Original Article

Expression of Serotonin Receptors in the Colonic Tissue of Chronic Diarrhea Rats

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ABSTRACT

Background/Aims: This study aimed to investigate the difference among the expression of serotonin receptors (5-HT_3, 5-HT_4, and 5-HT_7 receptors) in colonic tissue of chronic diarrhea rats. **Materials and Methods:** A rat model of chronic diarrhea was established by lactose diet. The expression of 5-HT_3, 5-HT_4, and 5-HT_7 receptors in the colonic tissue was detected using immunohistochemistry, real-time PCR and Western blotting techniques. **Results:** There is no significant difference on the protein expression of 5-HT_3 receptor between the normal group and the chronic diarrhea model group. The mRNA expression of 5-HT_3 receptor in the chronic diarrhea model group was significantly lower than that in the normal group (n = 10; $P < 0.01$). The protein and mRNA expression of 5-HT_4 receptor in the chronic diarrhea model group were significantly higher than those in the normal group (n = 10; $P < 0.05$, $P < 0.01$). On the contrary, the protein and mRNA expressions of 5-HT_7 receptor in the chronic diarrhea model group were significantly decreased compared with the normal group (n = 10; $P < 0.01$, $P < 0.01$). **Conclusions:** The results suggested the receptors of 5-HT_4 and 5-HT_7 may be involved in inducing diarrhea by lactose diet.

Key Words: 5-HT_3, 5-HT_4, and 5-HT_7 receptors, chronic diarrhea, colonic tissue, lactose

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Diarrhea is a common gastrointestinal disease throughout the world. In developing countries every year, approximately 1.5 million children die because of diarrhea.[1] Although medical conditions have improved, diarrhea is one of the most important causes of death in children.[2] Diarrhea is a troubling issue in children because they are susceptible to diarrhea. Diarrhea is characterized by a gastrointestinal function disorder, an increase of stool frequency, and a change of fecal consistency. Chronic diarrhea has been defined as diarrhea lasting for 14 days or longer.[3,4] Recently, mortality resulting from acute diarrhea has been decreased by using oral rehydrating therapy.[5] However, chronic diarrhea is still a problem that threatens the lives of children. Chronic diarrhea is induced by some factors, such as lactose, castor oil and rhubarb. Diarrhea caused by lactose occurs in over 50% of the world's population.[6] The morphological structure of a chronic diarrhea model intestine that has been induced by lactose is similar to that in children with diarrhea.[7]

5-Hydroxytryptamine (5-HT) is mostly synthesized and released from enterochromaffin (EC) cells of the intestinal mucosa.[8,9] It is an important brain–gut axis neurotransmitter that can regulate gut function. The function of 5-HT is activated in combination with receptors located in different tissues and cells. 5-HT receptors are extensively expressed in the central nervous system, peripheral nervous system, and gastrointestinal tract and are largely divided into seven distinct classes (5-HT_1R–5-HT_7R) according to their structural and operational characteristics.[10,11] The five families of 5-HT receptors, 5-HT_1R, 5-HT_2R, 5-HT_3R, 5-HT_4R, and 5-HT_7R, are expressed in the gut and are related with gut functions. The 5-HT_3, 5-HT_4 and 5-HT_7 receptors

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were studied in the gut and were found to play a role in regulating smooth muscle motility in the gastrointestinal tract.[12] However, a comparison of the expression of 5-HT_3, 5-HT_4, and 5-HT_7 receptors in diarrhea induced by lactose has not been reported. Therefore, our study will investigate the expression of 5-HT_3, 5-HT_4, and 5-HT_7 receptors in colonic tissue with chronic diarrhea that has been induced by lactose.

MATERIALS AND METHODS

Animals

Weaned Wistar rats (21 days) were supplied by the Experimental Animal Center of Dalian Medical University, Dalian, China. Animals were kept at a temperature of 23°C ± 2°C and a relative humidity of 55% ±2% on a 12 hour light-dark cycle for 3 days before the experiments.

