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Basic Study

Nitrergic Neurons Involvement in the Colonic Motility in Rat Model of Ulcerative Colitis

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Abstract

BACKGROUND

The mechanisms underlying gastrointestinal (GI) dysmotility with ulcerative colitis (UC) have not been fully elucidated. The enteric nervous system (ENS) plays an essential role in the gastrointestinal motility. As a vital neurotransmitter in the ENS, the gas neurotransmitter nitric oxide (NO) may impact the colonic motility. In this study, dextran sulfate sodium (DSS)-induced UC rat model was used for investigating the effects of NO by checking the effects of rate-limiting enzyme nitric oxide synthase (NOS) changes on the colonic motility as well as the role of the ENS in the colonic motility during UC.

AIM

To study and reveal the relationship between the effects of NOS expression changes in NOS-containing nitrergic neurons and the colonic motility with the NO function in the rat UC model.

METHODS

Male rats (n = 8/each group) were randomly divided into a control (CG), a UC group (EG1), a UC + NOS agonist thrombin derived polypeptide 508 trifluoroacetic acid (TP508TFA) group (EG2), and a UC + NOS inhibitor NG-monomethyl-L-arginine monoacetate (L-NMMA) group (EG3). The UC was only induced by administering 5.5% DSS in drinking water without any other treatment (EG1), TP508 TFA, and L-NMMA gavage for the EG2 and EG3, respectively. The Disease Activity Index (DAI) and histological assessment were recorded for each group, whereas the changes in the proportion of colonic nitrergic neurons were counted using immunofluorescence histochemical staining, Western blotting, and ELISA techniques, respectively. In addition, the contractile tension changes in the circular and longitudinal muscles of the rat colon were investigated *in vitro* using an organ bath system.

RESULTS

The proportion of NOS-positive neurons within the colonic myenteric plexus (MP), the relative expression of NOS, along with the NOS concentration in serum and colonic tissues were significantly elevated in EG1, EG2, and EG3 compared with CG rats. In UC rats, stimulation with agonists and inhibitors led to variable degrees increase and decrease for each indicator in EG2 and EG3 group. When the rats in EGs developed UC, the mean contraction tension of the colonic smooth muscle were detected *in-vitro* and were higher in the EG1, EG2, and EG3 than the CG group. Compared with the EG1, the contraction amplitude and mean contraction tension of the circular and longitudinal muscles of the colon in the EG2 and EG3, were enhanced and attenuated, respectively. Thus, during UC, regulation of the expression of NOS within MP improved the intestinal motility, thereby favoring the recovery of intestinal functions.

CONCLUSION

In the UC rats, increased number of nitrergic neurons in the colonic MP leads to the attenuation of colonic motor function. To intervent the NOS activity might modulate the function of nitrergic neurons in colonic MP and prevent the colonic motor

dysfunction. These results might provide clues for a novel approach to alleviate diarrhea symptoms of the UC patients.

Key Words: Nitrergic neurons; Nitric oxide; Nitric oxide synthase; Ulcerative colitis; Colonic motility; Colonic myenteric plexus

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Core Tip: This study focused on the effects of nitrergic neurons in the myenteric plexus (MP) on colonic motor function in rats with ulcerative colitis. The results suggest that an increased number of nitrergic neurons in the colonic MP of the ulcerative colitis (UC) rats leads to reduced colon contractile function. Therefore, the regulation of the activity of nitrergic neurons in the colonic MP through interference with the activities of nitric oxide synthase might be a novel potential and prospective way to reduce the diarrhea symptom in the UC patient.

