PHARMACOLOGICAL TREATMENTS AFFECTING GASTRO-INTESTINAL MOTILITY IN MAN

EDITED BY: Emidio Scarpellini, Gianluca Ianiro and Jan Tack
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PHARMACOLOGICAL TREATMENTS AFFECTING GASTRO-INTESTINAL MOTILITY IN MAN

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EDITORIAL
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Editorial: Pharmacological Treatments Affecting Gastro-Intestinal Motility in Man

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Keywords: esophageal motility, gastrointestinal motility, diarrhea, constipation, gut microbiota

Editorial on the Research Topic

Pharmacological Treatments Affecting Gastro-Intestinal Motility in Man

Motility of the upper and lower gastrointestinal (GI) tract is a complex of processes regulated by several receptors detectable along the GI tract e.g., cannabinoid, opioid, TRPV1 (Janssen et al., 2011), nerve endings and receptors belonging to the enteric nervous system (ENS), and hormones such as motilin, ghrelin, cholecystokinin (Janssen et al., 2011; Tack et al., 2016).

The upper and lower GI tract move in a highly coordinated fashion with periods of silence and periods of increased or maximum activity. This has several physiological functions: food boluses can progress from the esophagus to the anus. On the other hand, transient lower oesophageal sphincter relaxations guarantee the maintenance of the gastro-esophageal barrier towards acid and/or non-acid reflux of gastric/duodenal content (Tack and Pandolfino, 2018). Gastric relaxation, gastric meal accommodation, and colonic relaxation allow storage of larger amounts of luminal content. In the upper gastrointestinal tract, phases of interdigestive motility complex participate in the regulation of hunger, return of hunger, and satiety and appetite (Carbone and Tack, 2014). Continuous phasic motility of the stomach, small bowel, and colon favor digestion and absorption of vitamins and nutrients (Janssen et al., 2011). Several neuronal and non-neuronal receptors determine GI sensitivity to intestinal content, food ingredients, and, last but not least, gut microbiota and its products (Ianiro et al., 2014).

The gut microbiota is a "microorganism" itself with the GI tract, including the buccal cavity. It is composed of bacteria, viruses, protozoa, archaea, fungi (Scarpellini et al., 2015). It has metabolic, immune-modulating, and absorptive/digestive functions and regulates the secretion of GI hormones, hunger, and appetite in obese and non-obese animal models. There is a close interaction between digestive, sensory, and propulsive processes in the gastrointestinal tract and the resident microflora (Gibiino et al., 2017). Derangements of gut microbiota composition have been implicated in the pathogenesis of functional GI disorders such as irritable bowel syndrome, FD, and chronic constipation (Tait and Sayuk, 2021). Thus, the gut microbiota is a notable therapeutic target for the treatment of upper and lower functional GI disorders (e.g., irritable bowel syndrome). Gut microbiota can be modulated via absorbable and non-absorbable antibiotics, and pre- and probiotics. The latter has been deeply investigated in the last 3 decades by the pharmacological industry (Ortigão et al., 2020).

Special attention is needed for potential developments of fecal microbiota transplantation (FMT). This treatment, now commonly used to treat *Clostridium difficile* infections, seems to pave the road to its usage in functional GI disorders too (e.g. IBS) (Cammarota et al., 2019).

IBS, FD, and GERD show impairments of GI motility at various extents (Black et al., 2020). GERD often benefits from PPI treatment. However, about one-third of patients results to be refractory to treatment. Several prokinetic and reflux inhibitor remedies have been ruled out with alternate and

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incomplete success (Black et al., 2020). FD treatment is as complex as its physiopathology and prokinetics, drugs acting on GI sensitivity and CNS have an incomplete response. IBS, diarrhea, constipation treatment accounts of pre-, probiotics, antibiotics, antisecretory, prokintecis drugs, awaiting a personalized flow-chart.

Thus, several grey zones continue to exist in the neuro-gastroenterology field, such as treatment of oesophageal motility disorders (e.g., ineffective oesophageal motility disorders (IEM), achalasia, diffuse esophageal spasm, etc.) and refractory GERD (Mittal and Vaezi, 2020), and treatment of gastric (e.g., FD, gastroparesis) and lower GI tract motility disorders (e.g., IBS, diarrhea, constipation) (Camilleri, 2020; Scott et al., 2020).

Several prokinetic and non-prokinetic remedies have been studied and developed for the modulation of upper and lower GI motility. Their impact on the grey zones of neuro-gastroenterology was shown to be significantly different and heterogeneous (Black et al., 2020). These findings are increasingly allowing a patient-specific therapeutic approach.

The present article collection was selected to address several of the grey zones in clinical GI motility modulation, by pharmacological as well as non-pharmacological treatments.

We hope that this special issue will be useful for different specialists in the field (biologists, bio-engineers, doctors). It provides an extensive and updated review of the literature on the evidence on pharmacological and non-pharmacological treatments affecting gastrointestinal motility in humans. This collection can also serve as a basis for all future researchers in this expanding and rapidly changing area of biomedical investigation.

AUTHOR CONTRIBUTIONS

ES and JT wrote the Editorial article. GI revised the references. JT revised the entire manuscript.

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ORIGINAL RESEARCH
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Impact of Opioid Consumption in Patients With Functional Gastrointestinal Disorders

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Objective: We aimed to determine the burden of opioid consumption in a cohort of patients with functional gastrointestinal disorders.

Methods: All patients diagnosed with functional gastrointestinal disorders and referred to our university hospital were evaluated from 2013 to the beginning of 2019. Irritable bowel syndrome and functional dyspepsia diagnoses were determined according to Rome criteria and severity according to irritable bowel syndrome severity scoring system. Vomiting was quantified using a 5-point Likert scale, and constipation severity was measured using the Knowles-Eccersley-Scott-Symptom questionnaires. Quality of life was quantified by the GastroIntestinal Quality of Life Index. Patients were categorized as being treated on a chronic basis with either tramadol, step II opioids, step III opioids or as being opioid-free.

Results: 2933 consecutive patients were included. In our cohort, 12.5% had only irritable bowel syndrome, 39.3% had only functional dyspepsia, 24.9% had a combination of both, and 23.4% had other functional gastrointestinal disorders. Among them, the consumption of tramadol, step II (tramadol excluded) and step III opioids was 1.8, 1.3 and 0.3 % respectively in 2013 and 4.3, 3.4 and 1.9% in 2018 ($p < 0.03$). Opioid consumption was associated with increased vomiting ($p = 0.0168$), constipation ($p < 0.0001$), symptom severity ($p < 0.001$), more altered quality of life ($p < 0.0001$) and higher depression score ($p = 0.0045$).

Conclusion: In functional gastrointestinal disorders, opioid consumption has increased in the last years and is associated with more GI symptoms (vomiting, constipation and GI severity), higher depression and more altered quality of life.

Keywords: opioid, tramadol, constipation, vomiting, quality of life, functional gastrointestinal disorders

INTRODUCTION

Opioids are mostly prescribed for acute or chronic pain associated or not with cancer referring to the World Health Organization guidelines for pain evaluation. Step III (morphine, oxycodone, fentanyl, etc.) and step II (tramadol, codeine, etc) opioid consumption increased by 45% and 65% respectively between 2006 and 2017 in the United States (Vadivelu et al., 2018). Increasing opioid prescription in the US led to 30,000 deaths due to opioid overdoses in 2015 alone (Vadivelu et al., 2018). In France, the same trend is observed with an increase of 16% from 2004 to 2017 (Chenaf et al., 2018), with a consumption associated with increased morbidity and mortality (Chenaf et al., 2018).

Opioids are known to have gastrointestinal side effects such as nausea, abdominal pain, gas and constipation (Cook et al., 2008). Among side effects, opioid-induced constipation (OIC) is the most frequent side effect and is defined by Rome IV criteria (Mearin et al., 2016). Considering their gastrointestinal (GI) side effects, and the lack of efficacy to relieve pain in functional GI disorders (FGID), opioids are not recommended in the treatment of painful FGID (Szigethy et al., 2018). On the other hand, opioids may be prescribed to treat other associated conditions in FGID patients, which may worsen their GI symptoms or trigger additional GI symptoms. However, there are currently few studies reporting opioid consumption trends in FGID patients, and the impact of opioids on GI symptoms and quality of life in FGID patients still needs to be assessed.

Therefore, our aim was to determine the burden of opioid consumption in a FGID cohort and to assess its impact on GI symptoms and quality of life.

MATERIAL AND METHODS

Study Design and Ethics

We conducted a retrospective single center study in a university hospital in Normandy, France. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki (6th revision, 2008) and was approved by the local human research committee (E2020-51) as required by national legislation. The use of informatic data was declared to the Commission Nationale de l'Informatique et des Libertés (CNIL) (*n* 817.917), in compliance with French legislation. Written informed consent was obtained for all patients regarding the use and informatic storage of their medical data for research purposes, including score and Quality of Life questionnaires.

Patients

All patients diagnosed with FGID and referred for GI motility tests were retrospectively evaluated from January 1st 2013 to March 18th 2019. Clinical data (age, gender, weight, height and body mass index (BMI)) were collected. Opioid consumption was evaluated by reviewing medical charts. Patients were

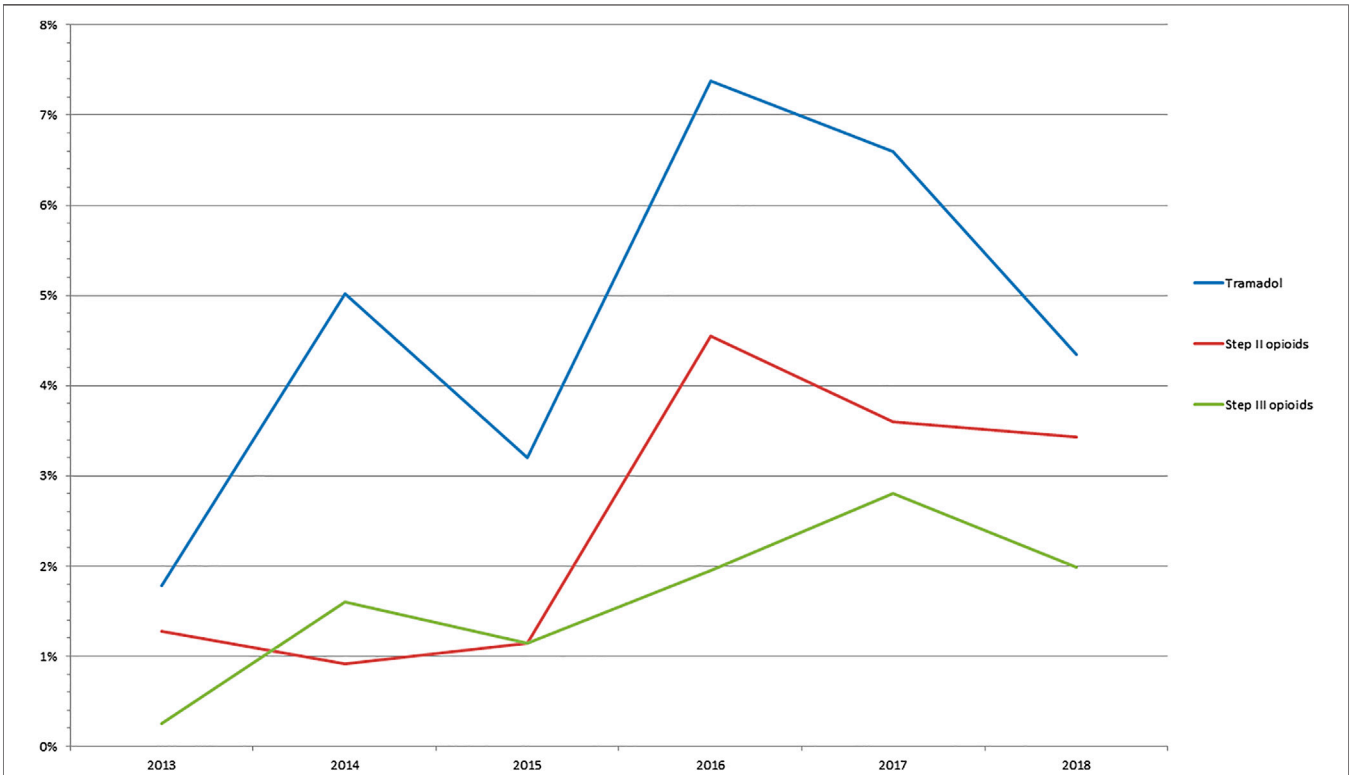

FIGURE 1 | Prevalence of opioid consumption between 2013 and 2018 in patients referred for functional gastrointestinal disorders.

