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Folic acid attenuates chronic visceral pain by reducing clostridiales abundance and hydrogen sulfide production

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Abstract

Irritable bowel syndrome (IBS) related chronic visceral pain affects 20% of people worldwide. The treatment options are very limited. Although the scholarly reviews have appraised the potential effects of the intestinal microbiota on intestinal motility and sensation, the exact mechanism of intestinal microbiota in IBS-like chronic visceral pain remains largely unclear. The purpose of this study is to investigate whether Folic Acid (FA) attenuated visceral pain and its possible mechanisms. Chronic visceral hyperalgesia was induced in rats by neonatal colonic inflammation (NCI). 16S rDNA analysis of fecal samples from human subjects and rats was performed. Patch clamp recording was used to determine synaptic transmission of colonic-related spinal dorsal horn. Alpha diversity of intestinal flora was increased in patients with IBS, as well as the obviously increased abundance of *Clostridiales* order (a main bacteria producing hydrogen sulfide). The hydrogen sulfide content was positive correlation with visceral pain score in patients with IBS. Consistently, NCI increased *Clostridiales* frequency and hydrogen sulfide content in feces of adult rats. Notably, the concentration of FA was markedly decreased in peripheral blood of IBS patients compared with non-IBS human subjects. FA supplement alleviated chronic visceral pain and normalized the *Clostridiales* frequency in NCI rats. In addition, FA supplement significantly reduced the frequency of sEPSCs of neurons in the spinal dorsal horn of NCI rats. Folic Acid treatment attenuated chronic visceral pain of NCI rats through reducing hydrogen sulfide production from *Clostridiales* in intestine.

Keywords

Chronic visceral pain, folic acid, hydrogen sulfide, *clostridiales*, intestinal flora

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Introduction

Irritable bowel syndrome (IBS) affects ~15% of people worldwide and is one of the most common functional bowel disorders. The condition is characterized by abdominal pain, in association with defecation or a change in bowel habit.[1,2] Emerging evidence showed that IBS, a hitherto enigmatic disorder thought to be predominantly related to psychological factors, has a microorganic basis in a subset of patients with IBS.[3] In the past few years, a microorganic basis for IBS, including small intestinal bacterial overgrowth (SIBO), post-infectious aetiology, altered gut permeability, immune activation and dietary factors is being understood.[4] Scholarly

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reviews have appraised the potential effects of the intestinal microbiota on intestinal motility and sensation, autonomic nervous system, hypothalamic-pituitary-adrenal axis, mucosal barrier and neuroimmune signaling.[5]

The role of gut microbes in health and disease has often been surmised from stool, which is easily sampled and rich in microbial diversity, density, and abundance. Faecal microflora analysis has been accepted as a measure to determine the relationship between intestinal microbiota and host health.[6,7] Studies suggested that fundamental differences in the composition of the human microbiome may be associated with the IBS disease phenotype in adults.[8] However, more information is needed about how the human microbiome contributes to the constellation of symptoms in IBS. In the animal model of folic acid (FA) deficiency, Zhang et al.[9] showed that intestinal flora was imbalanced in FA deficiency in rats and that intestinal microorganisms and FA level in rat serum were returned to normal after folic acid supplementation. In addition, FA treatment can affect the structural distribution of mammalian intestinal flora and the concentration of short-chain fatty acids to promote intestinal microbial balance.[10] However, whether FA can alleviate visceral pian is still unclear and needs to be further studied.

In the present study, we speculated that supplementation of folic acid may attenuate visceral pain by regulating intestinal microbial balance. We showed that FA supplement alleviated visceral pain and normalized the *Clostridiales* frequency in the gut microbial, and significantly reduced the frequency of sEPSCs of neurons in the spinal dorsal horn of NCI rats. Our findings might shed light on new thoughts and potential targets for the therapy of chronic visceral pain in patients with IBS.

Methods and materials

Induction of chronic visceral hyperalgesia in rats

Experiments were performed on male Sprague-Dawley (SD) rats (180 ± 20 g). Care and handling of these animals were approved by the Institutional Animal Care and Use Committee of the Soochow University and were in accordance with the guidelines of the International Association for the Study of Pain. Chronic visceral hyperalgesia was induced in rats by neonatal colonic inflammation (NCI).[11–13] Briefly, 10-day-old pups received an infusion of 200 μL of 0.5% acetic acid solution in saline into the colon (2 cm from the anus). Controls received an equal volume of normal saline (NS). Chronic visceral hyperalgesia was measured by colorectal distention (CRD) threshold. Experiments were performed in these rats at 6 weeks of age.

Human subjects

Twenty-seven IBS patients who met the Rome IV criteria and 27 healthy volunteers at the same time were recruited. Exclusion criteria: patients who had taken antibiotics, pregnant, immune diseases, surgical trauma, severe mental illness, alcohol abuse, intestinal vascular diseases, gastrointestinal prokinetic drugs, or enteroscopy showed organic lesions of intestinal mucosa and severe heart, lung and kidney diseases within 8 weeks before entering the group. One hundred milligrams of stool were diluted in 0.8 mL of phosphate buffered saline (PBS) and homogenized using a pellet pestle (Sigma-Aldrich, stokes Louis, Missouri, USA). After centrifuging at 5000 g at 4°C for 10 min, supernatants were filtered with 0.22 μm Spin-X tube filters (Corning Life Sciences, Durham, North Carolina, USA). Then previous step was repeated. Fecal supernatants were freshly made prior to each experiment.[14] The Ethics Committee of the People's Hospital of Suzhou New District approved the study and the informed consent was given.

Visceral pain score in IBS patients

The gastrointestinal symptom rating scale (GSRS) is a standard for assessing visceral pain in patients with IBS.[15] Abdominal pain is graded according to its degree, frequency, duration, relieving factors and influence of social activities (Table 1).