Chronic diarrhea model

Two rats were housed in each individual cage. Prior to inducing diarrhea, all rats (n = 20) were fed a standard American Institute of Nutrition AIN93G diet ad libitum and were provided with free access to water for three days.[13] The ingredients of the AIN93G diet were corn starch 52.9%, sugar 10.0%, fiber 5.0%, casein 20%, soy oil 7.0%, l-cystein 0.3%, mineral, and vitamin mix 4.7%. The AIN93G diet contained the following chemical composition (%): Protein 20.3, carbohydrate 67.9, fat 7, and minerals 4.7. A high-lactose diet (HLD) containing 52.9% lactose in place of starch was fed to rats.[7,14,15] The rats were randomly separated into two groups: The normal group (n = 10) and model group (n = 10). The rats in the normal group were fed an AIN93G diet *ad libitum*, whereas the model group was fed an HLD. Within 14 days of consuming the diet, chronic diarrhea was induced. The changes in stool and fecal fluid of the rats were observed in the process of molding. All of the procedures of this study were approved by the local Animal Care Committee and in accordance with the Guide for the Care and Use of Laboratory Animals published by the Science and Technology Commission of P.R.C. (STCC Publication No. 2, revised 1988).

Tissue preparation

After anesthetization with 10% chloral hydrate (350 mg/kg) using an intraperitoneal injection, the colonic tissues of rats were taken out of the body and were washed with ice-cold PBS. The connective tissues of the colon were removed under a low temperature condition. The colonic tissue stored in liquid nitrogen was used for RNA and protein extraction. The colonic tissues were fixed immediately in a 10% formaldehyde solution at 4°C and were used for immunohistochemistry.

Immunohistochemical staining for 5-HT_3, 5-HT_4, and 5-HT_7 receptors

The protein expression of 5-HT_3, 5-HT_4, and 5-HT_7 receptors in the colon tissue was detected by the method of immunohistochemical ABC.

The colonic tissues fixed in 10% formaldehyde solution for 24 h were embedded in paraffin. Five micrometer paraffin sections were deparaffinized in xylene and were rehydrated in an alcohol solution washed in water. Antigen retrieval and blocking of endogenous peroxidase activity were performed before immunostaining. After blotting with normal goat serum (Leica, Germany), tissue sections were incubated with the primary antibodies rabbit monoclonal anti-5-HT_3 receptor (1:200; Abcam, USA), rabbit monoclonal anti-5-HT_4 receptor (1:2000; Abcam, USA), and rabbit monoclonal anti-5-HT_7 receptor (1:2000; Abcam, USA) at 4°C for 24 h. Then, tissue sections were incubated with a biotin-labeled goat anti-rabbit antibody (Leica, Germany) at room temperature for 1 h. Finally, tissue sections were stained with 3,3-diaminobenzidine tetrahydrochloride (Leica; Germany) for approximately 2 min at room temperature; the nuclei were counterstained with hematoxylin. The staining slide was examined using a LEICA DM 2500 light microscope (Leica, Germany). Positive immunostaining was labeled with a brown color.

Western blotting analysis for 5-HT_3, 5-HT_4, and 5-HT_7 receptors' protein expression

The protein expression of 5-HT_3, 5-HT_4, and 5-HT_7 receptors in the colon tissue was observed by Western blotting.

The colonic tissues of rats were homogenized with lysis reagent supplemented with phenylmethylsulfonyl fluoride (PMSF) (Beyotime Chemical Co., China) according to the manufacturer's instructions. The concentration of protein was determined by a BCA protein assay kit (Pierce Biotechnology, USA).