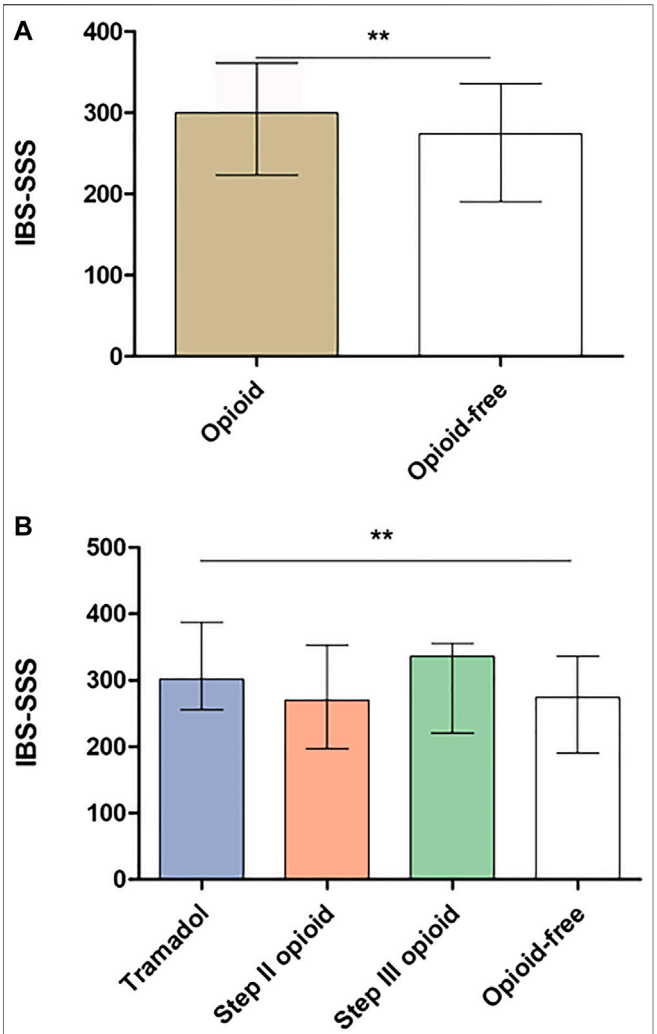

FIGURE 2 | Irritable bowel syndrome severity scoring system according to opioid consumption. **(A)** IBS severity according to global opioid consumption **(B)** IBS severity according to opioid subtype. IBS-SSS: Irritable bowel syndrome severity scoring system. Results are presented in mean ± SD. $^{**}p < 0.001$ in comparison with opioid-free patients. Kruskal-Wallis test and Dunn's test were performed to compare groups of continuous variables and Mann-Whitney test to compare opioid with opioid-free groups.

categorized as being treated on a chronic basis with either tramadol (tramadol or an association of tramadol and paracetamol), another step II opioid (codeine, or an association of codeine and paracetamol, caffeine, or an association of caffeine and paracetamol, or an association of – codeine, caffeine and paracetamol, or an association of codeine, acetylsalicylic acid and caffeine, or an association of codeine, acetylsalicylic acid, paracetamol–, nalbuphine and opium, or an association of buprenorphine, paracetamol, opium and caffeine), a step III opioid (morphine, oxycodone, fentanyl, tapentadol) or as being opioid-free. Rheumatologic, neurologic comorbidities and migraine were assessed.

Questionnaires

Validated self-questionnaires were systematically proposed to all patients. Irritable bowel syndrome (IBS) and functional dyspepsia (FD) were determined according to Rome criteria. Rome III criteria were used between 2013 and 2016 and Rome IV criteria since 2016 (Longstreth et al., 2006; Mearin et al., 2016). To define IBS patients before 2016, we used the association of Rome III criteria with the presence of abdominal pain (to be closest to the Rome IV definition). Severity was assessed in all patients using IBS Severity Scoring System (IBS-SSS) with a maximum score of 500 (Francis et al., 1997). Remission, mild, moderate and severe cases were defined by scores <75, 75–175, 175–300 and >300 (Francis et al., 1997).

GI symptoms were analyzed using a five-point Likert scale for vomiting (0: absent to 4: severe) and using the Knowles-Eccersley-Scott-Symptom questionnaires (KESS) for constipation (Knowles et al., 2000). KESS score comprises 11 individual items with a maximum of 39 points, a score higher than 10 defining constipation (Knowles et al., 2000).

Quality of life was quantified by the GI Quality of Life Index (GIQLI) comprising 36 questions concerning among others digestive symptoms and effects of medical treatment (Slim et al., 1999). The score range is between 0 (worst) and 144 (best quality of life) (Slim et al., 1999). Anxiety and depression were evaluated using the Hospital Anxiety and Depression (HAD) scale (Zigmond and Snaith, 1983). The scale ranges from 0: absent to 21: maximum of anxiety or depression. A score higher than 10 out of 21 defines anxiety or depression (Zigmond and Snaith, 1983).

Statistical Analysis

Data are expressed as n (percentage) and median [Q1-Q3]. Opioid consumption was analyzed between 2013 and 2018; in 2019, patients were only included at the beginning of the year and were therefore not representative of a one-year period. Characteristics were compared using chi-squared test for qualitative variables. Non-parametric continuous variables were compared using Kruskal-Wallis test and Dunn's test for subgroup analysis. Prevalence trends were analyzed using a chi-squared test. Associations were considered statistically significant when p-value was <0.05.

RESULTS

Patients

Between January 1st, 2013 and March 18th, 2019, 3204 patients were referred for FGID. While 271 declined to give their informed consent, 2933 consecutive patients were evaluated. In our cohort, 1096 patients responded to the Rome questionnaire, 12.5% had only IBS, 39.3% had only FD, 24.9% had a combination of IBS and FD, and 23.4% had other FGID. Patients were mostly female (72.9% of the cases) with a mean age of 50.0 [36.0; 62.0] years and a mean BMI of 24.1 [21.0; 27.9] kg/m^2. Rheumatologic, neurologic

TABLE 1 | Patients' characteristics according to opioid consumption.

	Tramadol (*n* = 139)	Step II opioid (*n* = 78)	Step III opioid (*n* = 47)	Opioid-free (*n* = 2689)	*p*-Value
Age (years)	55.0 [41.0; 67.0][b]	49.0 [41.0; 62.0]	49.0 [44.0; 60.0]	50.0 [36.0; 62.0]	0.04
BMI (kg/m^2)	25.2 [22.0; 29.3][b]	27.7 [23.2; 32.0][c]	24.5 [21.3; 31.2]	23.9 [21.0; 27.7]	<0.0001
Female	112 (80.6)	65 (83.3)	38 (80.8)	1941(72.2)	0.0137
FGID[a]					0.6324
Only IBS	10.7%	15.8%	13.0%	12.6%	
Only FD	41.1%	31.6%	34.8%	39.4%	
Both	35.7%	26.3%	26.1%	24.1%	
Other FGID	12.5%	26.3%	26.1%	23.8%	

Results are presented in median [Q1; Q3] and n *(percentage). BMI, body mass index; FD, functional dyspepsia; FGID, functional gastrointestinal disorder; IBS, irritable bowel syndrome. Kruskal-Wallis test and Dunn's test were performed to compare continuous variables and chi-squared tests to compare prevalence.*

[a]*filed by 37.4% of the population*

[b]p *value <0.05 in comparison with opioid-free patients.*

[c]p *value <0.0001 in comparison with opioid-free patients.*

TABLE 2 | Patients' medication according to opioid consumption.

	Opioid-free *n* = 2689	Opioids *n* = 264	*p*
Antiemetics *n* = 108 (3.7%)	85 (3.2%)	23 (8.7%)	0.0001
Laxatives *n* = 287 (9.8%)	247 (9.2%)	42 (15.9%)[a]	0.0016
Loperamide *N* = 149 (5.1%)	131 (4.9%)	19 (7.2%)[b]	0.0662
NSAID *n* = 77 (2.6%)	44 (1.6%)	37 (14.0%)	<0.0001
PPI *n* = 578 (19.7%)	482 (17.9)	102 (38.6%)	<0.0001
Steroids *n* = 40 (1.4%)	31 (1.2%)	10 (3.8%)	0.0038
PAMORA *n* = 9 (0.3%)	5 (0.19%)	4 (1.5%)	<0.0001

Results are presented as n *(percentage). NSAID, Nonsteroidal anti-inflammatory drugs; PAMORA, Peripherally-Acting Mu-Opioid Receptor Antagonists; PPI, Proton pump inhibitor.*

In the tramadol group:

[a]*Laxatives were used in 18.7%.*

[b]*Loperamide in 5.8%.*

comorbidities and migraine were present in 10.4%, 6.1% and 1.5% of the patients, respectively. Patients had moderate IBS-SSS 275.0 [195.0; 339.8] with altered quality of life (GIQLI:83.0 [67.0; 99.0]). Constipation was a ubiquitous problem with a KESS score of 11.0 [7.0; 17.0] points. Patients had a mean score of HAD-anxiety of 9 [7.0; 12.0] points and of HAD-depression of 6.0 [3.0; 9.0] points. Anxiety was therefore present in 39.1% of the patients and depression in 14.9%.

Patients were on laxatives, Peripherally-Acting Mu-Opioid Receptor Antagonists (PAMORA), antiemetics, loperamide, PPI, steroids and NSAIDs in 9.8%, 0.3%, 3.7%, 5.1%, 19.7%, 1.4% and 2.6% respectively.

Opioid Consumption

The global consumption of tramadol, step II and step III opioids was 4.7%, 2.7% and 1.6%, respectively in our cohort. Rheumatologic, neurologic comorbidities and migraine were more frequent in opioid patients in comparison with opioid-free patients (33.2% vs. 8.3%, 8.6% vs. 5.8% and 3.3% vs. 1.0%, $p < 0.0001$). Opioid consumption between 2013 and 2019 is presented in **Figure 1.** Consumption of tramadol, step II and step III opioids significantly increased between 2013 and 2018 ($p < 0.03$, **Figure 1**). There were more female patients in the three opioid groups in comparison with the opioid-free group (**Table 1**). Patients with tramadol and step II opioids were overweight in comparison with opioid-free patients (**Table 1**). Other characteristics are presented in **Table 1**.

The consumption of tramadol, step II opioids and step III opioids was 15.0%, 8.5% and 5.2% in patients with rheumatologic comorbidities, 5.6%, 2.2% and 0.6% in patients with neurologic comorbidities, and 8.9%, 13.3% and 0% in patients with migraine. Patients on opioid were also significantly more frequently on other drugs treating or affecting the GI tract in comparison with opiod-free patients (**Table 2**).

Gastrointestinal Symptoms According to Opioid Consumption

Opioid consumption was associated with more severe GI symptoms in comparison with opioid-free patients (**Figure 2A**) but also with more constipation and vomiting than opioid-free patients (**Figure 3A** and **Figure 3C**). Opioid consumption was associated with a poorer quality of life (**Figure 4A**) and a higher depression score (**Figure 5A**) while anxiety was similar between opioid and opioid-free patients (**Figure 5C**).

Chronic consumption of tramadol was associated with more severe GI symptoms (IBS-SSS: 313.7 ± 98.2 vs. 262.5 ±104.9, $p < 0.001$, **Figure 2B**), with more severe constipation and vomiting (**Figures 3B,D**) and with a poorer quality of life (**Figure 4B**), in comparison with opioid-free patients. Step III opioids were associated with a higher vomiting score (**Figure 3B**) and a poorer quality of life (**Figure 4B**). Opioid subgroups were not associated with depression or anxiety (**Figures 5B,D**).

DISCUSSION

Our study is the first to assess the effect of opioid consumption in a large cohort of patients with FGID. As reported in Europe in a global population (Bosetti et al., 2019), we have shown an increase in opioid consumption over the years in FGID patients. Opioid

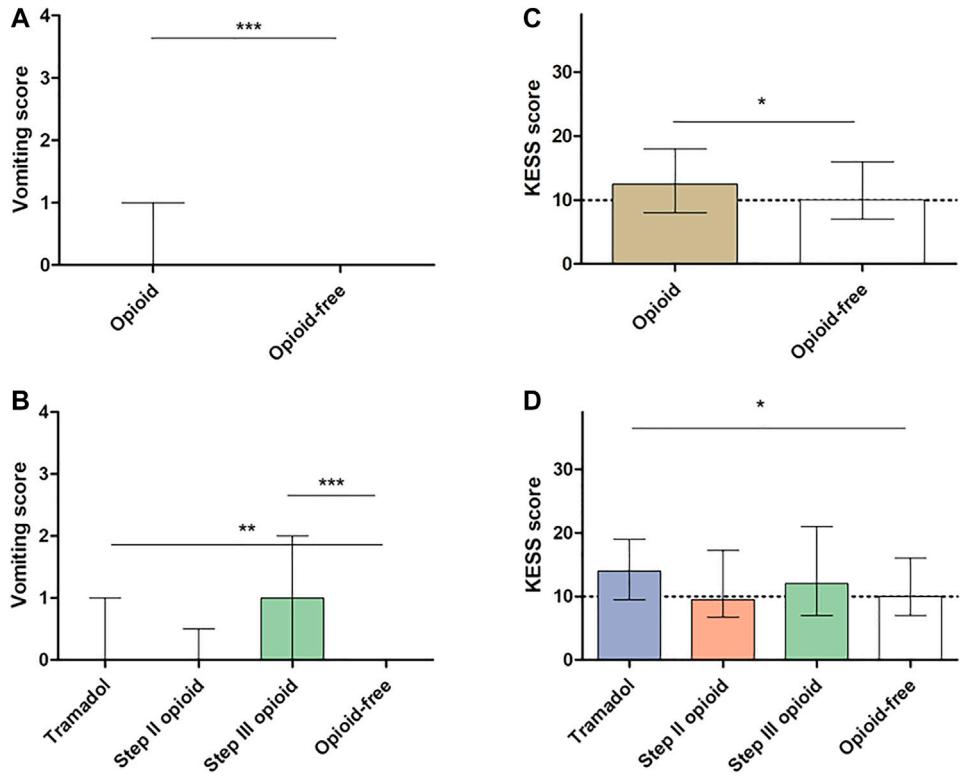

FIGURE 3 | Gastrointestinal symptoms according to opioid consumption. **(A)** Vomiting score according to global opioid consumption **(B)** Vomiting score according to opioid subtype **(C,D)** Constipation score according to global opioid consumption **(C)** Constipation score according to opioid subtype **(D)**. The dotted line defines constipation (higher score) and no constipation (lower score), according to KESS definition. KESS: Knowles-Eccersley-Scott-Symptom questionnaires. Vomiting score ranges from 0 (absence of symptoms) to 4 (very severe symptoms). *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ in comparison with opioid-free patients. *Kruskal-Wallis test and Dunn's test were performed to compare groups of continuous variables and Mann-Whitney test to compare opioid with opioid-free groups.*

consumption was associated with more severe GI symptoms (vomiting, constipation and GI severity), higher depression score and a poorer quality of life.