INTRODUCTION

Ulcerative colitis (UC) is a nonspecific inflammatory disorder of the intestine that primarily involves the rectum, sigmoid colon, and/or, the whole colon, in severe cases. Clinical features include recurrent episodes of abdominal pain, diarrhea, and mucopurulent stools [1], unfortunately the disease course is permanent and causes great suffering to the patients. UC can occur at any age (mainly in young adults), and in recent years, its incidence has increased worldwide [2]. The etiology of UC should be remains complex, however its pathogenesis might be related to the genetic[3], immunological, psychiatric depression & anxiety[4], environmental[5], dietary allergy, intestinal flora[6], and other factors. Therefore, it leads to treatment difficulty, a prolonged treatment course, and easy disease recurrence. Although current pharmacological treatments in the clinic might improve patient's symptoms, but they still cannot achieve satisfactory results. In recent years, the research on the aspects of gastrointestinal motility disorders in UC patients has advanced, and therefore the abnormal intestinal dynamics have become a focus of research on the pathogenesis of UC [7-9].

The enteric nervous system (ENS), as an essential component of the peripheral nervous system (PNS),and is independent of the central nervous system (CNS), including the submucous plexus (SP) and myenteric plexus (MP) [10,11], and is involved in the regulation of intestinal secretion, absorption, motility [12]. Neurons in the ENS, depending on their neurotransmission function, are divided into sensory neurons, motor neurons, and interneurons. Further, motor neurons are divided into excitatory and inhibitory ones, regulating the systolic and diastolic function of the intestine, respectively. Typically, the ENS plays a vital role in maintaining gut homeostasis, however gut motility gets impaired once systolic, and diastolic functions of the gut become imbalanced.

As the primary inhibitory gas neurotransmitter within the ENS, nitric oxide (NO), regulates several gastrointestinal functions, such as vascular permeability, mucosal defense, immune regulation, and gastrointestinal motility [13]. Moreover, nitric oxide

synthase (NOS), the rate-limiting enzyme of NO synthesis, is widely distributed in endothelial cells, macrophages, neuro-phagocytes, and neuronal cells. Further NOS can be divided into neural NOS (nNOS) and endothelial NOS (eNOS), existing in normal, and injury-induced NOS (iNOS). Out of these, nNOS is located within the nervous tissue of both CNS and PNS. NO is one of the major inhibitory neurotransmitters of non-adrenergic noncholinergic (NANC) nerves in the ENS and its releasing by NOS-positive neurons play an essential role in stimulating gastrointestinal secretion and relaxing gastrointestinal smooth muscle. Changes in the NOS expression level causes alterations in gastrointestinal motility, however the impact of such changes in UC on colonic motility have not been clearly defined. Therefore, this study aimed to observe the changes of NOS within the colonic MP in dextran sulfate sodium (DSS)-induced UC rats, to further understand the underlying mechanisms of colonic motility in UC for delineating a new direction for the treatment of UC.

MATERIALS AND METHODS

Reagent

Dextran sulfate sodium (DSS) (PC-99017), NG-monomethyl-L-arginine monoacetate (L-NMMA) (PC-45273) and thrombin derived polypeptide 508 trifluoroacetic acid (TP508TFA) (PC-50991) were purchased from PlantChemMed Co., Ltd (Shanghai, China); Rabbit-anti-HuD + HuC (ab184267) and Goat-anti-nNOS (ab1376) were purchased from Abcam (Cambridge, United Kingdom); Donkey anti-Goat Alexa Fluor 488 (a11055) and Donkey anti-Rabbit Alexa Fluor 594 (A21207) were purchased from Invitrogen Co., Ltd (Carlsbad, United States of America); Anti-β-Actin antibody, Mouse monoclonal (A1978) and Immobilon Forte western HRP substrate (Cat.No.WBLUF0020) were purchased from Merck KGaA (Darmstadt, Germany); HRP-labeled Goat Anti-Rabbit IgG (H + L) (ZB-2306) and HRP-labeled Goat Anti-Mouse IgG (H + L) (ZB-2305) were purchased from ZhongShan GoldBridge Biotechnology Co., Ltd (Beijing, China); SDS-PAGE Gel Preparation Kit (P0012A) was purchased from Beyotime Biotechnology Co., Ltd (Shanghai, China); BCA Protein Concentration Assay Kit (AR0146) was

purchased from BOSTER Biological Technology Co., Ltd (Wuhan, China); NOS1/nNOS ELISA Kit (E-EL-R1438C) were purchased from Elabscience Biotechnology Co., Ltd (Wuhan, China). In addition, all other reagents and chemicals used in this study are commercially available.