16S rDNA analysis of fecal samples

The 16S rRNA analysis of the fecal pellets was performed by OE Biotech Co., Ltd (Shanghai, China). DNA extraction was performed using QIAamp 96 PowerFecal QIAcube HT kit (QIAGEN, Duesseldorf, Germany) following the manufacturer's instructions. Genomic DNA was then amplified in 50-μL triplicate reactions with primers specific to the V3-V4 region of the bacterial 16S rRNA gene: 343F (5′-TACGGRAGGCAGCAG-3′) and 798R (5′-AGGGTATCTAATCCT-3′). The reverse primer contained a sample barcode, and both primers were connected with an illumina sequencing adapter (Illumina Company, San Digeo, CA). The PCR products were purified, and the concentrations were adjusted for sequencing on an Illumina Miseq PE300 platform.

Raw sequencing data were converted to the FASTQ format. The resulting paired-end reads were then preprocessed using Trimmomatic software to detect and cut off ambiguous bases. After trimming, the reads were subsequently assembled by FLASH software. Sequences were

Table 1. Gastrointestinal symptom scale IBS abdominal pain item.

Degree of abdominal pain	Score
No pain or temporary pain	0
Occasional pain, affecting partial social activities	1
Chronic pain, affecting many social activities	2
Severe pain, affecting all social activities	3

conducted further denoising by the use of QIIME software (version 1.8.0). Next, clean reads were subjected to primer sequences removal and clustering to generate operational taxonomic units (OTUs) using VSearch software at a similarity threshold of 97%. The representative read of each OTU was selected using the QIIME package. All representative reads were annotated and blasted against Silva database (version 123) using RDP classifier (confidence threshold was 70%).

Patch clamp recording on spinal cord slices

Rats were deeply anaesthetized with pentobarbital sodium. After removing of vertebral plate, the dorsal aspect of the vertebral column was exposed. The T13-L2 lumbar region of the spinal cord was removed and immersed in ice-cold sucrose-based artificial cerebral spinal fluid (SACSF) saturated with 95% O_2/5% CO_2 (carbogen). The SACSF contained (in mM): 50 sucrose, 95 NaCl, 1.8 KCl, 1.2 NaH_2PO4, seven $MgSO_4$, 0.5 $CaCl_2$, 26 $NaHCO_3$ and 15 Glucose. The colon-related part of the spinal cord was exposed and carefully removed with T13-L2 segments dorsal roots attached. Spinal cord was rapidly removed and embeded with 3% high strength agarose (type I-B, Sigma, USA). Spinal cord slices (450 μm) were cut with vibroslicer VT1200 S (LEICA, Germany) and transferred to 31°C SACSF solution to recover for 30 min and kept for the next 4–5 h. Spinal cord slices were then transferred to the recording chamber and continuously perfused with recording ACSF with the following composition (in mM): 127 NaCl, 1.8 KCl, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, and 15 Glucose. The flow rate of perfusion is about 2 mL/min. Neurons in the lamina II of spinal dorsal horn were selected for recording. Each new experimental protocol used a fresh slice.

Neurons used for recording in lamina II of spinal dorsal horn were visualized using infrared differential interference contrast (IR-DIC) video microscopy with a ×40 magnification water-immersion objective (B×51WI, Olympus, Shinjuku-ku, Tokyo, Japan). Patch electrodes (4–8 MΩ tip resistance) were made using a Flaming/Brown P-97 micropipette puller (Sutter Instruments Co.), from borosilicate glass capillaries. The internal solution of the electrodes for recording spontaneous excitatory post-synaptic currents (sEPSCs) and action potentials (APs) contained (in mM): 140 K-Gluconate, four NaCl, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Na-GTP, pH adjusted to 7.2–7.3 with KOH. After giga ohm (GΩ) seals (usually >4 GΩ) were formed and the whole-cell configuration was obtained, neurons were tested if the resting membrane potential was more negative than −50 mV and direct depolarizing current injections evoked APs overshooting 0 mV. We only included data of excitatory neurons according to the electrophysiological characteristics described by Washburn and Moises[16] in response to intracellular injection of a depolarizing current (100–300 pA, step 50 pA, duration 1000 ms) in the further analyses of sEPSCs. The holding potentials were −70 mV for recording sEPSCs. All drugs were dissolved in recording ACSF on the day of experiment and incubated by perfusion.

Data were acquired using a Digida 1440A interface, MultiClamp 700B amplifier and pClamp10 software. Data were sampled and filtered at 10 kHz with Bessel filter of amplifier. To ensure high-quality recordings, series resistance (<20 MΩ) was checked using membrane test function of pClamp10 software throughout the experiment. Data were stored on a computer and analyzed offline.

Folic acid measurement

Venous blood (3 mL) was collected from healthy donors and IBS patients in the early morning after fasting overnight. The blood samples were centrifuged. The serum was collected and detected in strict accordance with the laboratory operating rules to determine the concentration of FA in the serum of the two groups. The measuring instrument was Roche Cobase602 automatic electrochemiluminescence analyzer (Roche, Germany), and the reagents and calibrators were produced by Roche Diagnostic products (Shanghai Co., Ltd)

Hydrogen sulfide measurement in feces of IBS patients and NCI rats

Homogenized slurries were prepared from freshly-passed human or rat feces. After centrifuge for 10 min with 200 gravities, the upper suspension was taken. Two hundred and 50 μL of zinc acetate (1% wt/vol in water; Wuxi Co., Ltd) was added into fecal supernatant (200 μL) in Eppendorf vials followed by addition of *N*,N-dimethyl-p-phenylenediamine sulphate (133 μL, 20 mmol/l in 7.2 mol/l HCl; Shanghai Co.,Ltd) and $FeCl_3$ (133 μL, 30 mmol/l in 1.2 mol/l HCl; Shanghai Co., Ltd). After centrifuged at 5000 g for 10 min, the absorbance of the supernatant fraction at 670 nm was recorded according to the standard curve of H_2S.[17,18]

FA treatment in NCI rats

Three doses of FA (10, 80 or 150 μg/kg, Sigma-Aldrich, F8758, Pteroyl-L-glutamic acid) were used for intraperitoneal (i.p.) injection of rats. A dose of 80 μg/kg were used daily for a consecutive 7 days. Pain threshold was determined immediately after a single injection or the seventh injection.