Our cohort is similar to our previously described cohort of FGID patients with middle-age female predominance (Melchior et al., 2018). Our population is typical of FGID followed in a tertiary center and well-characterized with validated questionnaires. An overlap between IBS and FD was less frequent than reported in the literature. Indeed, previous studies showed an overlap in 23%–64% of cases, according to clinical routine and tertiary center and using Rome III criteria (von Wulffen et al., 2019). The prevalence of FGID is lower using Rome IV criteria than Rome III criteria (Bai et al., 2017; Saps et al., 2018). The use of Rome IV criteria since 2016 in our cohort, may explain a lower association between IBS and FD as reported in another tertiary center (von Wulffen et al., 2019).

In our cohort, the rate of consumption of tramadol, step II opioids and step III opioids rose to 4.3%, 3.4% and 1.9% respectively and was associated with female gender. The rate of consumption of opioids was higher than in the global population and increased over the years as in other French studies (Chenaf et al., 2018; Hider-Mlynarz et al., 2018; Bosetti et al., 2019). Global consumption of opioids increased over 16% and step III opioids from 0.5% to 1.1 between 2004 and 2017 using a nationally representative sample of the French Claims database (Chenaf et al., 2018). In contrast, there was a global decrease in the consumption of step II opioids, explained by dextropropoxyphene withdrawal in 2011 in France, while consumption of mild opioids including tramadol increased (Hider-Mlynarz et al., 2018). However, opioid consumption fell after a regulatory measure in July 2017 (i.e., a medical prescription is now needed for codeine). As in our cohort, female patients were more frequently opioid users (Chenaf et al., 2018). France is in the top three countries for analgesic consumption in Europe, with paracetamol leading (Hider-Mlynarz et al., 2018). Increased opioid use is associated with increased morbidity and mortality (Chenaf et al., 2018).

Opioid consumption is associated with OIC and vomiting and altered quality of life. Indeed, in our cohort we reported more severe constipation in particular in patients treated with tramadol in comparison with opioid-free patients and higher vomiting score in both tramadol and step III opioid patients. This result cannot be explained by associated drugs, while patients with tramadol consumed more laxatives than the other group. Constipation and vomiting are both adverse events known to be induced by opioids (Els et al., 2017) and both were specifically associated with tramadol (Montastruc et al., 2018). Despite opioid efficacy, the occurrence of side

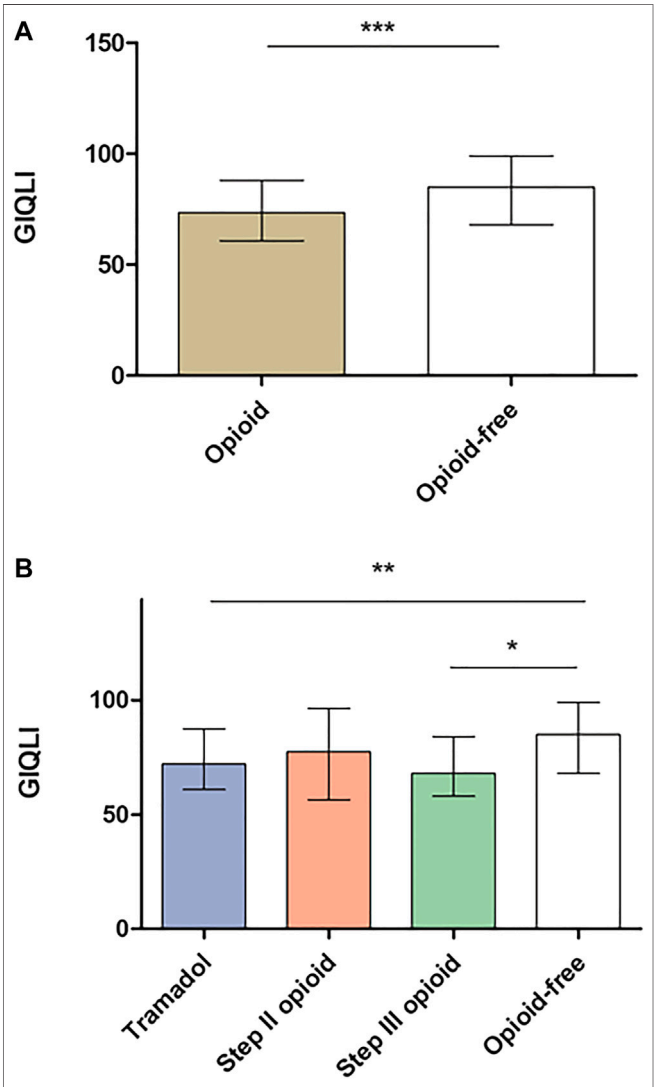

FIGURE 4 | GIQLI according to opioid consumption. **(A)** Quality of life according to global opioid consumption **(B)** Quality of life according to opioid subtype. GIQLI: Gastrointestinal Quality of Life Index. Results are presented as mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$ in comparison with opioid-free patients. *Kruskal-Wallis test and Dunn's test were performed to compare groups of continuous variables and Mann-Whitney test to compare opioid with opioid-free groups.*

effects could lead to opioid withdrawal in one third of patients (Furlan et al., 2006). OIC is the most frequent and known side effect of opioids, in particular for step III opioids (Farmer et al., 2019). OIC could be treated with laxatives which are often insufficient or PAMORA (Farmer et al., 2019). OIC prevalence was higher in patients treated with tramadol in our cohort but was not increased with step II and step III opioids. One explanation could be the weaker efficacy of PAMORA on tramadol and step II opioids (Halawi et al., 2018). The use of PAMORA to block opioid receptors is probably safe in FGID, the best option is probably to stop opioid use for these disorders but in the meantime their use in FGID warrants further studies (Corsetti et al., 2019). Another explanation could be that constipation induced by step III opioids is more recognized by clinicians and therefore probably associated with better clinical management than constipation associated with step II opioids.

Nausea and vomiting are well-known side effects of tramadol and increase with dosing (DeLemos et al., 2017). Nausea and vomiting symptoms were mostly assessed in postoperative or in cancer patients, where the causes can be various (Tsukuura et al., 2017; Shin et al., 2019). Consumption of tramadol and step III opioids was associated with a poorer quality of life in our cohort, probably linked with opioid-induced GI side effects. Mechanisms of opioid-induced nausea and vomiting are multifactorial, with central effects on the area postrema and gastric emptying modulation being key factors. Specific management of these side effects may reduce opioid withdrawal, optimize opioid efficacy and improve patients' quality of life. PAMORA efficacy has only been suggested in opioid-associated nausea and vomiting in animal models and in retrospective studies (Kanemasa et al., 2020; Sato et al., 2020). Further randomized controlled studies are therefore warranted on this particular condition.

Opioids are known to be ineffective to relieve pain symptoms in FGID (Tennant, 2015) and are therefore not a recommended therapeutic option to treat these patients. In our study, rheumatologic, neurologic comorbidities and migraine were strongly associated with opioid use, confirming that opioids in FGID patients are primarily prescribed for associated conditions. Despite their side effects, opioids are more commonly prescribed to patients with FGID than patients with identified organic gastrointestinal diseases (Sayuk et al., 2018). In addition, opioid misuse was associated with FGID in a cohort of inflammatory bowel disorders (Crocker et al., 2014; Sayuk et al., 2018). Opioids were more frequently prescribed in presence of personality disorders, psychiatric diagnoses, history of drug abuse, homelessness and/or IBS (Long et al., 2011; Sayuk et al., 2018). This association could explain the increase in depression score in the opioid group. But we have to consider that HADS is a questionnaire to screen anxiety and depression, and the diagnosis should be confirmed by a specialist, which was not the case in our study. Since 2013 in the US, gastroenterologists have prescribed fewer opioids in response to the opioid epidemic (Chen et al., 2020). In our cohort, the increased trend of opioid consumption is not likely to originate from gastroenterologists as opioid prescription was strongly associated with other GI conditions, including rheumatologic or neurologic comorbidities. Nevertheless, gastroenterologist-related initiation of opioid treatment remains to be assessed in Europe.

Of course, our study has several limitations mostly due to the retrospective analysis. The cumulative dose and duration of the opioids were not available and therefore not analyzed.

We searched for associated drugs that may have had an effect on the GI tract, but it is difficult to know whether these drugs affected our results. Indeed, the dosage and duration of all these drugs were not available for all the patients and therefore not analyzable. We have no information regarding the condition for which the drug was prescribed. Another limitation is the

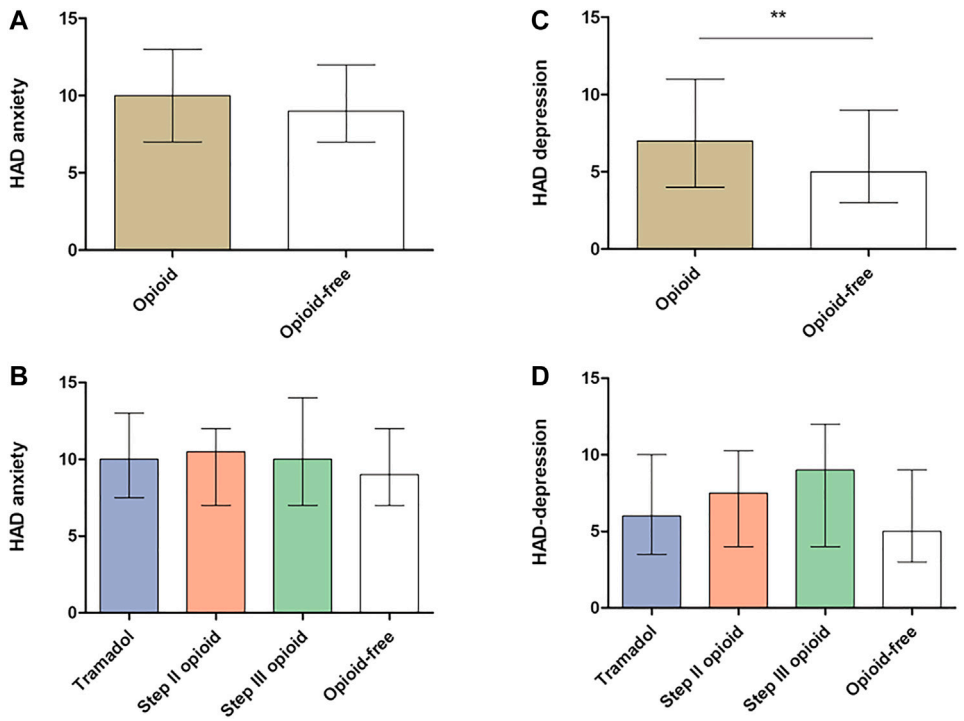

FIGURE 5 | Hospital anxiety and depression scale according to opioid consumption **(A,C)** Anxiety and depression according to global opioid consumption **(B,D)** Anxiety and depression according to opioid subtype. HAD, Hospital Anxiety and Depression scale. Results are presented as mean ± standard deviation (SD). $^{**}p < 0.001$. *Kruskal-Wallis test and Dunn's test were performed to compare groups of continuous variables and Mann-Whitney test to compare opioid with opioid-free groups.*

possibility that some patients used over the counter drugs. In the United States, half of patients with constipation used over the counter medication and only two patients out of five sought the advice of a healthcarer (Oh et al., 2020). In France many laxatives can be used without prescription, to avoid this problem we looked for all reported treatment in the medical chart but patients may have omitted to report some of them.