The total protein of colonic tissues was separated using 10% polyacrylamide gels and was then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). After blocking with 5% skim milk powder, the membrane was incubated with the primary antibodies, rabbit monoclonal anti-5-HT_3 receptor (1:100; Abcam, USA), rabbit monoclonal anti-5-HT_4 receptor (1:1000; Abcam, USA), rabbit monoclonal anti-5-HT_7 receptor (1:1000; Abcam, USA), and a rabbit polyclonal anti-β-actin (1:1000; Biosynthesis Biotechnology, China) overnight at 4°C. After washing three times, the membrane was incubated with a secondary antibody, goat anti-rabbit IgG (1:5000; ZSGB-BIO, China) -linked horseradish peroxidase (HRP) against 5-HT_3, 5-HT_4, and 5-HT_7 receptors, and β-actin. Finally,

the protein bands were detected using Investigator ProImage (FluorChem FC3, USA). The intensity of the bands was analyzed using Quantity One software (Bio-Rad Laboratories, USA); then, the intensity of the bands was normalized to that of β-actin.

Real-time PCR analysis for 5-HT_3, 5-HT_4, and 5-HT_7 receptors mRNA expression

The mRNA expression of 5-HT_3, 5-HT_4, and 5-HT_7 receptors in the colon tissue was analyzed using real-time fluorescence quantitative PCR (real-time PCR). Total mRNA was extracted from colonic tissues with RNAiso Plus (Takara Bio Inc., China) and was reverse transcribed to cDNA with a PrimeScript™ RT reagent Kit with a gDNA Eraser (Takara Bio Inc., China) according to the manufacturer's instructions. RT-PCR was conducted with SYBR® Premix Ex TaqTM II (Takara Bio Inc., China) following the manufacturer's protocol. Primers for the 5-HT_3, 5-HT_4, and 5-HT_7 receptors and β-actin were designed and are shown in Table 1. The procedure for real-time PCR was two steps: Stage 1: Reps 1, 95°C, 30 s and Stage 2: Reps 40, 95°C, 5 s; 60°C, 30 s. The expression of 5-HT_3, 5-HT_4, and 5-HT_7 receptor mRNA were normalized to the expression of β-actin.

Statistical analysis

All data are expressed as mean ± standard deviation (SD), and a $P < 0.05$ or <0.01 was regarded to have statistical significance. The significance of differences was carried out by Student's *t*-test and one-way ANOVA using SPSS 10.0. All experimental data were repeated at least three times.

RESULTS

Establishment and identification of a chronic diarrhea animal model

The chronic diarrhea rat model was established using a 14-day lactose diet. Rats of the model group appeared to have diarrhea after the first day of feeding high lactose diet. The duration of chronic diarrhea lasted for 14 days. The rats of the model group produced watery, soft, and yellowish stools, whereas the normal group rats produced pliable, soft, and well-formed pellet stools.

Table 1: The sequence of primers used for real-time polymerase chain reaction

Gene	Sequence (from 5′ to 3′)	GenBank accession number
β-actin-F	TCGTGCGTGACATTAAAGAG	NM_031144
β-actin-R	TGCCACAGGATTCCATACC	
5HT_3R-F	ACCGCCTGTAGCCTTGAC	NP_077370
5HT_3R-R	TGCTCTTGTCCGACCTCA	
5HT_4R-F	TAATGTGAGTTCCAACGAGGGT	NP_036985
5HT_4R-R	CAGCAGGTTGCCCAGGAT	
5HT_7R-F	GAGTGGCTTCCTAGAGGTGAC	NP_075227
5HT_7R-R	CAGGATGGAGCCGATCACAA	

Immunohistochemical staining for 5-HT_3, 5-HT_4, and 5-HT_7 receptors

The results of immunohistochemistry showed that the number of 5-HT_4 receptor-positive cells in the model group (M) was higher than in the normal group (N) in colonic tissues [Figure 1]. The results of the 5-HT_3 and 5-HT_7 receptor expression in the model group were opposite those of the 5-HT_4 receptor [Figure 1].