Statistical analyses

All values are showed as mean ± standard error. Statistical analyses were done using Prism 7 (Graph Pad, San Diego, California, USA) and OriginPro 8 (OriginLab, Northampton, MA) software. Gaussian distribution test was conducted before analysis. Two sample t-test or Mann-Whitney test was used to determine the significance of changes between two

groups. Two-way ANOVA followed by Tukey's post hoc test or one-way ANOVA analyses followed by Dunnett test was performed when appropriate. $p < 0.05$ was considered statistically significant.

Results

Alpha diversity of intestinal flora is increased in feces of IBS patients

16S rDNA gene sequencing was used to detect alterations in the gut microbiota composition between healthy donors (HD) and IBS patients (IBS). As depicted in Figure 1(a), the gut microbiota compositions of IBS patients ($n = 11$) were very different from that of the healthy donors ($n = 11$). Venn chart showed that IBS patients contained 3873 Operational Taxonomic Units (OTUs) in common with healthy volunteers, while alone possessed 3287 OTUs. HD alone possessed 1618 OTUs. Additionally, α-diversity of microbiota means the diversity of bacteria or species within a community or habitat. The ACE index, Chao one index, Richness index, Shannon index and Simpson index are commonly used indicators in evaluating the α-diversity. Our analysis indicated that IBS patients significantly increased the ACE index (Figure 1(b), $^{**}p < 0.01$, $n = 11$ for HD, $n = 11$ for IBS) and Chao one index (Figure 1(c), $^{*}p < 0.05$, $n = 11$ for HD, $n = 11$ for IBS) compared with those in the healthy volunteer donors. Although the Richness index (Figure 1(d), $p > 0.05$) and Shannon index (Figure 1(e), $p > 0.05$) were increased in IBS group ($n = 11$) compared with those in HD group ($n = 11$), but they had no statistical significance. The Simpson index (Figure 1(f), $p > 0.05$) was decreased in IBS patients ($n = 11$) compared with those in HD ($n = 11$), but it also had no statistical significance. These results suggested the alterations in the gut microbiota composition in patients with IBS.

Abundance of Clostridiales *order is increased in feces of IBS patients*

Next, we explored the relative abundance percent of intestinal flora composition at order levels. As shown in Figure 2(a), *Clostridiales* was the main flora in both HD and IBS groups. The frequency of *Clostridiales* was markedly increased in IBS group ($n = 11$) when compared with HD group ($n = 11$).

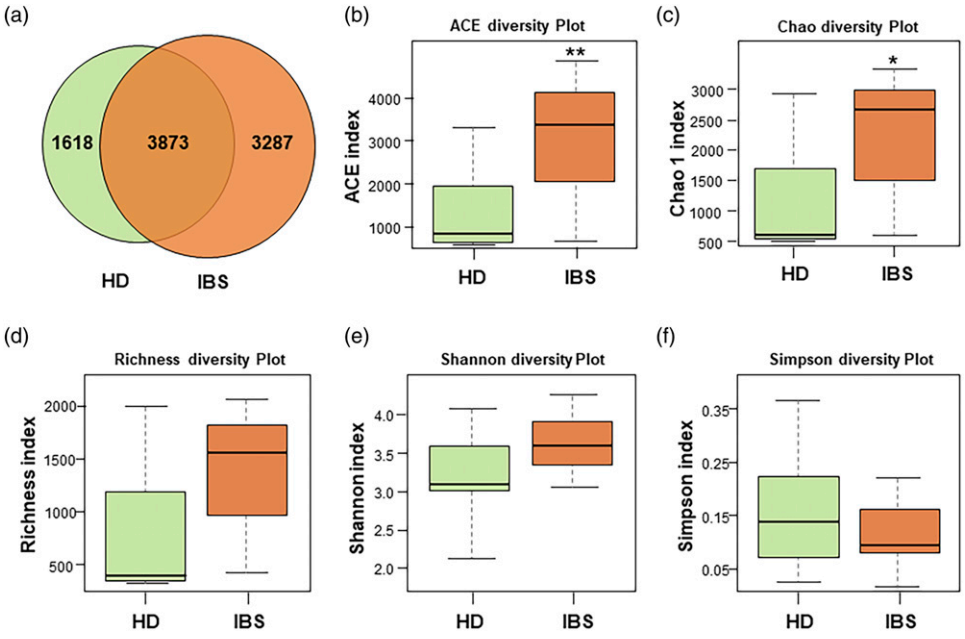

Figure 1. The diversity of intestinal flora was increased in IBS patients. (a) Compared with the Venn diagram of gut microbiota at the OTU level between IBS patients (IBS) and healthy donors (HD), there were more unique operational classification units in IBS than in HD ($n = 11$ for each group). (b) ACE index of microbial diversity was estimated, and the ACE value was proportional to the microbial diversity. Compared with HD, the ACE index of IBS was higher, which indicated that the diversity of microflora was high ($n = 11$ for each group, $^{**}p < 0.01$). (c) Chao one index of microbial diversity was estimated, and the Chao one value was proportional to the microbial diversity. Compared with HD, the Chao one index of IBS was higher, which indicated that the diversity of microflora was high ($n = 11$ for each group, $^{*}p < 0.05$). (d) Richness index of microbial diversity was estimated, and the Richness value was proportional to the microbial diversity. Compared with HD, the Richness index of IBS was higher, which indicated that the diversity of microflora was high ($n = 11$ for each group, $p > 0.05$, no statistical significance). (e) Shannon index of microbial diversity was estimated, and the Shannon value was proportional to the microbial diversity. Compared with HD, the Shannon index of IBS was higher, but it did not show statistical significance ($n = 11$ for each group, $p > 0.05$). (f) Simpson index of microbial diversity was estimated, and the Simpson value was negatively correlated with the microbial diversity. Compared with HD, the Simpson index of IBS was lower, but it did not show statistical significance ($n = 11$ for each group, $p > 0.05$).