CONCLUSION

In FGID, opioid consumption has more than tripled over a 5 year period and is higher than in the global population. Opioid consumption is associated with more severe GI symptoms (vomiting, constipation and GI severity), higher depression score and more altered quality of life. Opioid prescription should take into account worsening of GI symptoms and quality of life.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Rouen local human research committee (E2020-51). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GG is the guarantor of the article. CM, FW, CD, GG, and A-ML performed the research, CM, A-ML, and GG collected and analyzed the data, AL and GG designed the research study and CM and GG wrote the paper, and CM, FW, CD, GG, A-ML, and AL contributed to the design of the study. All authors approved the final version of the article.

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Conflict of Interest: CM and GG were scientific experts for Kyowa Kirin in 2018.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Corrigendum: Impact of Opioid Consumption in Patients With Functional Gastrointestinal Disorders Research Topic: Pharmacological Treatments Affecting Gastro-Intestinal Motility in Man

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Keywords: opioid, functional gastrointestinal disorder, tramadol, constipation, vomiting, quality of life

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An author name was incorrectly spelled as Goucerol. The correct spelling is Gourcerol. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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ORIGINAL RESEARCH
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Oceanapia magna Sponge Presents Dual Effect on the Gastrointestinal Motility of Rodents: *In Vitro* and *In Vivo* Assays

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Oceanapia magna Santos-Neto, Nascimento, Cavalcanti and Pinheiro sponges are distributed across tropical worldwide seas. Some studies of marine products have shown interesting activities in smooth muscle models. Hence, we assessed the effect of the ethanolic extract of *Oceanapia magna*. (OC-EtOH) on acute toxicity and gastrointestinal motility (*in vitro* and *in vivo*) in rodent models. On guinea pig ileum, OC-EtOH induced a concentration dependent contraction on basal tonus, which was not inhibited by atropine, but in the presence of pyrilamine or verapamil, the effect was antagonized. Contrastingly, on KCl- or histamine-induced contractions, OC-EtOH presented a transient contraction followed by a concentration-dependent relaxation. Moreover, OC-EtOH presented a relaxant profile on cumulative curves to $CaCl_2$ and tonic contraction induced by S-(-)-BayK8644, through Cav blockade. The acute toxicity assay showed that OC-EtOH (2,000 mg/kg, p.o.) did not present any sign of toxicity in female mice. Additionally, OC-EtOH presented antidiarrheal effect in mice, increased the intestinal normal transit and reduced the castor oil-induced intestinal transit. Thus, OC-EtOH presented a dual effect on guinea pig ileum promoting contraction through activation of H_1 and Ca_V, and relaxation through Ca_V blockade, besides the effect on upper gastrointestinal transit in mice, showing a potential medicinal use of this sponge in intestinal diseases such as diarrhea.

Keywords: marine sponge, *Oceanapia magna*, spasmolytic, spasmogenic, intestinal transit

INTRODUCTION

Natural products are considered a natural library of combinatorial chemistry that can provide substances with chemical and pharmacological diversity (Wang et al., 2011). The vast range of products available in the nature can be considered an important source of substances with potential therapeutic interest (Butler et al., 2014). Among the natural products diversity, several researchers

are interested in studying natural marine products due to the varied production of compounds that may be used to treat various diseases (Movahhedin et al., 2014).

Marine natural products are sources of compounds that have demonstrated a plethora of biological activities, in both pre-clinical and clinical research, such as antifungal (D'auria et al., 2007), anticoagulant (Jung et al., 2007), antibacterial (Horie et al., 2008), anti-inflammatory (Chao et al., 2008), immunomodulatory (Yamada et al., 2007; Courtois et al., 2008), antiviral (Artan et al., 2008) and anticancer (Malyarenko et al., 2018; Rath et al., 2018). Additionally, spasmolytic activity has been described for the brown alga *Dictyota pulchella* (Queiroz et al., 2011), the green alga *Hydrodictyon reticulatum* (Gutierrez et al., 2012), and caulerpina, an alkaloid isolated from algae of *Caulerpa* genus (Cavalcante-Silva et al., 2013; Cavalcante-Silva et al., 2016).

Oceanapia genus (Oceanapiidae) presents about 100 sponges species distributed across the world's tropical seas (Van Soest et al., 2015). Studies on *Oceanapia* genus are scarce in literature and only few biological activities have been described. *Oceanapia* species produce different classes of metabolites, including alkaloids, sphingolipids, steroids, acetylene, thiocyanate, among others (Ibrahim et al., 2013) that showed antifungal effect (Nicholas et al., 1999) and anticancer activity (Kijjoa et al., 2007).

Oceanapia magna Santos-Neto, Nascimento, Cavalcanti and Pinheiro is a recently described endemic species in Brazil. The word *magna* is an adjective derived from Latin and refers to the large size of this specimens (Santos-Neto et al., 2018).

There is considerable interest in investigating drugs obtained directly from natural products or their derivatives that act on the smooth muscle, once this type of muscle is present in the walls of various body organs, including blood vessels, stomach, bladder, airways and intestine. The regulation/deregulation of smooth muscle contractility plays a role in many pathophysiological processes such as hypertension, asthma, erectile dysfunction, renal and uterine cramping, diarrhea, and constipation (Webb, 2003).

Diarrhea is a process characterized by the increase of defecation frequency (more than 3 times in 24 h period), which may be watery or semi-liquid (World Health Organization, 2016). This disorder causes thousands of deaths per year, mainly in children under 5 years of age, which implies in a high cost for healthcare systems (Ndikubwimana and Ngendahimana, 2020). On the other hand, constipation is a condition that the individual presents less than three defecation episodes per week or evacuates hard, dry, and small stools, in a painful and difficult process to eliminate feces (Bielefeldt et al., 2016). This condition represents a significant economic burden. In the United States, the medical costs associated with constipation were estimated at United States $235 million in 2001, and about 95.3% of this costs were applied in the outpatient care (Nag et al., 2020). Therefore, there is an unexplored field that stimulates researchers to study natural products as tools to obtain new treatments and/or preventions to gastrointestinal diseases (Guo et al., 2014). Moreover, as described before, there is evidence that marine natural products present action on intestinal smooth muscle, what could represent an important contribution for human health, since marine products are known as a rich secondary metabolites source. Based on this, we decided to investigate a possible intestinal smooth muscle tone and/or antidiarrheal activities of the ethanolic extract from *Oceanapia magna* (OC-EtOH).

MATERIALS AND METHODS

Product-Test

The ethanolic extract obtained from *Oceanapia magna* sponge (OC-EtOH) was used in this study. The sponge was collected by Petrobras (Petróleo of Brazil S/A), in May 2011, in Potiguar Basin/Rio Grande do Norte (04° 44.8945′ S/036° 25.4571′ W), with a 108 m depth, as part of Project: Inter-institutional Network Benthic Algae and INCT in Tropical Marine Environments - AmbTropic (CNPq No. 610013/2011-4) and identified by Professor PhD. Ulisses dos Santos Pinheiro, Federal University of Pernambuco, with the number of fall UFPEPOR 1551. After collected, the material was washed in running water and, then, separated. After, the sponge specimens were lyophilized and weighed. Subsequently, about 2 kg of lyophilized material was triturated and exhaustively extracted with ethanol and vacuum filtered with filter paper into a celite pad on Buchner funnel. The extraction solution was dried using a rotaevaporator to yield 10 g of material, providing a yield of 0.5%.

HPLC Analysis

The *Oceanapia* extract was extracted in a solid phase extraction cartridge (SPE C-18), which was previously activated using 10 ml of methanol and 10 ml of ultrapure water. Approximately 128.0 mg of the extract was solubilized with 50 µL of MeOH and H_2O (1:1) and added to the cartridge, washed with ultrapure water (10 ml). The retained material was eluted with methanol (10 ml) and the solvent was evaporated to obtain 13.9 mg of the methanolic fraction. This fraction was solubilized in MeOH (3.5 mg/ml) for analysis by HPLC-ELSD and quantification of β-sitosterol and stigmasterol.

Quantification of β-sitosterol and stigmasterol was performed using equipment from Shimadzu Prominence LC-20AT with an Evaporative Light Scattering detector–ELSD (Alltech Associates, United States), automatic injector SIL-20AC, oven CTO-20^a and degasser DGU-20A5. The chromatographic separation was performed with a column Luna C-18 (150 mm × 4.6 mm × 5 µm, Phenomenex). Methanol (HPLC grade) was used as mobile phase with flow of 1.0 ml/min, column temperature 40 °C, drift tube temperature of 70°C and a nitrogen flow at 2 ml/min. For filtering samples, 0.45 µm nylon filters were used (Whatman).

Animals

For the experimental protocols, 168 Swiss mice (*Mus musculus*) weighing 31.8 ± 0.4 g and 63 guinea pigs (*Cavia porcellus*) weighing 370.5 ± 6.1 g, of both sexes, were used. All animals were housed at "Professor Thomas George" Bioterium of the Instituto de Pesquisa em Fármacos e Medicamentos (IPeFarM)/

UFPB. The animals were kept in a 12-h light-dark cycle, with monitored temperature (21 ± 1°C), and unrestricted access to food and water. During *in vivo* experiments, researchers knew about the distribution of animals in the experimental groups. All the experimental procedures were formerly authorized by the Ethics Committee on Animal Use (CEUA)/UFPB, certificate no. 146/2015.

Solutions and Drugs

Magnesium sulfate ($MgSO_4$), potassium chloride (KCl), calcium chloride ($CaCl_2$), glucose, sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Vetec Química Fina Ltda. (Brazil). Atropine (99%), (S-(−)-Bay K8644), chloride verapamil, pyrilamine, carbamylcholine hydrochloride (CCh), Cremophor®, and histamine were obtained from Sigma-Aldrich (Brazil). Carboxymethylcellulose was obtained from Formula (Brazil). Castor oil was obtained from Farmax (Brazil). Loperamide (99%) was obtained from Janssen Cilag Farmacêutica Ltd. (Brazil) and activated charcoal was obtained from Proquímicos (Brazil). All substances were diluted in distilled water, and the extract was solubilized in Cremophor® (3%), whose concentration never exceeded 0.01% (v/v). At this concentration, this chemical is devoid of contractile or relaxant effects on the studied organ, according to previous obtained data (data not shown).

Measurement of Contractile Response

For isotonic contractions recording, segments of guinea pig ileum were suspended longitudinally by cotton yarn in organ baths (5 ml) and connected to an isotonic lever coupled to a smoked-drum kymograph (DTF, Brazil). Isometric transducers (TIM 05) coupled to an amplifier (AECAD04F) and connected to digital acquisition system AQCAD 2.1.6 from AVS Projetos (São Paulo, SP, Brazil) were used to record isometric contractions. To verify if the spasmolytic effect of OC-EtOH was reversible, the organ preparation was washed with the physiological solution and, after 30 min, a new contraction was induced in order to observe if organ responsiveness was altered (data not shown). In the cumulative concentration-response curves a new concentration of standard drugs or extract were only added when a plateau of the previous concentration was obtained.

Pharmacological Experiments

Effect of OC-EtOH on Basal Tonus of Guinea Pig Ileum

The animals were fasted for 18 h before the euthanasia and subjected to decapitation using a guillotine. The abdomen was opened and an ileum segment of approximately 15 cm in length was removed and placed in a Petri dish containing a modified Krebs nutrient solution at 37°C, at a pH of 7.4, and gassed with carbogen (95% O_2 and 5% CO_2). Segments of this organ measuring approximately 2 to 3 cm were suspended in organ baths (5 ml). During the stabilization period (30 min) the organs were washed every 15 min by modified Krebs solution (mM): NaCl (117.0), KCl (4.7), $MgSO_4$ (1.3), NaH_2PO_4 (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25.0) and glucose (11.0) at 37 °C and bubbled with a carbogen mixture in a resting tension of 1 g. After stabilization, an isotonic contraction with 40 mM KCl was induced to verify organ functionality. The preparations were washed just before adding OC-EtOH. Then, OC-EtOH was added and its effect on basal intestinal muscle tonus observed. A cumulative concentration-response curve to OC-EtOH was induced. The results were evaluated by contractile amplitude percentage response of OC-EtOH. The values of extract concentrations able to produce 50% of maximal effect (EC_{50}) were calculated by non-linear regression.

Effect of OC-EtOH on KCl, Carbachol or Histamine-Induced Tonic Contractions

To evaluate the relaxing effect of the extract against different contracting agents, guinea pig ileum was obtained as described above. After stabilization period, 40 mM KCl, 10^{-6} M histamine or 10^{-5} M CCh was added to promote an isometric contraction in different preparations. To obtain the contraction plateau, the preparation continued in the presence of the contractile substances (approximately 10 min) then the organ was washed. After 30 min elapsed, a new contraction was performed with the same contractile agents as before to add OC-EtOH cumulatively (0.1–2,187 μg/ml). Relaxation was expressed as the reverse percentage of initial contraction elicited by the contractile agents. EC_{50} was obtained graphically from the concentration-response curves.

