2.7mM, calcium chloride ($CaCl_2$)1.8mM, magnesium chloride ($MgCl_2$) 1 mM, sodium bicarbonate ($NaHCO_3$) 11.9 mM, sodium dihydrogen phosphate (NaH_2PO_4) 0.4mM and glucose 5.55mM. The electrolytes were dissolved in endotoxin free MilliQ® water and stored at -4 °C until further use.

2.5 Preparation of tissue (Rat Ileum)

The rats of an average body weight 150-200 g were euthanized by cervical dislocation and laparotomy was performed. One to two centimetres long ileum was excised and transferred to Tyrode solution kept at 37.2°C in a shallow Petri dish. The lumen and surface of ileum was washed and flushed with Tyrode solution. The fascia adhering to the tissue was carefully removed. One end of the ileum was fixed to tissue holder which was then mounted in a tissue chamber containing 20 ml Tyrode solution and the other end of ileum was tied to an isometric force transducer. The bath temperature was maintained at 37.2°C and continuously perfused with 95% $O_2$and 5% CO_2. The tension was recorded using a polygraph digital data acquisition system linked to isometric transducer connected to a recorder. The ileum tissue was mounted under 1 g resting tension and allowed to equilibrate for 60 minutes before the commencement of the experiment. During the equilibration period of 60 minutes, Tyrode solution was changed every 15 minutes. After equilibration, two phases of experiment were performed.

In the first phase, the concentration response curve of histamine alone and in presence of selective antagonists for histamine type-1 (chlorpheneramine maleate) and histamine type-2 (ranitidine hydrochloride) receptors respectively and vice-versa. In the second phase, the concentration-response curves of acetylcholine in the presence of submaximal dose of histamine receptor agonist histamine and H_1 receptor (H_1-R) antagonist chlorpheneramine maleate and H_2 receptor (H_2-R) antagonist ranitidine hydrochloride respectively were obtained. The contractile responses of the different drugs were recorded with isometric force transducer connected to a recorder (M/s INCO Pvt. Ltd, Ambala, India) (Apu *et al.*, 2016) [3]. The readings were recorded in INCO-Niviqure Digital Data Acquisition System software ver. 60.1.1.The drugs were applied as a cumulative dose application protocol and concentration response curve for agonist-evoked contraction was plotted to yield E_{max} and EC_{50}. The contractile response was expressed as a percentage of maximum response obtained. The tissue was flushed with fresh Tyrode solution after each recording and a resting time of 30 minutes was provided between the two recordings.

2.6 Procedure

2.6.1 Median effective concentration (EC_{50}) of histamine receptor agonist alone and in presence of selective histamine antagonists

In set I of phase I of the experiment, the concentration-response curve of histamine alone on rat ileum was obtained by adding increasing concentration ranging from 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} 10^{-4}, 10^{-3}, 10^{-2} of the histamine cumulatively in volumes of 100µl and 300µl.

In set II of phase I of the experiment, the concentration-response curves of histamine were obtained in presence of selective antagonists for H_1(H_1-R) chlorpheneramine maleate and H_2(H_2-R) ranitidine hydrochloride respectively. The agonist was used in volumes of 100µL and 300µL at

concentrations ranging from 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} 10^{-4}, 10^{-3}, 10^{-2} .The antagonists used were allowed to maintain contact with the tissue preparation for 15 minutes prior to addition of graded concentrations of the respective agonists. The concentration (M) of each selective antagonist used is presented in table 1.

Table 1: Concentrations of the histamine receptor agonist and antagonists used in calculation of EC_{50}

	Histamine agonist	Concentration of Histamine agonist(M)	Selective antagonist	Concentration of Selective antagonist(M)
	Phase I (Set I)			
I	Histamine ihydrochloride	10^{-8} to10^{-2}		
	Phase I (Set II)			
I	Histamine dihydrochloride	10^{-8} to10^{-2}	Chlorpheneramine maleate Ranitidine hydrochloride	100µL,10^{-4}

2.6.2 Median inhibitory concentration (IC_{50}) of histamine receptor antagonists in presence of histamine receptor agonist: In set III of phase I experiment, response was taken initially with an intermediate dose of histamine (100 µl,10^{-4} M) on ileal smooth muscle of rat. The responses of graded concentration of the antagonist in volumes of 100µL and 300µL at ascending concentrations ranging from 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} 10^{-4}, 10^{-3}, 10^{-2}. The tissue was incubated with submaximal concentration of the histamine agonist each time prior to adding increasing concentration of respective antagonists (Table 2).