Western blotting analysis for 5-HT_3, 5-HT_4, and 5-HT_7 receptors' protein expression

The expression of the 5-HT_3 receptor in the chronic diarrhea model group did not vary significantly in comparison with the normal group (n = 10; [Figure 2]); the expression of the 5-HT_4 receptor in the chronic diarrhea model group was significantly increased compared with the normal group (n = 10; $P < 0.05$; [Figure 2]), and the expression of the 5-HT_7 receptor in the chronic diarrhea model group was significantly decreased compared with the normal group (n = 10; $P < 0.01$; [Figure 2]).

Real-time PCR analysis for 5-HT_3, 5-HT_4, and 5-HT_7 receptor mRNA expression

The mRNA expression of 5-HT_3 and 5-HT_7 receptors in the chronic diarrhea model group was significantly decreased compared with the normal group ($n = 10$; $P < 0.01$; [Figure 3]); the mRNA expression of the 5-HT_4 receptor in the chronic diarrhea model group was significantly increased compared with the normal group ($n = 10$; $P < 0.01$; [Figure 3]).

DISCUSSION

Chronic diarrhea treated by high lactose is regarded as an osmotic diarrhea.[13] A high-lactose diet has often been used for inducing persistent diarrhea. A high lactose diet was chosen as the method to induce a diarrhea model in the study. The results showed that the rats were considered to have diarrhea as previously described by other researchers.[16,17]

5-HT plays an important physiological role by activating 5-HT receptors in the contraction and relaxation of smooth muscle, stimulation of propulsive, and segmentation motility, and so on.[18] 5-HT receptors are G-protein-coupled receptors, except for the 5-HT_3 receptor. The 5-HT_3 receptor is unique among all of the 5-HT receptors, which are composed of multi-subunits; the 5-HT_3 receptor functions as a ligand-gated ion channel.[19,20] The 5-HT_3 receptor is involved in the regulation of GI motility and it causes the contraction and relaxation of intestinal smooth muscle by