Evaluation of Muscarinic and Histaminergic Receptors and Ca_V Participation in Spasmogenic Effect of OC-EtOH

After the stabilization period, isotonic contractions were induced with 40 mM KCl to check the organ's functionality and, after 15 min, two cumulative concentration-response curves of amplitudes similar to histamine, CCh (positive controls) or OC-EtOH were induced. Then, in the absence of the contractile agents or the extract, atropine (muscarinic antagonist, 10^{-9} and 3×10^{-8} M), pyrilamine (H_1 receptor antagonist, 10^{-9}–3×10^{-7} M) or verapamil (Ca_V blocker, 10^{-7}–10^{-6} M) was incubated for 15 min, in independent experiments (Ghayur and Gilani, 2005). After this period, a new cumulative concentration-response curve for CCh, histamine or OC-EtOH was obtained in the presence of atropine, pyrilamine or verapamil, in order to evaluate the participation of the muscarinic and histaminergic receptors, as well as th Ca_V.

The results were evaluated by comparing the percentage of contractile response in presence and absence of antagonists and blocker. The inhibitory effect was evaluated based on the analysis of EC_{50} values and E_{max} of OC-EtOH, which were calculated from the concentration-response curves obtained as described above.

Effect of OC-EtOH on KCl-Induced Tonic Contraction in Presence of Pyrilamine

Guinea pig ileum was obtained as described. After the stabilization, the preparation was washed and, after 15 min, pyrilamine was pre-incubated for 30 min to observe if the spasmogenic component of extract relaxation was removed. Then, an isometric contraction was induced with 40 mM KCl

and, during sustained tonic phase (8–10 min), OC-EtOH was added cumulatively (0.1–729 μg/ml). The relaxation produced by OC-EtOH was expressed as percentage contraction reverse produced by contractile agent. The EC_{50} values were calculated by nonlinear regression.

Effect of OC-EtOH on $CaCl_2$-Induced Contractions in Despolarizing Medium Nominally Without Ca^{2+}

After the stabilization period, a nominally Ca^{2+}-free depolarization solution (with 70 mM KCl in equimolar exchange for NaCl) was used to swap the modified Krebs solution, for a period of 45 min, to obtain an isotonic contraction. Then, $CaCl_2$ was added cumulatively to obtain a concentration-response curve, this step was performed in duplicate. Subsequently, OC-EtOH was pre-incubated at different concentrations and a new contraction curve was performed in the presence of $CaCl_2$ (Van Rossum, 1963) to assess the involvement of Ca_V in the relaxing effect of the extract. The maximal contraction obtained with the first $CaCl_2$ concentration-response curve was considered to be 100% (control), and all contractions were assessed in reference to it.

Effect of OC-EtOH on S-(-)-Bay K8644-Induced Tonic Contractions

To determine the Ca_V subtype involved in the extract relaxation activity, guinea pig ileum was obtained as described above. After stabilization period, pyrilamine was added for 30 min, to remove the spasmogenic component of the extract, then, 15 mM KCl was added for 10 min to promote partial depolarization of the ileum (Conte-Camerino et al., 1987). Subsequently, S-(-)-Bay K8644 3×10^{-7} M, a selective Ca_V1 agonist, induced a isometric contraction. During tonic phase of contraction, verapamil (positive control) or OC-EtOH (0.1–729 μg/ml) were added cumulatively. The relaxation was expressed as percentage of initial contraction reversal produced by S-(-)-Bay K8644 and EC_{50} value was calculated by nonlinear regression.

Behavioral Pharmacological Screening and Evaluation of Acute Toxicity of OC-EtOH in Mice

For evaluation of acute toxicity, we followed the methodology described by Organization for Economic Co-operation and Development (OECD) no. 423/2001 (OECD, 2001). Initially, three female mice were used, which, after fasting for 4 h, were treated with OC-EtOH (single dose of 2,000 mg/kg, p.o.) or saline +0.9% Cremophor® (control, p.o.). Animals were also evaluated for 14 days for survival analysis purposes, in order to estimate the extract dose that kills 50% of animals tested (LD_{50}).

After administration of OC-EtOH, a series of behavioral parameters were observed during first 4 h, such as hyperactivity, aggression, tremor, convulsion, piloerection, sedation, ataxia, catatonia, analgesia, loss of corneal and headset reflex, dyspnoea, ambulation, scaling, abdominal contractions, bleeding, self-mutilation and nausea-like behaviour, such as pica (adapted from Almeida et al., 1999; Batra and Schrott 2011). The ponderal evolution was also measured before treatment, 7 and 14 days after treatment. This protocol was performed twice, in different experiments.

Effect of OC-EtOH on Upper Gastrointestinal Transit in Mice

After 12 h of food restriction, 0.9% NaCl plus Cremophor® (10 ml/kg, negative control), atropine (2 mg/kg, positive control) or OC-EtOH (31.25; 62.5; 125, 250, 500 and 1,000 mg/kg) were administrated orally in male and female mice separated into three groups (n = 6, each). After 30 min, 5% activated charcoal solubilized in carboxymethylcellulose 0.5% (0.01 ml/g) was administered orally. After 30 min of administration of the marker (activated charcoal), the animals were euthanized by decapitation using guillotine, and an incision was made in the animal's abdomen to remove the small intestine and measure the distance traveled by the marker. The same methods were used as previously, but 30 min before the administration of the activated charcoal, the castor oil (0.01 ml/g) was added orally (Hsu, 1982; Than et al., 1989). The results were expressed as a percentage of the distance covered by the marker in relation to the total length of the small intestine. The inhibitory effect that was exerted by OC-EtOH was evaluated based on an ED_{50} analysis.

Effect of OC-EtOH on Castor Oil-Induced Diarrhea in Mice

Animals were treated orally with 0.9% NaCl plus Cremophor® (10 ml/kg, negative control), loperamide (10 mg/kg, positive control) or OC-EtOH (62.5; 125, 250, 500 and 1,000 mg/kg) in different groups (n = 6, each). Then, to induce diarrhea, castor oil (0.01 ml/g) was administered by gavage after 30 min of the initial treatments. These same animals were placed in boxes lined with white paper, separately, and observed for 4 h uninterrupted. The number of stools were assessed, as well as their consistency, and classified as solid or liquid to determine the total number of stools and the total quantity of liquid stool episode (Awouters et al., 1978). The inhibitory effect of OC-EtOH was evaluated based on the ED_{50} value, which is the dose of a drug that produces 50% of its maximal effect.

Statistical Analysis

The results were expressed as a percentage of the mean ± standard error of the mean (SEM) and statistically analyzed by the one-way ANOVA followed by Bonferroni's post-test for multiple comparisons. The null hypothesis was discarded when $p < 0.05$. The EC_{50} and ED_{50} values were calculated by nonlinear regression. The data were analyzed using GraphPad Prism® software version 5.01 (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

HPLC Analysis

In the chromatogram regarding the quantification of β-sitosterol and stigmasterol steroids, it was possible by high performance liquid chromatography–evaporative light-scattering detector (HPLC-ELSD). The chromatogram shows that the concentration of stigmasterol is greater than β-sitosterol and can be considered as chemical marker for this specie.

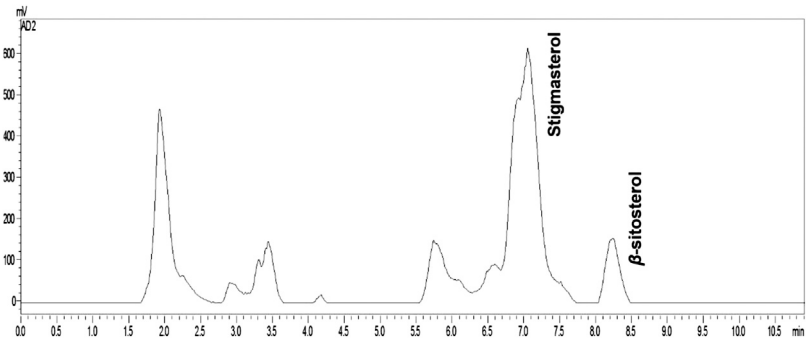

FIGURE 1 | Chromatogram (HPLC-ELSD) of *Oceanapia magna* fraction.

TABLE 1 | Quantification of stigmasterol and β-sitosterol from Oceanapia magna extract.

	100.0 (μg)of extract	R.T.	R^2
Stigmasterol	1.97	7.052	0.996
β-sitosterol	0.26	8.236	0.995

R.T., retention time; R^2, correlation coefficient.

Curiously, these compounds are isolated from plant species always together, however in this work it was possible to analyze and quantify the two separate constituents using C-18 column coupled to HPLC-ELSD (**Figure 1**; **Table 1**).

The calibration curve was based on peak area and using external standard in concentrations ranging from 0.0156 to 0.125 mg/ml, for both β-sitosterol (Sigma) and stigmasterol (Merck), both in triplicate. The limit of detection (LOD) and the limit of quantification (LOQ) for β-sitosterol were 0.135 and 0.408 μg, respectively, and for stigmasterol were 0.131 and 0.396 μg, respectively.

Effect of OC-EtOH on Basal Tonus of Guinea Pig Ileum

OC-EtOH (0.1–2187 μg/ml, n = 5), cumulatively added on basal tonus, contracted guinea pig ileum in a concentration-dependent manner (EC_{50} = 48.6 ± 2.7 μg/ml) with a similar amplitude to previous contraction induced by KCl 40 mM (**Figure 2A**). Maximum contractile effect (E_{max}) of the OC-EtOH was obtained with 729 μg/ml (**Figure 2**).

Evaluation of Muscarinic Receptors Participation in Spasmogenic Effect of OC-EtOH

In presence of atropine (10^{-8} and 3×10^{-8} M), a non-selective muscarinic antagonist (positive control), OC-EtOH-induced cumulative contraction curve (EC_{50} = 48.6 ± 2.7 μg/ml, n = 5) was not shifted (EC_{50} = 43.3 ± 2.6 and 41.4 ± 3.8 μg/ml, respectively, n = 5) and E_{max} was not modified (**Figure 3A**).

Differently, in presence of atropine (positive control), CCh-induced cumulative contraction curve (10^{-8}–3×10^{-5} M, n = 5) was shifted to the right in a parallel manner, without E_{max} reduction. EC_{50} values for CCh were $1.9 \pm 0.1 \times 10^{-7}$ M in the absence and $2.4 \pm 0.2 \times 10^{-7}$; $1.5 \pm 0.07 \times 10^{-6}$; $5.3 \pm 0.4 \times 10^{-6}$ and $2.3 \pm 0.04 \times 10^{-5}$ M in the presence of 10^{-9}; 3×10^{-9}; 10^{-8} and 3×10^{-8} M of atropine, respectively (**Figure 3B**).

Evaluation of Histamine Receptors Participation in Spasmogenic Effect of OC-EtOH

In presence of pyrilamine, a H_1 histamine receptors antagonist (positive control), the OC-EtOH-induced cumulative contraction curve (10^{-9}–3×10^{-7} M) was inhibited and shifted to the right in a non-parallel manner and presented E_{max} reduction of 100% (control) to 96.7 ± 1.1; 81.4 ± 3.6; 61.3 ± 2.8; 35.5 ± 2.1; 19.7 ± 2.7 and 0%, respectively (n = 5). The EC_{50} values of OC-EtOH were altered from 48.6 ± 2.7 μg/ml (control) to 41.3 ± 3.2; 88.6 ± 10; 131.2 ± 11 and 255.2 ± 25.7 μg/ml using the following pyrilamine concentrations 10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7} and 3×10^{-7} M, respectively (**Figure 3C**).

In the same way, in pyrilamine presence (positive control), histamine-induced cumulative contraction curve (10^{-9}–10^{-3} M) was shifted to the right in a parallel manner without E_{max} reduction (n = 5). The EC_{50} values to histamine was $2.3 \pm 0.2 \times 10^{-7}$ M in absence and $2.5 \pm 0.3 \times 10^{-7}$; $3.9 \pm 0.3 \times 10^{-7}$; $8.3 \pm 0.3 \times 10^{-7}$; $5.0 \pm 0.2 \times 10^{-6}$; $9.5 \pm 0.2 \times 10^{-6}$ and $3.1 \pm 0.3 \times 10^{-5}$ M in presence of 10^{-9}; 3×10^{-9}; 10^{-8}; 3×10^{-8}; 10^{-7} and 3×10^{-7} M of pyrilamine, respectively (**Figure 3D**).

Evaluation of Ca_V Participation in OC-EtOH Spasmogenic Effect

OC-EtOH-induced cumulative contraction curve (EC_{50} = 48.6 ± 2.7 μg/ml, n = 5) was inhibited in the presence of 10^{-7}, 3×10^{-7} and 10^{-6} M of verapamil, a Ca_V blocker, with abolition of OC-EtOH contractile effect (**Figure 4**).