Table 2: Concentrations of the histamine receptor antagonists and agonists used in calculation of IC_{50} for histamine receptors

	Antagonist	Concentration of antagonist(M)	In presence of Agonist	Concentration of agonist(M)
	Phase I (Set III)			
I	Chlorpheneramine maleate	10^{-8} to10^{-2}	Histamine dihydrochloride	100µL,10^{-4}
II	Ranitidine hydrochloride	10^{-8} to10^{-2}	Histamine dihydrochloride	100µL,10^{-4}

2.6.3 Median effective concentration (EC_{50}) of acetylcholine alone and in presence of histamine receptor agonist and selective histamine antagonist

In the set I of phase II experiment, acetylcholine cumulative contractile dose response curves were obtained with graded doses of acetylcholine (10^{-8} to 10^{-2}M) and corresponding median effective concentration (EC_{50}) and pD2values were calculated. For each concentration, 100µl and 300µl volumes were added to the tissue bath. The concentration-response curves of the muscarinic agonist Ach was obtained in presence of non-selective muscarinic antagonist atropine sulphate, selective antagonist for H_1R (chlorpheneramine maleate) and H_2 R ranitidine hydrochloride respectively. The agonist drug histamine and antagonists were allowed to maintain contact with the tissue preparation for 15 minutes prior to addition of graded concentrations of ACh. Acetylcholine was used in volumes of 100µL and 300µL at concentrations ranging from 10^{-8},10^{-7},10^{-6},$10^{-5}10^{-4}$,10^{-3},10^{-2}. The concentration (M) of each drug used is presented in table 3.

Table 3: Concentrations of the histamine receptor agonist, antagonist and muscarinic receptor agonist acetylcholine and antagonist for the calculation of EC_{50} of acetylcholine

	Muscarinic receptor agonist	Concentration of Ach (M)	Muscarinic /Histamine receptor agonist/antagonist agonist	Concentration of drugs
	Set I Phase VI			
I	Acetylcholine	10^{-8} to10^{-2}	-	-
II	Acetylcholine	10^{-8} to10^{-2}	Atropine sulphate	100µL,10^{-6}
III	Acetylcholine	10^{-8} to10^{-2}	Histamine dihydrochloride	100µL,10^{-4}
IV	Acetylcholine	10^{-8} to10^{-2}	Chlorpheneramine maleate	100µL,10^{-4}
V	Acetylcholine	10^{-8} to10^{-2}	Ranitidine hydrochloride	100µL,10^{-4}

2.6.4 Median inhibitory concentration (IC_{50}) of atropine sulphate, histamine and selective histamine receptor antagonists in presence of Ach

The pre-contractile response was taken initially with submaximal dose of acetylcholine (100 µl, 10^{-4} M) on ileal smooth muscle of rat. The concentration of the agonist at which an approximately 80 per cent contractile response is attained is taken as the submaximal dose. The tissue was incubated for 2 min with submaximal concentration of the muscarinic agonist acetylcholine each time prior to adding increasing concentration of atropine sulphate, histamine and respective antagonists. The drugs were used in volumes of 100µL and 300µL at concentrations ranging from 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} 10^{-4}, 10^{-3},10^{-2}.The concentration (M) of each drug used is presented in table 4. The corresponding median inhibitory concentration (IC_{50}) values and pD2values were determined (Ghosh, 2011) [15].

Table 4: Concentrations of histamine receptor agonist, antagonist and muscarinic receptor agonist acetylcholine for the calculation of IC50

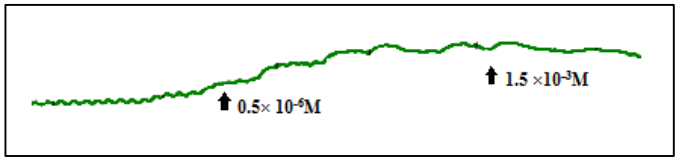
	Agonist/antagonist	Concentration of drug(M)	Muscarinic agonist	Concentration of Muscarinic agonist
	Set II Phase VI			
I	Atropine	10^{-8} to10^{-2}	Acetylcholine	100µL, 10^{-4}
II	Histamine dihydrochloride	10^{-8} to10^{-2}	Acetylcholine	100µL, 10^{-4}
III	Chlorpheneramine maleate	10^{-8} to10^{-2}	Acetylcholine	100µL, 10^{-4}
IV	Ranitidine hydrochloride	10^{-8} to10^{-2}	Acetylcholine	100µL, 10^{-4}

2.7 Analysis of Functional Responses
Contractile responses obtained to drugs used in rat ileum were converted to percentage response and plotted against the log drug concentrations. The EC_{50} and IC_{50}were calculated by non-linear regression analysis using GraphPad Prism 5.0.Mean and standard errors for the results were determined by one-way ANOVA (Snedecor and William, 1989) [23]

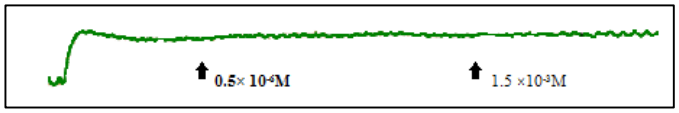
3. Results
3.1 Response of histamine receptors to known agonist and antagonists
Histamine hydrochloride didn't produce any modulation in the normal response as represented in the physiographic recording (Fig. 1). Also the histamine H_1-R antagonist chlorpheneramine maleate and H_2-R antagonist ranitidine didn't bring any change in the normal ileal motility.