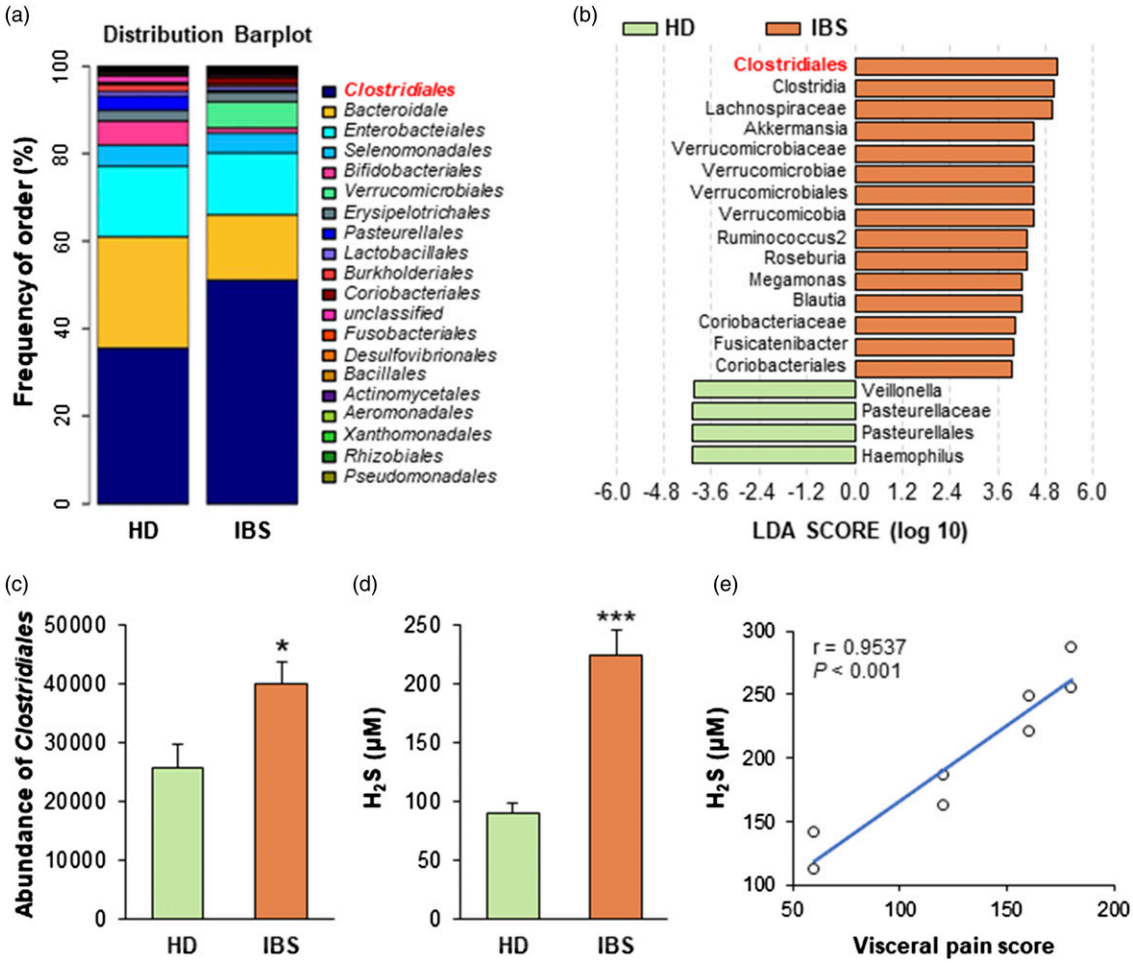

Figure 2. Abundance of *Clostridiales* and the concentration of H_2S in feces were increased in IBS patients. (a) The transverse axis is HD and IBS, and the longitudinal axis is the relative abundance ratio. The color corresponds to the name of each flora under this taxonomic level, and the width of different color blocks indicates the relative abundance ratio of different microflora. Compared with HD, the frequency of *Clostridiales* in feces of IBS increased (n = 11 for each group). (b) Compared with HD, Linear discriminant analysis Effect Size (LEfSe, LDA score >3.0) confirmed that there were more *Clostridiales* of IBS (n = 11 for each group). (c) PERMANOVA assay revealed enhancement of the frequency of *Clostridiales* in IBS compared with HD (n = 11 for each group, *p < 0.05). (d) Compared with HD, the concentration of H_2S in feces of IBS significantly increased (n = 6 for each group, ***p < 0.001). (e) Pearson correlation was used to analyze the correlation between the concentration of H_2S in feces and the visceral pain score of IBS patients. The results showed that there was a positive correlation between the concentration of H_2S in feces and the symptom score of IBS patients (n = 6 for each group, r = 0.9537, ***p < 0.001).