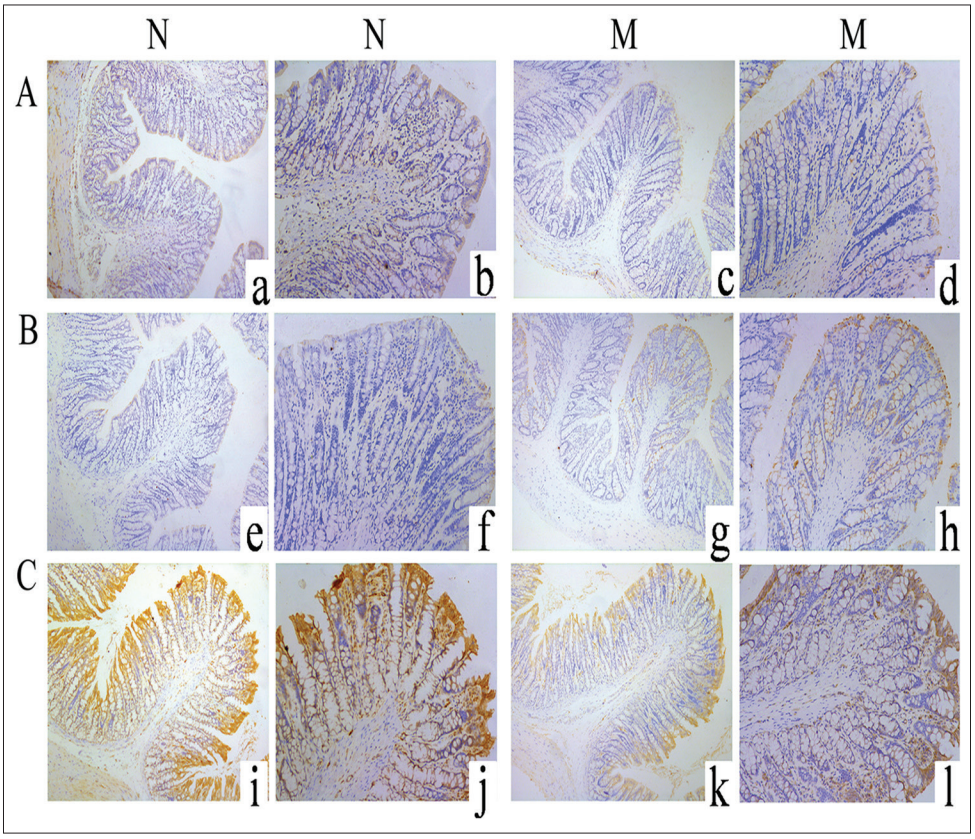

Figure 1: Immunohistochemical staining analysis of 5-HT_3, 5-HT_4, and 5-HT_7 receptors in colonic tissues of the model group (M) and the normal group (N). (a) The relative expression of 5-HT_3R. (a and c): ×100 (b and d): ×200; (b) the relative expression of 5-HT_4R. (e and g): ×100 (f and h): ×200; (c) the relative expression of 5-HT_7R. (i and k): ×100 (j and l): ×200

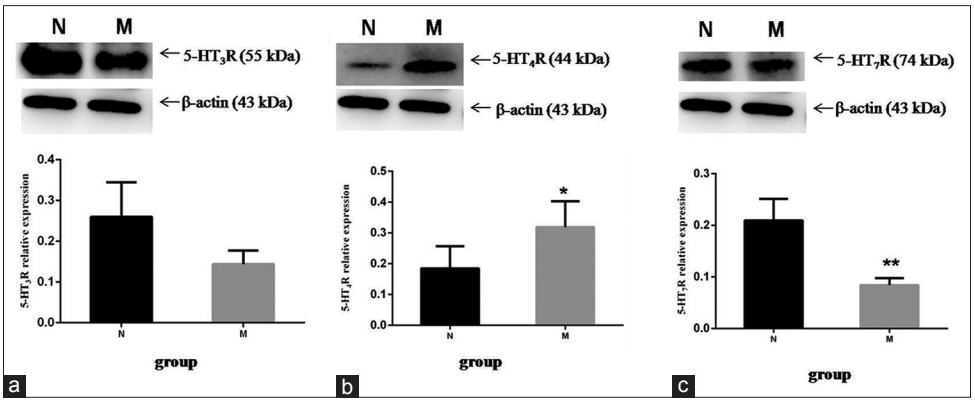

Figure 2: Western blotting analysis of 5-HT_3, 5-HT_4, and 5-HT_7 receptors in colonic tissues of the model group (M) and the normal group (N). (a) The relative expression of 5-HT_3R; (b) the relative expression of 5-HT_4R; (c) the relative expression of 5-HT_7R. Bars represent data that is presented as the mean ± standard error. *$P < 0.05$, **$P < 0.01$

depolarizing the cell.[21] 5-HT_4 and 5-HT_7 receptors belong to a type of metabotropic receptor.[22] The 5-HT_4 receptor is activated and contributes to the regulation of gut propulsive motility.[23] The 5-HT_7 receptor participates in regulating smooth muscle relaxation in the gastrointestinal tract[24,25] and restraining gut peristalsis.[25,26]

In this study, we showed 5-HT_3, 5-HT_4, and 5-HT_7 receptors' expression in colonic tissue in chronic diarrhea rats compared with normal rats. It is suggested that they may be involved in the pathway of hyperactive bowel movements with diarrhea. However, the variation of expression in 5-HT_3, 5-HT_4, and 5-HT_7 receptors was different. From