Animals and Animal Tests

A total of 32 male, 8-week-old Sprague-Dawley rats with weights of 200 ± 20 g, were housed in an SPF grade animal house. The animals were kept at a standard room temperature of 24°C, with 40%-60% relative humidity, 12-hour light-dark alternation, and a standard laboratory diet containing 23% protein and water. All animals were provided by the Animal Center of the Fourth Military Medical University and divided into four major groups. All experimental procedures were conducted in accordance with the Principles of Laboratory Animal Care and approved by the University Ethics Committee and performed as per institutional guidelines. Efforts were made to minimize the number of animals used.

The rats were randomly divided into four groups (n = 8/each group), including the control (CG), UC (EG1), UC + NOS agonist TP508TFA (EG2), and UC + NOS inhibitor L-NMMA group (EG3). The CG group was housed as described above, whereas the animals in rest of the three experimental groups (EG1-3), were given tap water containing 5.5% DSS. The CG group was fed in the same way as the experimental groups except that DSS was not added in the tap water. All animals were given free access to water for 7 days, and the water was changed to tap water at day 15. Further, EG2 rats were treated with 3 mL of 0.01 mmol/L TP508 TFA i.g daily for 15 days and EG3 rats were treated with 3 mL of 0.01 mmol/L L-NMMA i.g daily for 15 days. Animals were regularly monitored for the general conditions, body weights, stool characteristics, occult blood, and hematochezia for the evaluation of the disease condition. The scoring criteria of the Disease Activity Index (DAI) were as follows [14]: Body weight: no loss, 0 point; loss by 1-5%, 1 point; loss by 6-10%, 2 points; loss by 11-15%, 3 points; loss by more than 15%, 4 points. Stool characteristics: normal (well-shaped), 0 points; bondless (mushy and semi-formed stool that does not adhere to the

anus), 2 points; loose (dilute watery stool that can adhere to the anus), 4 points. Fecal occult blood or macroscopic hematochezia: normal, 0 points; occult blood (+), 2 points; macroscopic hematochezia, 4 points. DAI score was calculated as equal to the average value of the sum of the above scores.

Rats in each group were individually subjected to the open field test on day 14 of the experiment. The rats were randomly placed into boxes with a height of 30-40 cm and a length of 100 cm on the bottom side. The box's inner walls were darkened, and the bottom surface was divided on the average 25 squares (4 cm × 4 cm) with a digital camera set 2 meters above each side. The data was acquired automatically and recorded for 15 min. This technique was used, to test the spontaneous activity of the animals and their anxious behavior in an open environment.

On the 15th day of the experiment, rats in each group were anesthetized with an intraperitoneal injection of 7% chloral hydrate (0.4 mL/100 g). Further, the whole colon was removed to compare the colon length for rats in each group.

Histological Evaluation of the Colon

Eight rats in each group were used to histological evaluation of the colon. Following the whole colon removal, the intestinal lumen was flushed using 0.01 M PBS buffer, and transected 6-9 cm from the anus. Colons were fixed in 4% paraformaldehyde, dehydrated in graded alcohols, and then embedded in the paraffin. The block was cut into 5 µm thick sections, hematoxylin-eosin stained. Afterwards the slides were mounted with neutral gum and dried at 37°C overnight. A whole slide was observed under the scanning biomicroscope (SLIDEVIEW VS200, Olympus, Tokyo, Japan). Histological changes were noted based on the staining results, and the histological index (HI) scoring was performed, with the following criteria [15]: 0 points for no damage; 1 points for basal 1/3 crypts disappearance; 2 points for basal 2/3 crypts disappearance; 3 points for crypts disappearance with intact epithelial cells; and 4 points for crypt and epithelial cell disappearance.