The same result was confirmed by Linear discriminant analysis Effect Size (LEfSe, LDA score >3.0, Figure 2(b)) and PERMANOVA assay (Figure 2(c), *p < 0.05, n = 11 for HD, n = 11 for IBS). Since *Clostridiales* is one of the main florae producing hydrogen sulfide (H_2S),[19–21] we, therefore, measured the concentration of H_2S in feces of both groups. As expected, IBS group produced much more H_2S than HD group (Figure 2(d), ***p < 0.001, n = 6 for HD, n = 6 for IBS). Importantly, correlation analysis showed that the concentration of H_2S was positively correlated with visceral pain scores of IBS patients (Figure 2(e), ***p < 0.001, n = 6 for HD, n = 6 for IBS). The above data indicate that H_2S-producing *Clostridiales* may be involved in the pathological process of visceral pain in patients with IBS.

NCI increases Clostridiales *frequency and* H_2S *concentration in rat feces*

To further investigate the role of *Clostridiales* and H_2S on chronic visceral pain related to IBS, we studied the flora composition in a known rat model of chronic visceral pain induced by neonatal colonic inflammation (NCI). As depicted in Figure 3(a), the gut microbiota compositions of NCI rats (n = 12) were different from that of CON rats (n = 12). Venn chart showed that NCI rats contained 262 Operational Taxonomic Units (OTUs) in common with CON rats, while alone possessed 369 OTUs. CON rats alone possessed 356 OTUs. The α-diversity was not altered (Figure 3(b)–(d), p > 0.05, n = 12 for CON, n = 12 for NCI). As shown in Figure 3(e), the

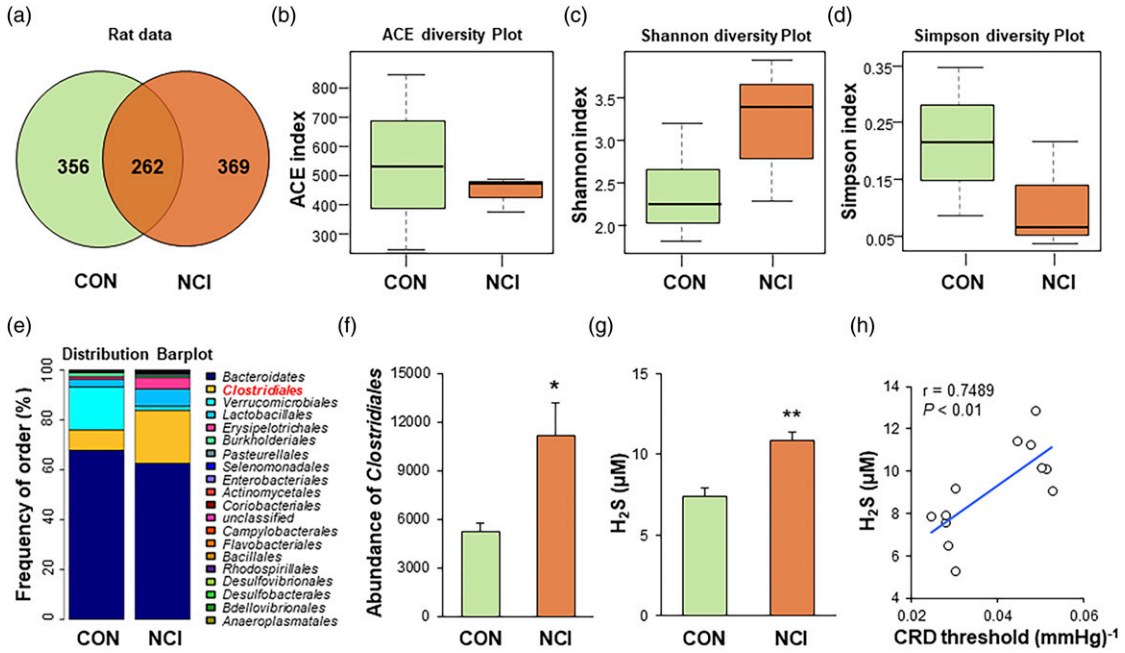

Figure 3. Abundance of *Clostridiales* and the concentration of H_2S in feces were increased in NCI rats. (a) Compared with the Venn diagram of gut microbiota at the OTU level between NCI and CON rats, there were more unique operational classification units in NCI than in CON rats (n = 12 for each group). (b) ACE had no statistical significance (n = 12 for each group, $p > 0.05$). (c) Shannon had no statistical significance (n = 12 for each group, $p > 0.05$). (d) Simpson had no statistical significance (n = 12 for each group, $p > 0.05$). (e) The transverse axis is CON and NCI rats, and the longitudinal axis is the relative abundance ratio. The color corresponds to the name of each flora under this taxonomic level, and the width of different color blocks indicates the relative abundance ratio of different microflora. Compared with CON rats, the frequency of *Clostridiales* in feces of NCI rats increased (n = 12 for each group). (f) PERMANOVA assay revealed enhancement of the frequency of *Clostridiales* in NCI rats compared with controls (n = 12 for each group, $^*p < 0.05$). (g) Compared with CON rats, the concentration of H_2S in feces of NCI rats significantly increased (n = 6 for each group, $^{**}p < 0.01$). (h) There was a significant negative correlation between pain threshold and H_2S concentration in NCI rats (n = 6 for each group, r = 0.7489, $^{**}p < 0.01$).

frequency of *Clostridiales* was markedly increased in NCI group ($n = 12$) when compared with CON group ($n = 12$). The frequency of *Clostridiales* was increased in feces of NCI rats by PERMANOVA assay when compared with CON rats (Figure 3(f), $^*p < 0.05$, n = 12 for CON, n = 12 for NCI). These results were consistent with the clinic results of the IBS patients. Additionally, the concentration of H_2S was remarkedly elevated in feces of NCI rats when compared with control ones (Figure 3(g), $^{**}p < 0.01$, $n = 6$ for CON, $n = 6$ for NCI). Furthermore, the correlation analysis demonstrated the significant negative correlation between H_2S concentration and pain threshold of NCI rats (Figure 3(h), $^{**}p < 0.01$, $n = 6$ for CON, n = 6 for NCI). These data indicate that H_2S-producing *Clostridiales* may be involved in the pathological process of visceral pain in NCI rats.