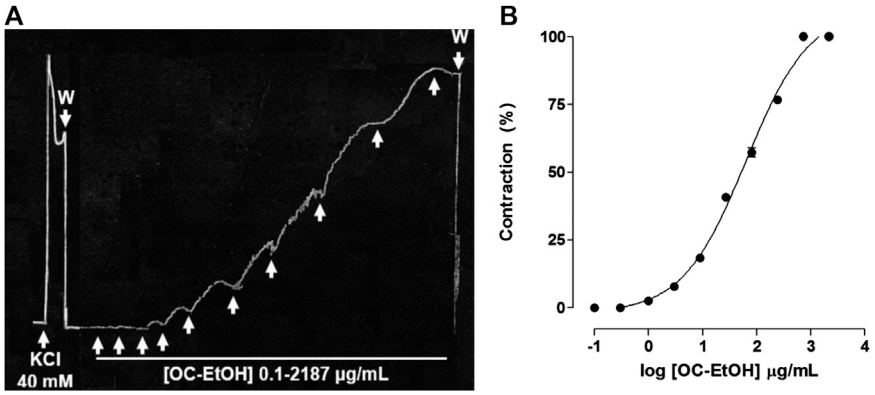

FIGURE 2 | Representative record of OC-EtOH (0.1–2,187 µg/ml, n = 5) cumulative contractile effect on basal tonus of guinea pig ileum **(A)**. Cumulative concentration-response curves to OC-EtOH (● on basal tonus of guinea pig ileum (n = 5) **(B)**. The upward arrows represent the concentration of 0.1; 0.3; 1; 3; 9; 27; 81; 243; 729 and 2,187 µg/ml of OC-EtOH. W, wash.

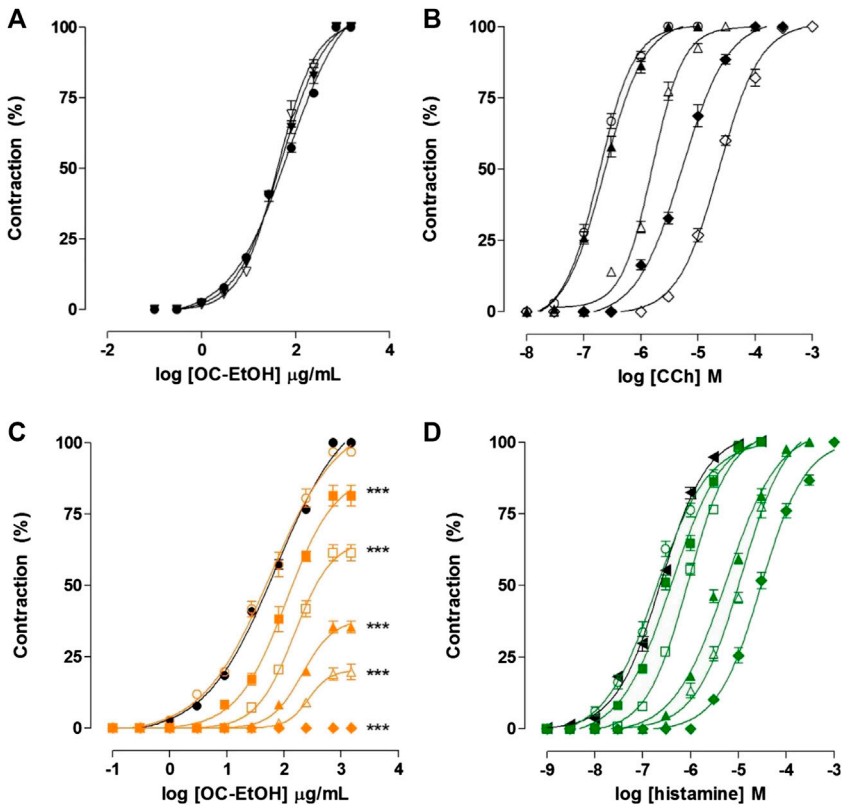

FIGURE 3 | Cumulative concentration–response curves to OC-EtOH **(A,C)**, CCh **(B)** and histamine **(D)** on guinea pig ileum in both absence (○, ●, ◄, ●) and presence of atropine 10^{-9} (▲), 3×10^{-9} (△), 10^{-8} (◆, ▼) and 3×10^{-8} M (◇, ▽) or pyrilamine 10^{-9} (green unfiled circle, orange unfiled circle), 3×10^{-9} (green filed square, orange filed square), 10^{-8} (green unfiled square, orange unfiled square), 3×10^{-8} (green filed triangle, orange filed triangle), 10^{-7} (green unfiled triangle, orange unfiled triangle) and 3×10^{-7} M (green filed diamond, orange filed diamond) on guinea pig ileum. Symbols and vertical bars represent the mean and S.E.M., respectively (n = 5). CCh = Carbachol. One-way ANOVA followed by Bonferroni's post-test: ***$p < 0.001$ (control vs. pyrilamine).

According to EC_{50} values of OC-EtOH, no differences between control curve (EC_{50} = 48.6 ± 2.7 µg/ml, n = 5) and those obtained in presence of 10^{-7} and 3×10^{-7} M of verapamil (EC_{50} = 55.9 ± 5.3 and 51.8 ± 3.7 µg/ml, respectively, n = 5) were observed. It was not possible to calculate the extract EC_{50} when the organ was incubated with 10^{-6} M of verapamil. E_{max} values were altered in the presence of 10^{-7}; 3×10^{-7} and 10^{-6} M of verapamil from 100% (control) to 90.0 ± 1.2; 53.2 ± 3.2 and 4.9 ± 1.3%, respectively (**Figure 4**).

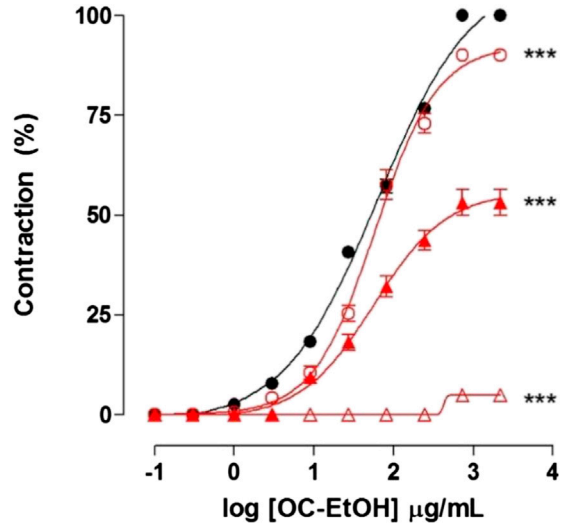

FIGURE 4 | Cumulative concentration–response curves to OC-EtOH in absence (●) and presence of verapamil 10^{-7} (x̄), 3×10^{-7} (▲) and 10^{-6} (△) on guinea pig ileum. Symbols and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test: ***$p < 0.001$ (control vs. verapamil).

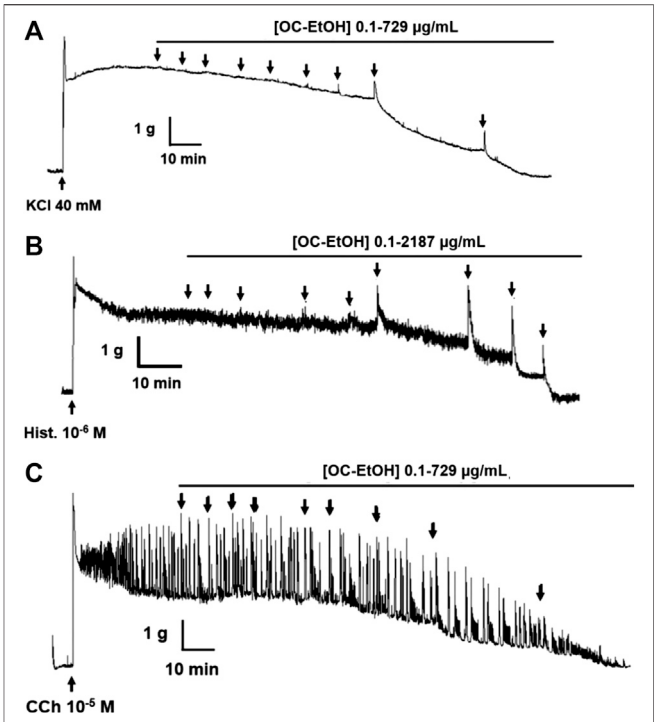

FIGURE 5 | Representative records of OC-EtOH relaxant effect on guinea pig ileum pre-contracted by KCl 40 mM **(A)**, histamine 10^{-6} **(B)** or CCh 10^{-5} M **(C)** (n = 5).

Effect of OC-EtOH on KCl-, Histamine- or Carbachol-Induced Tonic Contractions

OC-EtOH (0.1–2187 µg/ml, n = 5) induced a transient contractile effect (spasmogenic) followed by a relaxation (spasmolytic) in a concentration-dependent manner when guinea pig ileum was pre-contracted both with KCl 40 mM or histamine 10^{-6} M (**Figure 5**). Transient contraction induced by OC-EtOH at 27, 81, 243 and 729 µg/ml were 0.0; 24.2 ± 7.1; 83.1 ± 6.9 and 99.8 ± 0.1%, respectively, compared to tonic component induced by KCl and 12.4 ± 2.2; 59.8 ± 10.7; 86.5 ± 7.9 and 88.1 ± 5.8%, respectively, compared to histamine (**Figure 6**). OC-EtOH spasmogenic potency was around twice more potent when the contraction was induced by histamine (EC_{50} = 73.5 ± 6.9 µg/ml) than by KCl (EC_{50} = 176.6 ± 32.6 µg/ml) (**Figure 6**).

Differently, on tonic contraction induced by CCh 10^{-5} M, OC-EtOH (0.1–729 µg/ml, n = 5) showed only a relaxant effect profile (**Figure 5**). About OC-EtOH spasmolytic potency, it was equipotent to relax guinea pig ileum pre-contracted either by KCl (EC_{50} = 103.9 ± 8.6 µg/ml), histamine (EC_{50} = 90.1 ± 9.2 µg/ml) or CCh (EC_{50} = 97.1 ± 17.4 µg/ml). The OC-EtOH spasmolytic E_{max} (100%) was obtained at 729 µg/ml when guinea pig ileum was contracted by KCl or CCh, and at 2187 µg/ml when the organ was contracted by histamine (**Figure 7**).

Effect of OC-EtOH on KCl-Induced Tonic Contraction in the Presence of Pyrilamine

In the presence of pyrilamine (3×10^{-7} M), the transient contractile effect of OC-EtOH on KCl-induced tonic contraction was abolished (**Figure 8**). Interestingly, in presence of pyrilamine, OC-EtOH spasmolytic effect was potentiated (**Figure 9**).

According to EC_{50} values, OC-EtOH was about 2-fold more potent in relaxing KCl-induced tonic contraction in the presence of pyrilamine 3×10^{-7} M (EC_{50} = 62.9 ± 9.9 µg/ml, n = 5) than in its absence (EC_{50} = 103.9 ± 8.6 µg/ml, n = 5).

Effect of OC-EtOH on $CaCl_2$-Induced Contractions in Depolarizing Medium Nominally Without Ca^{2+}

OC-EtOH (27, 81, 243 and 729 µg/ml, n = 5) inhibited the contractions induced by cumulative increase in extracellular concentration of $CaCl_2$ in depolarizing medium nominally without Ca^{2+} (n = 5). $CaCl_2$ cumulative concentration-response curves were shifted to the right in a non-parallel manner and with E_{max} reduction from 100% (control) to 98.5 ± 0.9; 69.3 ± 4.8; 45.0 ± 4.4 and 1.8 ± 0.5%, respectively. EC_{50} values of the $CaCl_2$ were altered from $2.1 \pm 0.1 \times 10^{-3}$ M (control) to 2.5 ± 0.3; 3.9 ± 0.7 and $6.8 \pm 1.4 \times 10^{-3}$ M, respectively. The EC_{50} could not be determined when the organ was incubated with 729 µg/ml of the extract t (**Figure 10A**).

Similarly, verapamil, a Ca_V blocker (positive control; 3×10^{-8}–3×10^{-6} M) inhibited $CaCl_2$ cumulative concentration-response curves (10^{-5}–10^{-1} M). The curves were rightwards shifted, in a non-parallel manner, presenting E_{max} reduction from 100% (control) to 80.2 ± 3.7; 63.2 ± 0.9; 35.6 ± 2.9 and 0%, respectively. EC_{50} values of the $CaCl_2$ were altered

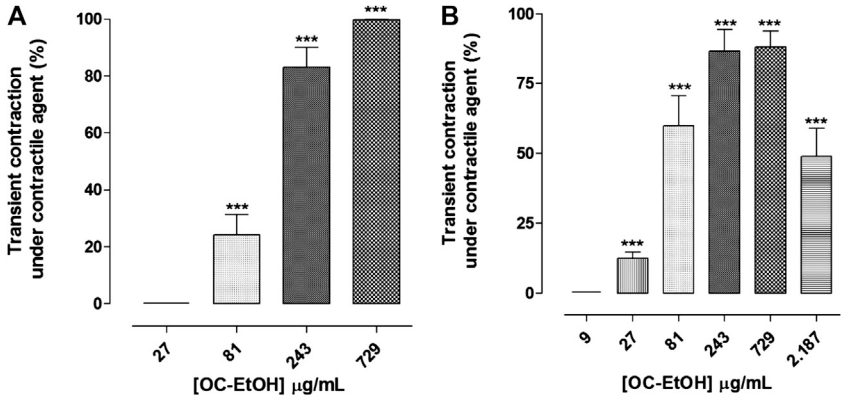

FIGURE 6 | OC-EtOH contractile effect under KCl-**(A)** and histamine-**(B)** induced tonic contractions on guinea pig ileum. Vertical bars represent the means and S.E.M., respectively. One-way ANOVA followed by Bonferroni's post-test, significant differences are indicated by ***$p < 0.001$ (n = 5).