Immunofluorescence Histochemical Staining

The distal colons of 8 rats in each group were dissected separately. The intestinal lumens were flushed in 0.01 M PBS buffer, then a colonic strip with a width of approximately 0.5-1.0 cm long was cut along the travel direction of the circular muscle, afterwards the dissected colon was fixed in the 4% paraformaldehyde. The mucosal layer was fixed upward and horizontally in PBS buffer (pH = 7.4). The mucosal, submucosal, and circular muscle layers were removed with the help of filament forceps to preserve the longitudinal muscle layer. The tissues were blocked in the 10% donkey serum for 30 min and then incubated with Rabbit-anti-HuD + HuC (1: 500) and Goat-anti-nNOS (1: 300) in a shaker overnight at 4°C. Slides were incubated with Donkey anti-Goat Alexa Fluor 488 (1: 500) and Donkey anti-Rabbit Alexa Fluor 594 (1: 500) for 4 h. The specimens were observed under eight different fields (approximately 1.0 cm × 1.0 cm) of view using confocal microscopy (FV-1000, Olympus, Tokyo, Japan) with the appropriate laser beams and filter settings for Alexa 488 (excitation 488 nm; emission 510-530 nm) and Alexa 594 (excitation 543 nm; emission 590-615 nm). Digital images were captured with an FV10-ASW 4.2 from Olympus, these images were eventually saved as TIFF files to calculate changes in the proportion of colon nitrogen neurons.

Western Blotting

Four rats in each group were anesthetized and perfused pre-cooled PBS buffer, and the terminal colon was transected. The mucosa and submucosa were separated by silk tweezers. The muscle layer was put into a pre-cooled EP tube. The tissue was homogenized using an ultrasonic grinder, in the lysis buffer (RIPA: protease inhibitor: phosphatase inhibitors = 100: 1: 1). After standing for 10 min on ice, the supernatant was centrifuged at 12000 rpm (10008 × g) for 10 min. The protein concentration was measured using a BCA protein concentration assay kit and FC microplate reader (1410101, Thermo Fisher Scientific, Shanghai, China). The protein samples were kept at -80°Cfor further use.

Gels were made using the SDS-PAGE gel preparation kit and electrophoresed at a constant voltage of 80 and 120 V. The membrane transfer was achieved under a constant current of 300 mA. First, the PVDF membranes were blocked with western

blocking solution (P0252, Beyotime Biotechnology Co., Ltd., Shanghai, China) for 15 min and then incubated with Rabbit-anti-nNOS (1: 1000), and Anti-β-Actin antibody, Mouse monoclonal (1: 5000) on a shaker overnight at 4°C. The PVDF membranes were then incubated with HRP-labeled Rabbit anti-Goat IgG (H + L) (1: 10000) and HRP-labeled Goat anti-Mouse IgG (H + L) (1: 10000) for 2 h. After 10 min × 3 rinses in TBST, the membranes were probed by Immobilon™ Western chemiluminescent HRP substrate (WBKLS0050, Merck KGaA, Darmstadt, Germany) and placed into ECL for the detection. The proteins were analyzed using Image-Pro Plus software (Image-Pro Plus Version 6.0, Media Cybernetics, Maryland, United States of America).

ELISA

Whole blood from four rats in each group were kept for 2 h at room temperature and then centrifuged at 3800 rpm (1000 × g) for 20 min, following the removal of supernatant. Next, the terminal colon tissues were grounded and disrupted with an appropriate volume of PBS (usually a weight to volume ratio of 1: 9) homogenized using a glass homogenizer, and then centrifuged at 8460 rpm (5000 × g) for 5 to 10 min, afterwards the supernatant was collected. The NOS1/nNOS ELISA kit was used to determine the concentration of NOS in the colon as well as in the serum.