Folic acid treatment decreases Clostridiales *frequency of NCI rats*

Since folic acid (FA) was reported to attenuate neuropathic pain in animal models,[22] we next investigated the role of FA on intestinal flora composition and visceral pain of NCI rats and IBS patients. As depicted in Figure 4(a), the concentration of FA in serum of IBS patients was significantly reduced when compared with health donors ($^{***}p < 0.001$, n = 10 for HD, n = 10 for IBS). Then we explored the effects of FA treatment. 16S rDNA gene sequencing indicated that FA treatment of NCI rats slightly reduced the OTU number (Figure 4(b)) but did not affect the α-diversity (Figure 4(c)–(e), $p > 0.05$, $n = 3$ for each group). As shown in Figure 4(f), the frequency of *Clostridiales* in NCI rats after FA treatment was markedly decreased (n = 3 for each group). This was further confirmed by PERMANOVA assay showing that *Clostridium* decreased significantly after FA treatment (Figure 4(g), $^*p < 0.05$, n = 3 for each group).

FA treatment alleviates visceral pain of NCI rats

Next, we investigated the effect of FA on H_2S concentration and visceral pain of NCI rats. After intraperitoneal injection of FA (80 μg/kg),[22,23] the concentration of H_2S in feces was decreased significantly in NCI rats (Figure 4(h), $^{***}p < 0.001$, n = 7). FA treatment also substantially increased the pain threshold in a dose-dependent manner. The analgesia effect was occurred from 0.5 h to at least 2 h after application of FA (80 or 150 μg/kg) (Figure 4(i)). Further, the effect lasts