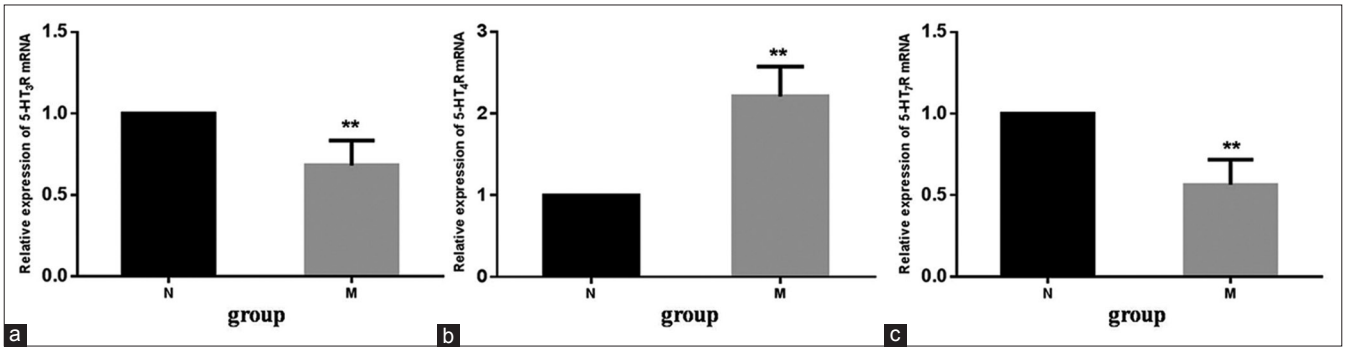

Figure 3: Real-time polymerase chain reaction analysis of 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptors in colonic tissues of the model group (M) and the normal group (N). (a) The relative expression of 5-HT$_3$R; (b) the relative expression of 5-HT$_4$R; (c) the relative expression of 5-HT$_7$R. Bars represent data that is presented as the mean ± standard error. $^{**}P < 0.01$

the immunohistochemical staining result, we observed that the positive rate of 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptors staining was extensively observed in colonic tissues. The positive rate of 5-HT$_3$ receptor and 5-HT$_7$ receptor staining was decreased between the normal group and the model group. However, the positive rate of 5-HT$_4$ receptor staining in the model group was obviously increased compared with that in the normal group. We acquired similar results in comparison with the expression of 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptors in chronic diarrhea rats compared with normal rats in Western blotting experiments. Besides, we detected the mRNA expression of the 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptors. We found that the mRNA expressions in 5-HT$_3$ and 5-HT$_7$ receptors with chronic diarrhea rats were lower than in normal rats. However, the mRNA expression of the 5-HT$_4$ receptor in chronic diarrhea rats was higher than in normal rats. We inferred that the 5-HT$_4$ and 5-HT$_7$ receptors may be related with diarrhea induced by lactose at the protein level. The role of 5-HT$_3$ receptor in diarrhea is uncertain because of the difference in protein and RNA expression in this study. Some research has reported that the 5-HT$_4$ receptor can accelerate the peristaltic reflex and promote normal motility.[27,28] Colonic motility became slow without the 5-HT$_4$ receptor.[29] Thus, more 5-HT$_4$ receptor should be expressed in chronic diarrhea rats. The expression of 5-HT$_4$ receptor in our study is consistent with previous reports. Therefore, the interaction between the 5-HT$_4$ receptor and its ligand is related with diarrhea by accelerating colonic motility. The 5-HT$_7$ receptor can mediate colonic relaxation[26] and inhibit colonic peristalsis.[30] The function of the 5-HT$_7$ receptor is opposite to the function of the 5-HT$_4$ receptor. Thus, less 5-HT$_7$ receptor should be expressed in chronic diarrhea rats. The expression of the 5-HT$_7$ receptor in the diarrhea model group is less than that in the normal group. The 5-HT$_7$ receptor is also involved in diarrhea, although it has a different role than the 5-HT$_4$ receptor. We conclude that the 5-HT$_4$ and 5-HT$_7$ receptors are involved in diarrhea that is induced by lactose. Future studies on the 5-HT$_3$ receptor will facilitate clarifying the relation among 5-HT, 5-HT receptors, and diarrhea.

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Conflicts of interest

There are no conflicts of interest.

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