In-vitro Gut Colonic Tension Detection

Four rats in each group were used to explore the changes of *in-vitro* gut colonic tension, including 8 circular and 8 Longitudinal colon muscle strips. Rats were anesthetized, and the abdominal cavity was exposed, then the intestinal tube was carefully separated with the forceps and was quickly freed. The colon was excised and placed in 37°C Krebs' fluid with a continuous supply of 95% O_2 and 5% CO_2 mixture. Then 3 mm × 10 mm circular and longitudinal muscle strips were cut, where both ends were anchored to tension receptors and platinum rings at the lower end, respectively. The signals acquired by the tension receptors were recorded and processed by a multi-channel physiological signal acquisition and processing system (RM6240E, INSTRUMENT FACTORY, Chengdu, China). The mean amplitude of spontaneous contractions was recorded in circular and longitudinal muscle strips obtained from control and UC rats

at rest, when the muscle strips were allowed to rest in the incubation solution for 30 min. After 10 min of recording, TP508TFA (1×10^{-4} mol/L) was added to the bath of the UC group, and the mean amplitude changes of circular and longitudinal muscle strips were recorded, respectively. The liquid in the bath was replaced after 10 min, and the bath was washed. After resting for 30 min, L-NMMA (1×10^{-4} mol/L) was added, and the corresponding mean amplitude changes were re-recorded.

Statistical Analysis

SPSS version 23.0 statistical software (SPSS Inc, Chicago, United States of America) was used for statistical analysis. Data were expressed as mean ± standard deviation, and the comparisons between groups were performed by one-way ANOVA. A *p value* of < 0.05 was considered statistically significant.

RESULTS

DAI Scores

Sloth, anorexia, emaciation, decreased fur gloss, and higher stool frequency was found in all EGs rats, with some developing mucopurulent bloody stools. From day 0 to 5, animals in all groups gained weight, *viz,* CG group (28.90 ± 2.43 g), EG1 group (18.10 ± 3.23 g), EG2 group (17.00 ± 6.17 g), and EG3 group (21.80 ± 3.63 g). The body weights of rats in each group were not statistically significant ($P > 0.05$); however, the bodyweight of EG1-3 group began to decrease from day 6. Until day 15, CG group (116.60 ± 2.76 g) and EG2 group (58.60 ± 7.79 g) gained, however EG1 group (26.00 ± 3.69 g) and EG3 group (60.40 ± 3.99 g) lost weight. The decrease in body weight was statistically significantly different between EGs and CG ($P < 0.05$); Differences in weight change between EG2-3 and EG1 were also statistically significant ($P < 0.05$) (Figure 1 A). As for the stool profiles of all the groups, 2 rats in CG group exhibited bondless and loose stools without hematochezia. For EG1 group, 3.25 ± 0.31 days to the occurrence of loose stools, 3.37 ± 0.37 days to fecal occult blood, and 4.1 2 ± 0.39 days to macroscopic hematochezia. For EG2 group, 3.85 ± 0.50 days to loose stools, 4.00 ± 0.37 days to fecal occult blood, and 4.62 ± 0.41 days to macroscopic hematochezia. For EG3 group, 2.87 ±

0.29 days to loose stools, 2.62 ± 0.26 days to fecal occult blood, and 3.37 ± 0.32 days to macroscopic hematochezia. Regarding the DAI scores, no statistically significant differences was found from day 0 to day 5 for any group ($P > 0.05$). After day 5, the intergroup differences in DAI scores were significantly increased. The DAI scores were (0.12 ± 0.12), (3.15 ± 0.11), (2.67 ± 0.12), and (3.69 ± 0.15) for the CG, EG1, EG2 and EG3 group, respectively at day 15. Moreover, the scores were significantly different between EGs and CG ($P < 0.0001$) and were also significantly different between EG2-3 and EG1 ($P < 0.05$) (Figure 1 B).

Comparison of Colon Length and Behavior

The colonic morphology of rats from each group was compared during the dissection (Figure 2 A). Compared with CG, the colon length in EGs showed various degrees of shortening, and the differences were statistically significant ($P < 0.0001$). The difference was also significant between the EG2-3 and EG1 ($P < 0.05$) (Figure 2 B).