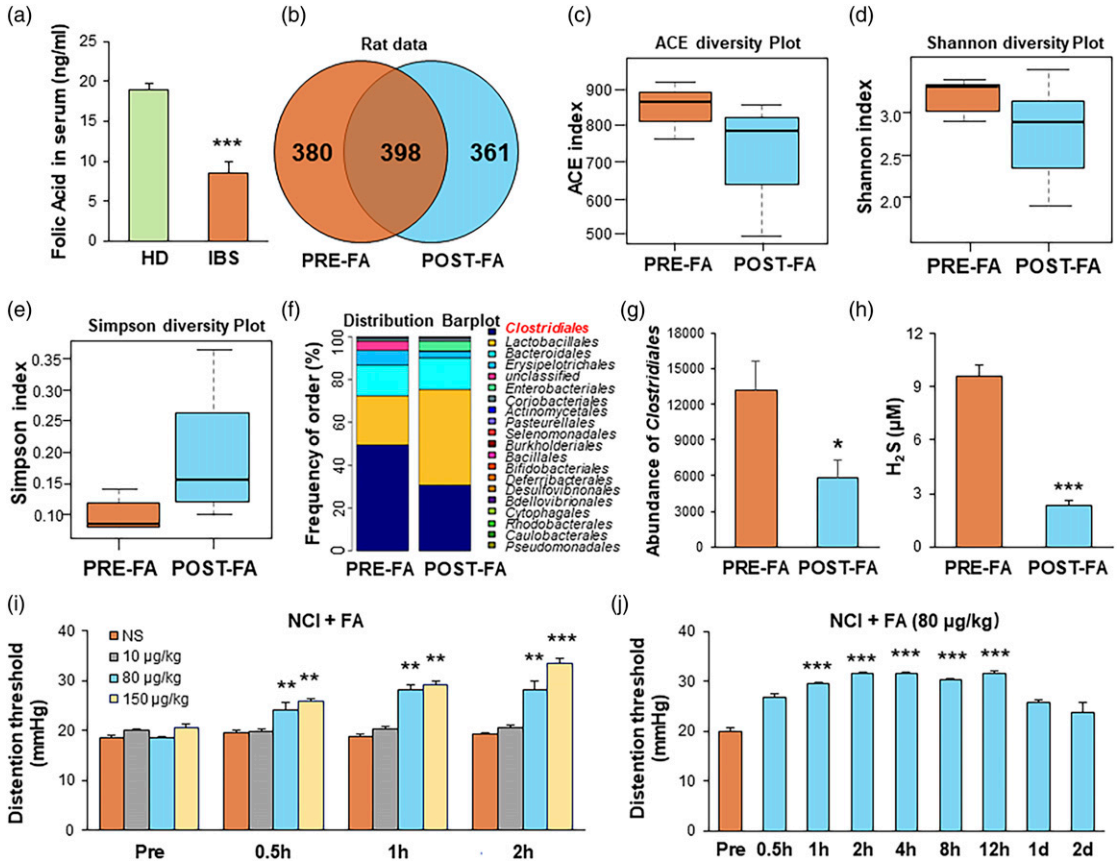

Figure 4. Folic acid (FA) treatment decreased the *Clostridiales* frequency of IBS patients and alleviated visceral pain of NCI rats. (a) Compared with HD, concentration of FA in serum was significantly reduced in IBS patients ($n = 10$ for each group, $^{***}p < 0.001$). (b) Compared with the Venn diagram of gut microbiota at the OTU level between NCI and FA treatment of NCI rats, there were more unique operational classification units in NCI than in FA treatment of NCI rats ($n = 3$ for each group). (c) ACE was no statistical significance ($n = 3$ for each group, $p > 0.05$). (D) Shannon had no statistical significance ($n = 3$ for each group, $p > 0.05$). (e) Simpson had no statistical significance ($n = 3$ for each group, $p > 0.05$). (f) The transverse axis is NCI rats pre and after FA injection, and the longitudinal axis is the relative abundance ratio. The color corresponds to the name of each flora under this taxonomic level, and the width of different color blocks indicates the relative abundance ratio of different microflora. Compared with pre, the frequency of *Clostridiales* in feces of NCI rats treated with FA decreased ($n = 3$ for each group). (g) FA injection slightly suppressed the abundance of *Clostridiales* in feces compared with pre group ($n = 3$ for each group, $^{*}p < 0.05$, PERMANOVA assay). (h) FA injection obviously suppressed the concentration of H_2S in feces compared with pre group ($n = 7$ for each group, $^{***}p < 0.001$). (i) The CRD threshold of NCI rats was significantly increased by 0.5 h after intraperitoneal injection of FA (10, 80 or 150 μg/kg) ($n = 11$ per group, $^{**}p < 0.01$, $^{***}p < 0.001$ vs NS, two-way ANOVA followed by Tukey's post hoc test). (j) The antinociceptive effect of FA persisted for 12h after the final injection of a 7-days daily series ($n = 11$ per each, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Pre, one-way ANOVA followed by Dunnett's test).

to 12 h after a daily intraperitoneal injection for a consecutive 7 days (Figure 4(j)). These results suggested that FA treatment had a great effect to attenuate chronic visceral pain of NCI rats.

FA treatment suppresses spinal synaptic transmission

Since our previous study showed that the AMPA receptor-mediated glutamatergic synaptic activity was significantly enhanced in SG of NCI rats,[24] we next study the effect of FA on spontaneous excitatory post-synaptic currents (sEPSCs) of neurons at spinal dorsal horn of NCI rats. As shown in Figure 5(a), the representative traces of sEPSCs were recorded from control (CON) and NCI rats, respectively. Compared with CON rats, the frequency of sEPSCs in NCI rats was significantly increased although the amplitude was not altered ($^{*}p < 0.05$, $n = 10$ cells for CON and $n = 9$ cells for NCI). This is consistent with our previous report.[24] Figure 5(b) was the representative traces of synaptic transmission in the spinal dorsal horn after a consecutive 7-days injection of FA or normal saline (NS). Compared with the NS group, the frequency of sEPSCs in the FA group was significantly decreased although the amplitude was not changed ($^{*}p < 0.05$, $n = 12$ cells for NS and 10 for FA). These data suggested that FA application reduced the spontaneous glutamatergic synaptic activity of SG neurons in NCI rats.

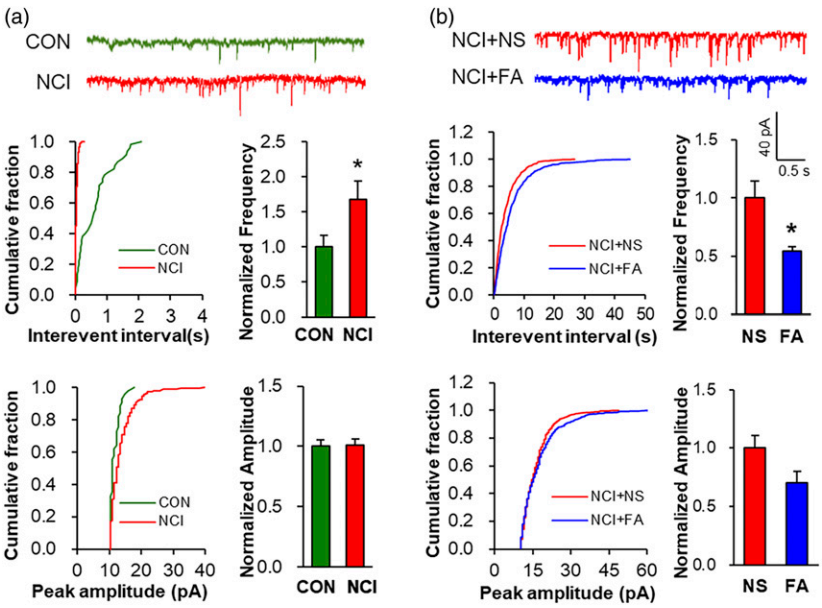

Figure 5. FA treatment reduced the frequency of spontaneous excitatory postsynaptic currents in the spinal cord of NCI rats. (a) Representative traces of sEPSCs in spinal dorsal horn neurons. Compared with CON rats, the frequency of sEPSCs in spinal dorsal horn neurons of NCI rats was significantly increased, but the amplitude was not altered (n = 10 cells for CON and n = 9 cells for NCI, *p < 0.05). (b) Representative traces of synaptic transmission in the spinal dorsal horn after injection of FA or normal saline (NS). Compared with the NCI + NS group, the frequency of sEPSCs in spinal dorsal horn neurons of the NCI + FA group was dramatically decreased, but the amplitude was not changed (n = 12 cells for NS and 10 for FA, *p < 0.05).

Discussion

Irritable bowel syndrome (IBS), is one of the common diseases of gastrointestinal dysfunction in clinical practice, characterized by abdominal discomfort or pain with which the pathogenesis is not clear.[25,26] In the present study, we showed that H_2S concentration increased in feces of both IBS patients and NCI rats and that H_2S concentration was correlated very well with visceral pain of both IBS patients and the NCI rats. Importantly, the abundance of *Clostridiales* order, a main bacterium producing H_2S, was obviously increased in both IBS patients and the NCI rats. Together with our previous studies that NCI enhanced expression of H_2S-producing enzyme CBS in dorsal root ganglion and that inhibition of CBS attenuated visceral pain of NCI rats,[24] we provided additional evidence to confirm that H_2S is involved in the development and maintenance of chronic visceral pain.