Fig 1: Representative physiographic recording of histamine alone. The arrows depict the point of application of the respective concentration of drug.

3.2 Response of acetylcholine in presence of histamine and atropine
The effect of histamine was compared with the prototype muscarinic receptor agonist acetylcholine. Acetylcholine caused a concentration dependent contraction of rat ileum. Representative physiographic recording of ACh cumulative dose response in presence of histamine and the response of histamine in presence of the submaximal dose of ACh are given in figure 2. The log dose response curve of ACh alone, in presence of non-selective antagonist (atropine) and histamine are represented in figure 3. The mean EC_{50} and pD2 values of ACh alone, in presence of antagonist atropine and histamine are represented in table 5 and 6. Both histamine and atropine showed a right ward shift in dose response curve of ACh maintaining the maximum, without change in slope and E_{max}. This rightward shift with atropine was most remarkable when compared to histamine. In presence of histamine, a higher concentration of Ach was required to produce the same response.



A. ACh in the presence of histamine (10^{-4}M)



B. Histamine in the presence of submaximal ACh (10^{-4}M.)

Fig 2: Representative physiographic recording (**A**) ACh cumulative dose response in presence of histamine and (**B**) the response of histamine in presence of the submaximal dose of Ach

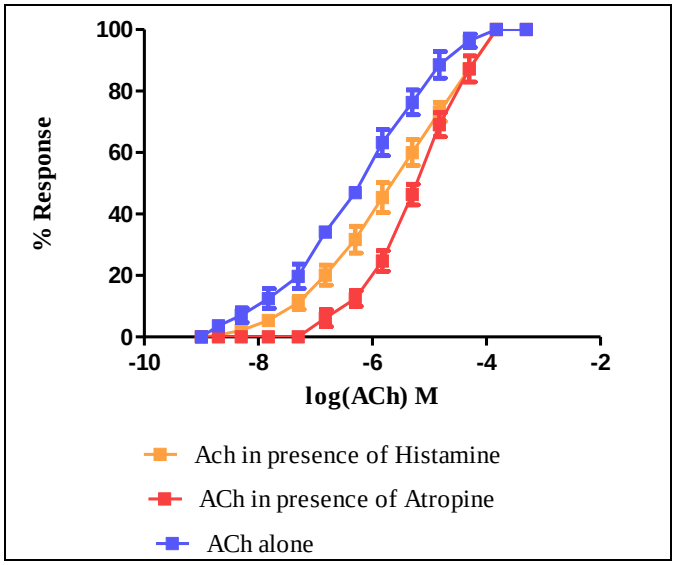


Fig 3: The log dose response curve of ACh alone, in presence of non-selective antagonist (atropine) and histamine

Table 5: EC_{50} and $logEC_{50}$ values of acetylcholine alone and in presence of histamine and atropine.

	LogEC50	EC50	95% Confidence Interval	
			LogEC50	EC50
ACh	-6.229	5.903×10^{-7}	-6.394 to -6.064	4.038×10^{-7} to 8.629×10^{-7}
ACh in presence of Atropine	-5.226	5.937×10^{-6}	-5.302 to -5.151	$4.988\times 10^{-6}$to 7.067×10^{-6}
ACh in presence of histamine	-5.752	1.772×10^{-6}	-5.901 to -5.602	$1.257\times 10^{-6}$to 2.498×10^{-6}

Table 6: Mean pD2 value of ACh and ACh in presence of histamine and atropine sulphate

	Mean pD2 value
ACh alone	6.24 ± 0.02^{a}
ACh in the presence of histamine	5.74 ± 0.02^{b}
ACh in the presence of Atropine	5.22±0.04c

n=6, values are Mean ±SE, One way ANOVA with Tukey's post Hoc analysis using Graph Pad Prism 5.0 with different superscript vary significantly ($P< 0.05$)

4. Discussion
Histamine, an aminergic neurotransmitter is abundantly secreted in the intestinal mucosa and produces different physiological response by its action on H_1 H_2, H_3 receptors. Histamine was believed to have an important impact on GI tract in at least three major functions: modulation of GI motility, enhancement of gastric acid production, and alteration of mucosal ion secretion. Many authors have reported the histamine induced contractions in caecum and colon of cat, guinea pig and rat colon, guinea pig small and large intestine, colon of simian species (Tidmarsh, 1932; Aguilar *et al*., 1986; Leurs *et al*., 1991; Kim *et al*., 2011) [24, 1, 18, 16]. A thorough understanding of the involvement of histamine in the regulation of gastrointestinal motility is deficient in literatures. However, there are evidences indicating that excess production of histamine by mast cells may be responsible for diarrhoea caused by increased neuronal secretomotor function (Fabisiak *et al.*, 2017) [13]. Studies have revealed different responses of acetylcholine and histamine in rectal and colonic tissues in rats (Aguilar *et al*.,

1986; Singh and Mandal, 2013) [1, 22].