On the 14th day of the experiment, the behavior of rats was examined using an open field test. The number of times the rats passed through the center of an open box, within 15 min period is shown as follows: 30.75 ± 1.46 times for CG, 18.38 ± 0.98 for EG1, 23.38 ± 1.40 for EG2, and 9.50 ± 0.86 for EG3. Differences were statistically significant between the EGs and CG ($P < 0.0001$). Additionally, EG2-3 showed statistically significant differences compared with the EG1 ($P < 0.05$) (Figure 2 C, D).

Colonic histological damage assessment

The results of H&E staining indicated that the colonic tissue structure of CG rats was normal, with the mucosal layer showing a well-arranged monolayer of columnar epithelial cells, clear intestinal glands, morphologically normal goblet cells, and no inflammatory cell infiltration. In the colon of EG1 rats, most crypts disappeared, with some broken or disappeared epithelia, accompanied by inflammatory cell infiltration. In the colon of EG2 rats, 1/3 - 2/3 basal crypts were disappeared, with the occasional destruction of epithelial cells. In the colon of EG3 rats, crypts were completely disappeared, with some broken or disappeared epithelia, accompanied by massive inflammatory cell infiltration (Figure 3 A). The HI scores for the colons of the rats in the

CG, EG1, EG2 and EG3 group were- 0.00 ± 0.00, 3.07 ± 0.25, 2.22 ± 0.17, and 3.71 ± 0.14, respectively. All differences were statistically significant between EGs and CG ($P < 0.0001$), and EG2-3 and EG1 ($P < 0.05$) (Figure 3 B).

Changes in the Proportion of NOS in Colonic Myenteric Neurons

The distal rat colons were double-stained with immunofluorescence histochemistry to observe the distribution and expression of NOS-positive neurons within the colonic MP. Within the colonic MP of rats, the proportion of these neurons in each group were counted. Enteric neurons exhibited a reticular distribution in colonic MP (Figure 4 B). Usually, NOS-positive neurons accounted for 31.38 ± 0.94% in CG but were more distributed within the ganglia's marginal side (Figure 4 A). Their somas were fusiform or star-shaped, and their nuclei were round with the several elongated protrusions. The protrusions of these neurons were interconnected with each other to form a dense and complex neural network. In EG1, the % of NOS-positive neurons to colonic neurons increased to 42.25 ± 0.88% under disease conditions. The distribution of these neurons was no longer confined to the edges of the ganglia and began to appear elsewhere within the ganglia (Figure 4 D). Moreover, the proportion of NOS-positive neurons in EG2 increased to 51.75 ± 1.22%. Meanwhile, these neurons in the ganglia were disorganized and widely distributed within the ganglia (Figure 4 G). Compared with the EG1 group, the proportion of NOS-positive neurons in EG3 group decreased to 37.25 ± 1.09%, with their distribution within the ganglia being predominantly marginal (Figure 4 J). The changes in the proportion of NOS-positive neurons to colonic neurons in the EGs group were statistically significant as comparison to CG ($P< 0.0001$); however, EG2 and EG3 were also significantly different from the EG1 ($P < 0.05$) (Figure 4 M).

NOS Protein Expression Level of Colonic MP

The expression level of NOS protein in colonic MP of EG1 rats was higher than CG group. The NOS expression in the EG2 rat colon was further increased, while the expression in the EG3 rat colon was lower than the EG1. Differences were statistically

significant between EGs and CG ($P < 0.0001$) and between EG2-3 and EG1 ($P < 0.05$) (Figure 5 A, B).

Concentration of NOS in Colonic Myenteric Tissue and Serum

The concentration of NOS was significantly increased in the colonic myenteric tissue of EGs compared with CG ($P < 0.0001$); EG2 had a higher NOS concentration than EG1, whereas EG3 had a lower concentration than EG1, and the difference between EG2-3 and EG1 was also statistically significant ($P < 0.05$) (Figure 6 A). In addition, the serum concentrations of NOS were significantly increased in rats with EGs compared with CG ($P < 0.0001$); however, EG2 had a higher NOS concentration than EG1, whereas EG3 had a lower concentration than EG1. The observed differences were statistically significant ($P < 0.05$) (Figure 6 B).