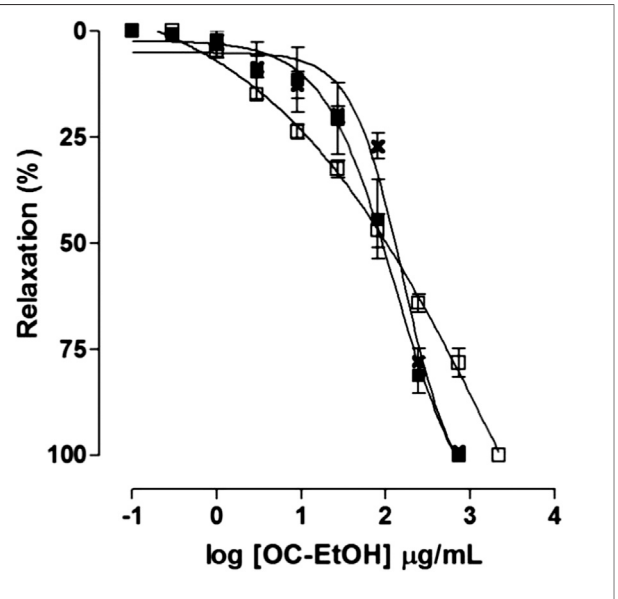

FIGURE 7 | Effect of OC-EtOH on guinea pig ileum pre-contracted with KCl 40 mM (✖), histamine 10^{-6} M (□) or CCh 10^{-5} M (■) (n = 5). Symbols and vertical bars represent the mean and S.E.M., respectively.

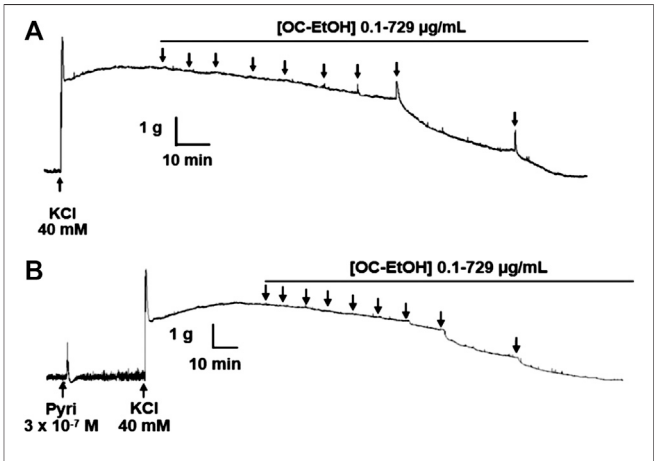

FIGURE 8 | Representative records of OC-EtOH relaxant effect on guinea pig ileum pre-contracted with KCl 40 mM in absence **(A)** and presence **(B)** of pyrilamine (n = 5). Pyri, pyrilamine.

from $2.1 \pm 0.3 \times 10^{-3}$ M (control) to 2.4 ± 0.2; 3.5 ± 0.4; $7.4 \pm 0.03 \times 10^{-3}$ and $1.2 \pm 0.01 \times 10^{-2}$ M; respectively (**Figure 10B**).

Effect of OC-EtOH on S-(-)-Bay K8644-Induced Tonic Contractions

OC-EtOH relaxed guinea pig ileum pre-contracted with S-(-)-Bay K8644 3×10^{-7} M, a Ca_V agonist, in pyrilamine presence (EC_{50} = 166.9 ± 17.8 μg/ml, n = 5) and the relaxant potency of the extract was reduced by 2.6 times when compared to pre-contracted ileum with KCl 40 mM (EC_{50} = 62.9 ± 9.9 μg/ml n = 5) (**Figure 11A**).

As positive control, verapamil relaxed guinea pig ileum pre-contracted with KCl 40 mM (EC_{50} = 0.0094 ± 0.0006 μg/ml) and S-(-)-Bay K8644 3×10^{-7} M (EC_{50} = 0.30 ± 0.02 μg/ml, n = 5), in medium partially depolarized with KCl 15 mM (**Figure 11B**).

Behavioral Pharmacological Screening and Evaluation of Acute Toxicity of OC-EtOH in Mice

After OC-EtOH oral administration (2,000 mg/kg), no behavioral changes were observed in female mice (n = 6) in the evaluated experimental conditions during 4 h of observation. Weight assessment were performed before the treatment, 7 and 14 days after the treatment and no changes in animals' weight were observed in both negative control animals

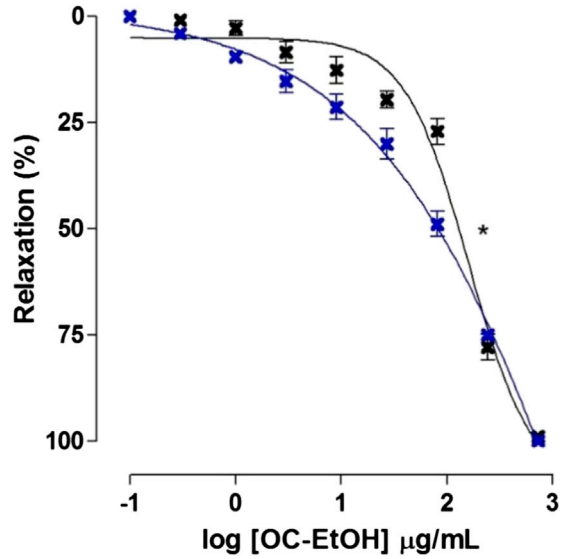

FIGURE 9 | Relaxant effect of OC-EtOH on guinea pig ileum pre-contracted with KCl 40 mM in absence (black cross) and presence (blue cross) of pyrilamine. Symbols and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test: *$p < 0.05$ (KCl vs. pyrilamine + KCl).

(33.5 ± 0.3, 33.7 ± 0.8 and 34.8 ± 0.6 g) and in the animals treated with OC-EtOH (29.0 ± 0.5, 30.0 ± 0.7 and 30.7 ± 0.6 g)., respectively. Interestingly, there were no death events during 14 days of observation.

Effect of OC-EtOH on Upper Gastrointestinal Transit in Mice

OC-EtOH (250, 500 and 1,000 mg/kg, p.o.) increased dose dependently the intestinal transit traveled by the activated charcoal (marker) (72.9 ± 1.2%) to 82.6 ± 1.8 and 90.5 ± 1.5%, respectively, showing an ED_{50} of 477.6 ± 14.8 mg/ml (n = 6). The distance traveled by the marker in the intestine of control animals (72.9 ± 1.2% of intestine total length) was decreased to 42.2 ± 1.4% when the animals were treated with atropine (2 mg/kg, p.o.) (**Figure 12A**).

In contrast, OC-EtOH (62.5; 125, 250, 500 and 1,000 mg/kg) inhibited the castor oil-induced intestinal transit, in a dose-dependent manner, reducing the distance in 41.3 ± 2.3; 55.6 ± 1.1; 73.2 ± 1.8; 88.5 ± 0.9 and 40.0 ± 2.8% (n = 6), respectively, in comparison to the negative control (0.9% saline + Cremophor®). It was also observed that atropine (2 mg/kg) inhibited 58.2 ± 3.1% of the distance traveled by the marker in the castor oil-induced intestinal transit as compared to the negative control (**Figure 12B**). OC-EtOH showed ED_{50} of 93.30 ± 7.2 mg/kg and the E_{max} (88.5 ± 0.9%) was observed at 500 mg/kg (n = 6).

Effect of OC-EtOH on Castor Oil-Induced Diarrhea in Mice

OC-EtOH (125, 250, 500 and 1,000 mg/kg, p.o., n = 6) inhibited castor oil-induced diarrhea equipotently and in a dose-dependent manner in terms of defecation frequency (15.4 ± 2.2; 29.8 ± 3.6; 59.8 ± 1.6 and 40.0 ± 5.3%, respectively) and number of liquid stools (30.0 ± 3.6; 66.7 ± 3.3; 85.0 ± 2.2 and 68.3 ± 5.4%) when compared to the negative control. It was also observed that loperamide (positive control, 10 mg/kg) inhibited 100% of castor oil-induced diarrhea (**Figure 13**). OC-EtOH showed ED_{50} and E_{max} 2-3-fold more potent in inhibiting liquid stool (ED_{50} = 189.1 ± 13.6 mg/ml, E_{max} = 15.0 ± 2.2%) compared to defecation frequency (ED_{50} = 387.7 ± 21.6 mg/ml, E_{max} = 40.2 ± 1.6%).

DISCUSSION

In this work, the effects of ethanolic extract obtained from *Oceanapia magna*. sponge (OC-EtOH) were investigated on

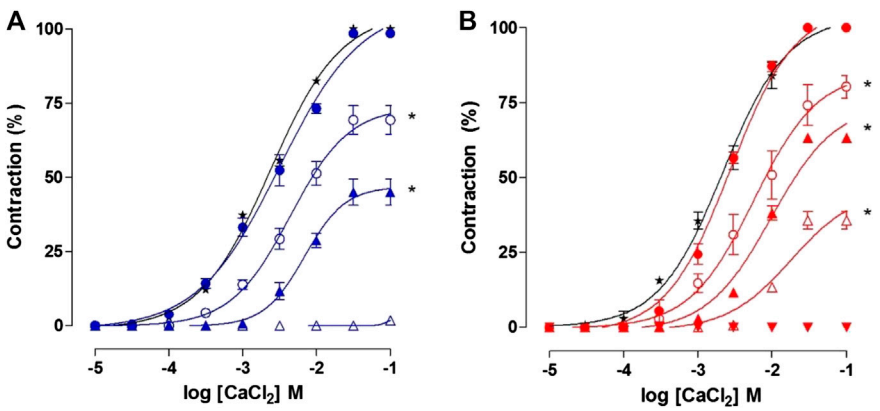

FIGURE 10 | Cumulative concentration–response curves to $CaCl_2$ in depolarizing medium nominally without Ca^{2+} in absence (★) and presence of 27 (blue filed circle), 81 (blue unfiled circle), 243 (blue filed triangle) and 729 µg/ml (blue unfiled triangle) of OC-EtOH **(A)** and in presence of 3×10^{-8} (red filed circle), 10^{-7} (red unfiled circle), 3×10^{-7} (red unfiled triangle10^{-6} (red filed triangle) and 3×10^{-6} M (red inverted filed triangle) of verapamil **(B)** on guinea pig ileum. Symbols and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test: *$p < 0.05$ (control *vs.* verapamil or OC-EtOH).

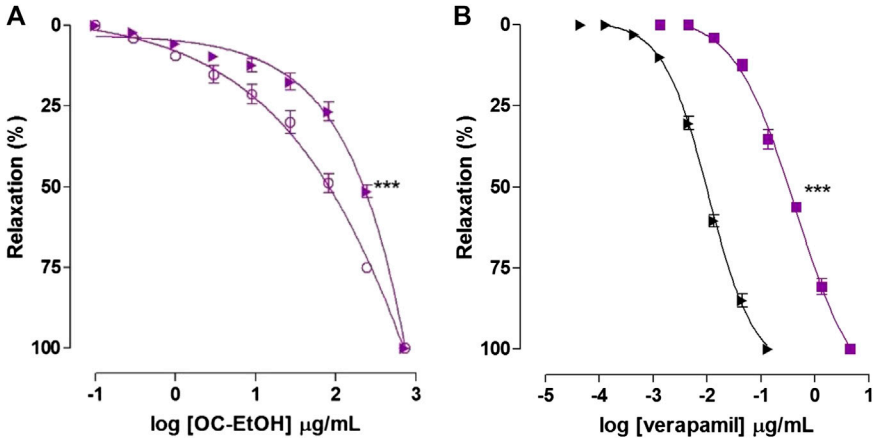

FIGURE 11 | Relaxant effect of OC-EtOH **(A)** on guinea pig ileum pre-contracted with KCl 40 mM (violet unfiled circle) and S-(-)-Bay K8644 3×10^{-7} M (violet right-pointing triangle) in pyrilamine presence and relaxant effect of verapamil **(B)** on guinea pig ileum pre-contracted with KCl 40 mM (black right-pointing triangle) and S-(-)-Bay K8644 3×10^{-7} M (violet filed square) in pyrilamine presence. Symbols and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test: ***$p < 0.001$ (S-(-)-Bay K8644 vs. KCl).