Human intestinal microorganisms are composed of about 1000–1150 species of bacteria, which play an important role in intestinal development and nutritional absorption.[27] The intestinal microorganisms also play a very important role in digestion, immunity, pathogen resistance, regulation of a variety of metabolic pathways and the production and secretion of neurotransmitters and hormones.[28,29] When the number and composition of bacteria change, it is considered to be intestinal flora imbalance.[30,31] And it has been reported that the abnormal distribution of intestinal flora in patients with IBS has an impact on the brain-gut axis, which can affect visceral sensitivity, neuroimmune signal transduction, intestinal motility, intestinal mucosal barrier permeability and hypothalamus-pituitary-adrenal axis dysfunction.[32–34] In the present study, 16S rDNA gene sequencing was used in the faeces of healthy humans and IBS patients. Although the α analysis was used to analyze the change in flora between two groups, the β analysis, which was not analyzed in the present study, is also an important index of gut microbiota.

In order to better understand the potential effects of intestinal microorganisms on visceral pain in IBS, people need to understand not only the bacteria in feces, but also bacterial metabolites such as short-chain fatty acid and H_2S because these metabolites can not only regulate mucosal epithelial cell renewal, repair intestinal barrier function, and prevent bacteria and their metabolites from entering the bloodstream as well. It can also regulate immunity, metabolism and the function of the central nervous system.[35] After discovering a variety of physiological functions of carbon monoxide and nitric oxide, researchers have also demonstrated that H_2S plays important roles in gastrointestinal, nervous, cardiovascular, respiratory, kidney, liver and other systems, especially in the pathogenesis of digestive tract disorders such as IBS.[36]

Our previous studies showed that neonatal colorectal acetic acid treatment led to visceral pain hypersensitivity in adult rats by regulation of multiple ion channels leading to peripheral pain sensitization.[24] We speculate that intestinal microorganisms and their metabolites (such as H_2S) may be

the "mastermind" of affecting gut-brain axis function and inducing chronic visceral pain. In this experiment, the frequency of *Clostridiales* in intestinal flora of NCI rats was significantly higher than that in the normal group. With the same research method, it was found that the frequency of *Clostridiales* in intestinal flora of IBS patients was significantly higher than that of healthy volunteers (Normal), and the concentration of H_2S in feces was significantly higher than that of Normal. After further analysis, we showed that there was a positive correlation between the concentration of H_2S in feces and the IBS scores of IBS patients. In fact, *Clostridiales* convert cysteine to H_2S by cysteine desulfhydrase activity. According to the above experimental results and literature reports,[37] we speculate that due to the disorder of intestinal flora, the change of the frequency of *Clostridiales* producing H_2S and the abnormal concentration of H_2S in intestine or body, the visceral sensitivity of IBS may be increased, and then affect the pathophysiological process of IBS.

One of the important findings is that FA treatment obviously attenuated the visceral pain. Although detailed mechanisms have not been investigated yet, the FA-induced analgesia is likely mediated by reduction of H_2S concentration. FA is a necessary nutrient for mammals, which promotes growth and development and maintains metabolism.[10,38] It also plays an important role in the central nervous system.[39] Zhang et al. showed that FA modulated matrix metalloproteinase-2 expression, alleviated neuropathic pain, and improved functional recovery in spinal cord-injured rats.[22] Other clinical studies have demonstrated that folic acid can reduce skeletal muscle pain.[40] However, whether FA can alleviate visceral pain is still unclear. It has been reported that FA can affect the structural distribution of mammalian intestinal flora and the concentration of short-chain fatty acids to promote intestinal microbial balance.[10] Some studies have demonstrated that the imbalance of intestinal flora will increase visceral sensitivity in rats, and the imbalance of IBS intestinal flora may be involved in the formation of visceral hypersensitivity. In the present study, we have provided additional evidence to confirm that folic acid may reduce chronic visceral pain by regulating intestinal flora. We first showed that the concentration of folic acid in the blood of IBS patients was significantly lower than that of healthy volunteers (Normal). FA supplement alleviated chronic visceral pain, normalized the *Clostridiales* frequency and reduced H_2S concentration in NCI rats.

In addition to reduce H_2S concentration and balance the intestinal microbial, FA supplement significantly reduced the frequency of sEPSCs of neurons in the spinal dorsal horn of NCI rats. It is generally believed that excitatory synaptic transmission is related to the transmission of the pain signals. Consistent with previous report,[24] we confirmed that NCI significantly increased the excitability of SG neurons. Very surprisingly, we showed that folic acid treatment reduced the excitability of SG neurons. It is, therefore, reasonable to conclude that FA-induced analgesia was likely mediated by suppression of spinal synaptic transmission. Clinically, FA may be a potential treatment strategy for chronic visceral pain in patients with IBS. Since we only looked at sEPSCs, it is difficult to determine whether the decrease in sEPSCs after folic acid treatment is due to reduced input or reduced sensitivity to input. Further experiments are of great help to record the evoked sEPSCs.

In conclusion, this study showed that folic acid treatment attenuates chronic visceral pain of NCI rats most likely through reducing H_2S production from *Clostridiales* in intestine. Modulation of spinal synaptic transmission might be an important mechanism to control the pain processing at the spinal cord level, which deserves a further investigation in the future. It is of great significance to deeply explore the mechanism of visceral hypersensitivity, to interfere with the occurrence and development of visceral hypersensitivity, and to research for an effective target for the clinical treatment of recurrent abdominal pain in patients with functional gastrointestinal disorders such as IBS.

Author contributions

R-X.W and Y-X.W performed experiments, analyzed data and prepared figures and the manuscript. Y-C.L performed experiments and analyzed data. X.X and J-B.Z revised the manuscript. G-Y.X designed experiments, supervised the experiments and finalized the manuscript. R.L designed experiments, analyzed data and revised the manuscript. All the authors have read and approved the paper.

Declaration of conflicting interests

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Ethics approval

Care and handling of the animals were approved by the Institutional Animal Care and Use Committee of Soochow University and were in accordance with the guidelines of the International Association for the Study of Pain.

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