Comparison of Contraction Tension of Isolated Rat Colonic Smooth Muscle

At rest, the contraction of the longitudinal muscle (LM) in the colon of CG rats appeared as a regular and sine wave-like curve with relatively neat amplitude (Figure 7 A). In EGs rats, the contraction was significantly more frequent, with the increased amplitude (Figure 7 B, C, D). The contraction tension of the longitudinal muscle of the colon was considerably more significant in the EGs than the CG rats ($P < 0.0001$). However, the contraction tensions of the colonic longitudinal muscle were significantly weakened and increased in the EG2 and EG3 rats when compared with EG1 rats ($P < 0.001$) (Figure 7 E).

At rest, the contraction of the colonic circular muscle (CM) in CG rats appeared as a regular and triangular wave-like curve. The amplitude was neat, with a contraction interval between the adjacent two waves (Figure 8 A). In EGs rats, contraction of the circular muscle of the colon was significantly accelerated, and its amplitude was increased (Figure 8 B, C, D). The contraction tension of the colonic circular muscle was significantly larger in the EGs than in the CG rats ($P < 0.0001$). The contraction tension of the colonic circular muscle appeared significantly weakened and increased in EG1 rats compared with EG2 and EG3 rats, respectively ($P < 0.0001$) (Figure 8 E).

DISCUSSION

An increase or decrease in the number of neurons and/or neuronal degeneration in the ENS can lead to various diseases. For instance, congenital dysplasia of ENS, leads to congenital megacolon (Hirschsprung disease) and primary achalasia, whereas neurodegenerative ENS alterations can lead to the disorders such as Alzheimer, Huntington, and Parkinson diseases [16,17]. The secondary alterations in the ENS also results in inflammatory infiltrates or immune system pathologies such as irritable bowel syndrome [18], Idiopathic enteric gangliosidosis [19], paraneoplastic syndrome [20], slow transit constipation [21], severe acute pancreatitis [22], diabetes mellitus [23,24], and UC [25].

The different types of ENS neurons have significantly different morphology and structure with the synthesis and secretion of neurotransmitters. However, by forming complex synaptic connections, these neurons participate in the structural basis, that mainly underlies the relatively independent reflex activity of the gut and can also regulate the diverse motor and sensory activities of the digestive tract.

Despite the unclear etiology of UC, numerous studies have shown that its pathogenesis might be associated with the interactions between genetic susceptibility, environmental provoking factors, and immune-mediated tissue damage. Additionally, the relationship between abnormal intestinal motility with UC has also gained attention. Abnormal ENS is an important mechanism contributing to the abnormal colonic motility of UC, which is an important factor for causing diarrhea in UC patients.

The onset of UC has a complex genetic background. Based on the gene polymorphism and heterogeneity, the impaired local barrier function of the intestinal mucosa, can be result of combination of factors, such as altered epithelial permeability, neuroendocrine regulation, and intestinal flora translocation [26]. This, in turn, provokes the inflammatory response with symptoms such as abdominal pain, diarrhea, and colonic motor dysfunction. As the rate-limiting enzyme of NO synthesis in the body, NOS has three sub-types: iNOS, eNOS and nNOS. Histological studies have identified intense focal iNOS expression by the inflamed bowel epithelium and in the mononuclear cell infiltrate in the intestinal tissues of both Crohn's disease and UC

patients [27]. A great number of studies suggest that iNOS in ENS may play a part in preventing activation of mast cells, reducing leukocyte adhesion to endothelium, and protecting the host from being invaded by colonic bacteria [28-30]. In normal and UC states, eNOS expression is limited to colonic vascular endothelium [31]. Baker *et al* [32]confirmed that during DSS induced UC, eNOS KO mice suffered less tissue damages and inflammations than wild-type mice, suggesting that eNOS is essential for maintaining the integrity of gastrointestinal mucosa. nNOS is one of the specific markers for the nitrergic neurons within the ENS, and the primary inhibitory neurons of the colonic MP [33]. By releasing inhibitory neurotransmitter NO, nitrergic neurons can regulate gastrointestinal motility. The changes in expression of nNOS in colonic MP of UC rats indicate that nitrergic neurons may be involved in NO-based neurotransmission and regulate gastrointestinal motility in UC state.