In the present study, it was observed that histamine did not elicit any response in the rat ileal tissue even at the highest concentration of 10^{-2}M added. The H_1 (chlorpheneramine maleate) and H_2 (ranitidine dihydrochloride) receptor antagonists were also without effect on the isolated longitudinal strip of rat ileum with prior exposure to histamine. These results may be due to either the absence of histaminergic receptor (H_1,H_2) or lack of the receptor in the rat ileum. The current findings corroborated with the results observed by Sakai *et al*. (1979) [21] and Hemedah *et al.* (2001) [15], where histamine and its analogues did not produce any effect on the ileum of rat. Similar findings were also reported by Aguilar *et al*. (1986) [1] in rat colon. This contrasts the previous results obtained on rat ileal tissue, where histamine induced the contractile response (Bigovic *et al*., 2010) [6]. Furthermore, another study has revealed the existence of a different cholinergic and histaminergic activity in adult and neonate as well as in rectal and colonic tissue (Singh and Mandal, 2013) [22]. This indicated that action of histamine was different and depended on the part of intestine it acted upon and the subtype of receptor present there. On the other hand, histamine triggered contraction and depolarization of longitudinal smooth muscle cells of guinea pig ileum by increasing Ca^{2+} partition through voltage-gated and receptor-operated Ca^{2+} channels (Yamanka *et al.,* 1987) [25]. Furthermore, for functional studies of histaminergic drugs, the guinea pig ileum was reported to be the best model (Leurs *et al*.,1991) [18].

The dose dependent contractile response evoked by acetylcholine (Ali *et al*., 2004) [2] and electrical stimulation (Blandina *et al*., 1984 [8]; Kurjak *et al*., 1999 [17]; Brankovic *et al*., 2011[10]) on isolated rat ileum was well recognized. In the present study, ACh also produced a dose dependent increase in the contractile response of the rat ileum tissue as reported earlier for other muscarinic agonists, carbachol or oxotremorine-M. Pre-treatment with submaximal doses of atropine and histamine attenuated the contractile response to acetylcholine. On the other hand, the acetylcholine induced contraction was not altered by adding graded doses of histamine. This indicates an involvement of H3 mediated pathway. The role of H_3 receptors located on the nerve terminals of myenteric plexus on the pre and post ganglionic NANC fibres in negatively modulating the excitatory neurotransmitter acetylcholine and substance was explained by Poli *et al.* (2001) [19].The results of the present study were supported by the findings of Bertaccini *et al.* (2000) [5], Curuzzi *et al*.(2000) [12] where the selective histamine H_3 receptor agonists (R)α-methyl histamine and Immepip prevented the contraction produced by acetylcholine (10^{-7} M) isolated cells from the longitudinal muscle of the guinea pig ileum. Further, they were inactive both on basal contractility and on acetylcholine-induced contractions. It was observed by Cooke *et al.* (1984) [11] that blockade of muscarinic receptor by atropine during the electrical field stimulation, increased the histamine evoked response but not otherwise. On the contrary, absence of H_3 receptor in rat ileum and rabbit colon are also reported with no role in the modulation of cholinergic neuronal function in the rat intestine unlike those in the guinea-pig (Pozzoli *et al*., 1997 [20]; Hemedah *et al*., 2001 [15]). Since specific H_3 receptor agonist and antagonist was not used, the present study could not confirm the involvement of post junctional H_3 receptor in preventing the contractile response to acetylcholine.

5. Conclusion

The present investigation revealed that the endogenous mediator, histamine did not elicit any response in the rat ileal tissue. In addition, the H_1-R antagonist (chlorpheneramine maleate) and H_2-R antagonist (ranitidine) also did not produce any modulation in the basal contractile response of ileum. This indicated the absence or meagre presence histamine H1 and H2 receptors in rat ileum. However, the modulation of contractile response to acetylcholine by histamine, indicates an involvement of H_3 receptor mediated pathway in rat ileal tissue. Further molecular and functional studies are needed to confirm its role.

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7. Conflicts of interest

The authors declare that there are not any conflicts of interests

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