The current study has shown that NO gas is the second messenger in the smooth muscle cells (SMCs) or interstitial cells of Cajal (ICCs) [34]. NO being highly lipid-soluble, reaches target cell ICCs in a freely diffusible manner after synthesis. It binds to the soluble guanylyl cyclase (SGC) in the cells to increase the enzymatic activity by altering its spatial configuration, which further leads to an increase in cyclic guanosine monophosphate (cGMP) within the cells, activating the cGMP protein kinase-dependent calcium pumps, and therefore finally participates in intercellular information transmission [35]. Therefore, it could be concluded that as a messenger of information transmission between the NOS-positive neurons and gastrointestinal SMCs, increased NO can reduce the Ca^{2+} influx and directly promotes the smooth muscle relaxation. In addition, studies have been confirmed that NO can inhibit the muscle contraction by inhibiting the release of excitatory transmitters [23,36]. Therefore, NO's reduced contractility of intestinal smooth muscle might be one of the important mechanisms contributing to the colonic dysmotility [9,37].

In the present study, DSS was applied to induce the UC rat model successfully. For the first time, the findings of a secondary increase in NOS expression in colonic MP of UC rats, combined with altered *in vitro* colonic contraction tension, suggest that the

increased NOS expression is associated with the altered colonic motility in UC rats. Increasing (or decreasing) the number of NOS-positive neurons might enhance (or attenuate) the diastolic function of colonic smooth muscle regulated by these neurons. However, the change in the number of NOS-positive neurons often due to changes in the concentration and release of neurotransmitter caused by changes in the amount of NOS contained in neurons under pathological conditions, rather than caused by neuronal degeneration and regeneration. The results of both agonists and inhibitors of NOS were administered in *in-vitro* studies of the colon, separately, further confirmed that the altered NOS expression regulate the colonic motility in UC. A previous similar study demonstrated that the NOS expression and NO concentration within the muscular layer of the stomach and small intestine was increased in the animal models of chronic pancreatitis disease[38]. These results suggested that the reduced contractility of the gastric circular muscle because of NO inhibition might be important mechanisms underlying gastric motor dysfunction in chronic pancreatitis [36,38]. Moreover, these findings can provide an interesting insight into the role of the ENS during GI dysmotility.

The initiation of colonic dysmotility in UC may be related to the structural alterations and abnormal numbers of ICCs, disturbed intestinal electrophysiology, changes in colonic pressure, and abnormal expression of gut-related neurotransmitters. Our present results have demonstrated that the increased NOS expression inhibits contraction motility of colonic smooth muscle. Therefore, appropriate adjustment in NOS levels can alter the expression of nitrergic neurons, control the motor movement of intestinal smooth muscle, and improve the UC colonic motor function. Of note, this could improve the symptoms of UC patients, providing a basis for the screening of novel agents against UC.

CONCLUSION

The increased number of nitrergic neurons in the colonic myenteric plexus (MP) in the UC rats, both *in vitro* and *in vivo*, diminished the colonic motor function. In contrast,

activation and inhibition activities of the NOS could induce, diminish, and enhance the colon motor function, respectively. Further, an increased number of nitrergic neurons in the colonic MP of the UC rats leads to reduced colon contractile function. Therefore, the regulation of the role of nitrergic neurons in the colonic MP through interference with the activities of NOS might be a novel potential and prospective way to reduce the diarrhea symptom in the UC patient.

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