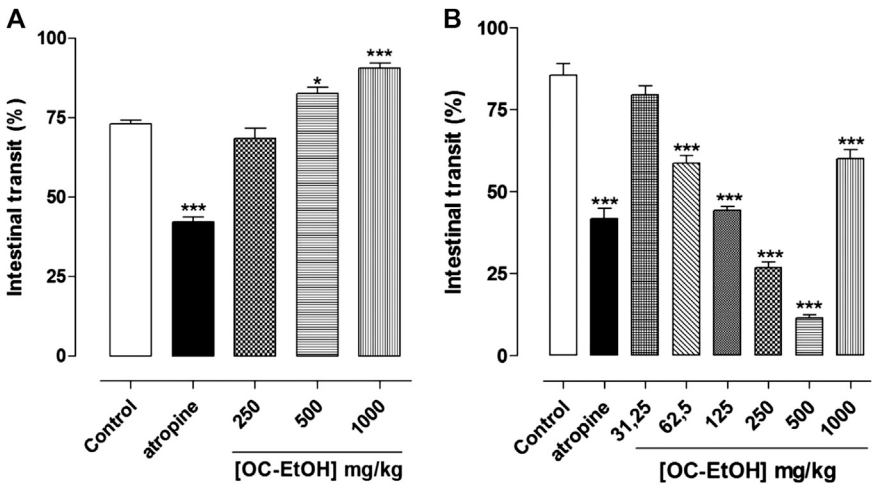

FIGURE 12 | Effect of OC-EtOH on normal **(A)** and castor-oil-induced **(B)** upper gastrointestinal transit in mice (n = 6). Columns and vertical bars represent the mean and S.E.M., respectively. One-way ANOVA followed by Bonferroni's post-test: *$p < 0.05$ and ***$p < 0.001$ (saline vs. atropine/OC-EtOH).

rodents' intestinal smooth muscle. It was demonstrated, for the first time, that OC-EtOH has dual effect on gastrointestinal tract, presenting spasmogenic action in guinea pig ileum, through histamine receptors (H_1 subtype) activation, and spasmolytic effect by blocking the influx of Ca^{2+} through voltage-gated calcium channels (Ca_V1). Therefore, *Oceanapia magna*. sponge demonstrated to be a potential source for medicinal metabolites that could be used for intestinal diseases treatment, such as constipation and/or diarrhea.

Initially, the effect of OC-EtOH was assessed on guinea pig ileum basal tonus in order to evaluate its possible activity on intestinal smooth muscle. Thus, the extract promoted intestinal contraction in a concentration-dependent manner (**Figure 2**), showing the spasmogenic effect of OC-EtOH.

In view of these results, an investigation of the mechanism that leads the tested product to promote a contraction was carried out. One of the possible spasmogenic mechanisms could involve the activation of M3 and/or H1 receptors. These receptors are characterized as receptors coupled to $G_{q/11}$ protein-PLCβ effector system, resulting in an increase of intracellular Ca^{2+} concentration, leading to muscle contraction (Ehlert et al., 1999; Berridge et al., 2003). To evaluate the muscarinic receptors participation in spasmogenic effect, we used the standard drug atropine, a nonselective muscarinic receptors antagonist (Bashir et al., 2006). According to the results, it was observed that atropine, in different concentrations, did not inhibit OC-EtOH-induced cumulative contraction curves. Thus, muscarinic receptors activation by OC-EtOH was discarded in its contractile effect (**Figure 3**).

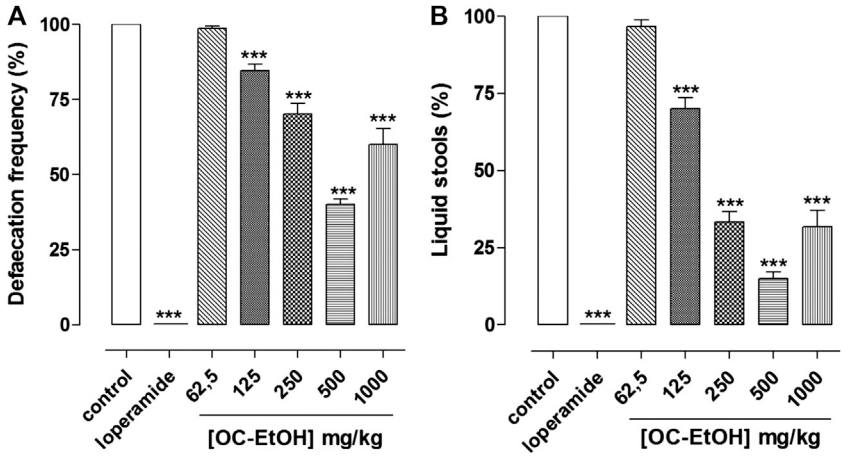

FIGURE 13 | Antidiarrheal effect of OC-EtOH on castor-oil-induced diarrhea in mice (n = 6). Percentage of total stools number **(A)** and percentage of liquid stools **(B)**. Columns and vertical bars represent mean and S.E.M., respectively. One-way ANOVA followed by Bonferroni's post-test: ***$p < 0.001$ (saline vs. loperamide/OC-EtOH).

H_1 histamine receptors are distributed in a variety of tissues, including airways, genitourinary, cardiovascular and gastrointestinal smooth muscle (Hill, 1990). To evaluate the hypothesis that OC-EtOH could act on H_1 receptors to produce its contractile effect, pyrilamine (H_1 receptor antagonist) was used as standard drug (Ghayur and Gilani, 2005). It was found that OC-EtOH-induced cumulative contraction curves in pyrilamine presence were shifted to right with reduction on both potency and efficacy. According to appearance of curves, it can be said that in OC-EtOH there are components that appear to act via H_1 receptors, however there are other components that act in other ways (**Figure 3**).

An increase of $[Ca^{2+}]_c$ in the smooth muscle is the main cause for contraction production (Hill-Eubanks et al., 2011). Ca_V are an essential element in initialization and/or maintenance of smooth muscle contraction. So, we decided to evaluate if Ca_V could be involved in the OC-EtOH spasmogenic mechanism of action. Verapamil, a Ca_V blocker, was used as a standard drug (Staneva-Stoytcheva and Venkova, 1992). OC-EtOH was added in a cumulative manner in the presence of different concentrations of verapamil and the curves were shifted to the right, with a reduction in both potency and efficacy of OC-EtOH (**Figure 4**). It can be suggested that the secondary metabolites found in OC-EtOH are activating Ca_V to promote its spasmogenic effect on guinea pig ileum.

Guinea pig ileum has a biphasic contraction (phasic and tonic) and, particularly, this organ depends on extracellular Ca^{2+} and the influence of this ion is higher on the tonic response (Triggle et al., 1979; Honda et al., 1996). Therefore, we decided to also evaluate the effect of OC-EtOH on tonic component of the contraction induced by different contractile agents such as KCl, histamine and CCh on guinea pig ileum.

The OC-EtOH presented a dual profile on tonic contraction induced by KCl and histamine: in the first moment, the extract induced a transient contraction followed by a slow relaxation, both in a concentration-dependent manner (**Figure 5**). No significant difference between the values for the spasmolytic extract potency was observed (**Figure 7**) and this indicates that OC-EtOH has secondary metabolites that act in a common pathway to modulate the intestinal smooth muscle motility. OC-EtOH showed a higher spasmogenic potency when compared to its spasmolytic effectiveness. These findings suggest, for the first time, that OC-EtOH, a marine natural product, presents dual effect (spasmogenic and spasmolytic) on guinea pig intestinal smooth muscle.

As showed until now, OC-EtOH possibly presents secondary metabolites with dual effect on intestinal smooth muscle. So, it was decided to proceed with the investigation of the mechanisms underlying the OC-EtOH spasmolytic activity.

To verify if the OC-EtOH spasmogenic component was interfering in the relaxant action, the experimental protocols were carried out in the presence of pyrilamine to avoid the OC-EtOH spasmogenic activity. The OC-EtOH-induced contraction was abolished in the presence of the H_1-antagonist, and the OC-EtOH relaxation effect on tonic contraction induced by KCl was about 2-fold more potent when compared to relaxation in antagonist absence (**Figures 8**,**9**). Therefore, this evidence suggests that the transient contractile effect promoted by OC-EtOH is due to a positive modulation of H_1 receptors on intestinal smooth muscle.

Smooth muscle is described as a biphasic organ, presenting two phases of contraction. In the first one, the contraction is rapid and transient. The second phase is characterized by a tonic contraction that is maintained for a while (Horie et al., 2005). Both phases are dependent on extracellular Ca^{2+}, as the whole contraction is inhibited by blocking Ca_V, which means that this is one of the main pathways involved in this entry (Rembold, 1996). Since OC-EtOH inhibited the tonic contractions induced by three distinct contractile agents in a concentration-dependent manner, presenting no difference among the pharmacological potencies (**Figure 7**), we theorized that OC-EtOH might be acting on Ca_V, by blocking Ca^{2+} influx once both coupling mechanisms, mixed

(histamine and CCh) and electromechanical (KCl), lead to tonic contractions almost exclusively by Ca^{2+} influx through Ca_V (Bolton, 1979; Rembold, 1996; Bolton, 2006).

Verapamil was used as positive control since it is a standard drug that blocks Ca_V. In presence of OC-EtOH, cumulative concentration-response curves to $CaCl_2$ were shifted to right, in a non-parallel manner, and reduced E_{max} (**Figure 10**). The results obtained were similar to the positive control. These findings support the hypothesis that OC-EtOH could inhibit Ca^{2+} influx through Ca_V to produce its spasmolytic effect.

To reinforce this hypothesis, OC-EtOH was assessed in relaxing the guinea pig ileum pre-contracted by S-(-)-Bay K8644 (Conte-Camerino et al., 1987), a Ca_V1 agonist (Ferrante et al., 1989). The experimental protocol was carried out in pyrilamine presence, in order to remove the spasmogenic effect of the extract, and verapamil was used as positive control. OC-EtOH relaxed the organ pre-contracted with S-(-)-Bay K8644 in a concentration-dependent manner and it was 2.6 times less potent when the organ was pre-contracted with KCl (**Figure 11**). So, surely, OC-EtOH blocked the Ca^{2+} channels to induce its spasmolytic effect on guinea pig ileum, and Ca_V1 is the channel subtype involved, but other targets are not discarded in the participation of OC-EtOH dual effect on intestinal smooth muscle.

Following the *in vitro* investigation, female mice received acute treatment of OC-EtOH 2,000 mg/kg (n = 6, p.o.) and animals were observed for 4 h. No behavioral changes were observed, discarding toxic effect on central and autonomic nervous systems. There was no death of animals during 14 days of observation and no interference in ponderal evolution of animals. This assay was divided into two groups with three animals each. So, in the dosing regimen used (2,000 mg/kg), OC-EtOH showed no toxic effect, and, according to 423/2001 Organization for Economic Cooperation and Development (OEDC) guideline, this result indicates a low acute toxicity and an estimate of LD_{50} greater than or equal to 5,000 mg/kg. This result shows that OC-EtOH can be considered safe for the following *in vivo* experimental protocols.

Since OC-EtOH presented spasmogenic and spasmolytic activity in intestinal smooth muscle and diarrhea and constipation can be caused, among other factors, due to changes in intestinal motility (Field and Semrad, 1993), we decided to investigate if OC-EtOH alters the intestinal motility in mice.

Interestingly, OC-EtOH did not inhibit normal upper gastrointestinal transit in mice, but an increase of the intestinal distance traveled by the marker was observed when animals were treated with doses of 500 and 1,000 mg/kg when compared to control group. Because at the dose of 500 mg/kg the observed effect was not 100%, the 1,000 mg/kg dose was used to make sure that the previous dose would be the maximum or not. The fact that the dose of 1,000 mg/ml gave a higher value can be explained by the desensitization of the organ or system, showing that the dose of 500 mg/kg is the one that produces the maximum effect. Given these results and corroborating data found *in vitro* assays, it may be suggested the OC-EtOH promotes a laxative effect under physiological conditions. Different results were observed in castor-oil-induced intestinal transit, since, in this biological context, OC-EtOH inhibited dose-dependently intestinal motility in mice (**Figure 12**). This fact may be directly related to an antidiarrheal effect and this might occur by intestinal motility inhibition, in pathological conditions, what could represent a relevant therapeutic effect. This effect can help to reduce the number of evacuations, increase water absorption due to slow passage of intestinal contents, and increase stool viscosity (Gurgel et al., 2007).

It is reported that castor oil inhibits Na^+/K^+-ATPase, reducing the absorption of normal liquid (Izzo et al., 1999), in addition to promoting the activation of adenylyl cyclase, stimulating the production of cAMP (Gaginella and Bass, 1978). Ricinoleic acid is also responsible for producing irritating and inflammation of the intestinal mucosa, leading to the release of several inflammatory mediators, such as prostaglandins, nitric oxide (NO), platelet activation factor (PAF) and tachykinins (Awouters et al., 1978). In addition, the ricinoleic acid is an agonist of prostaglandin E_2 (PGE_2) receptors, activating EP_3 and EP_4, and the activation of the former in intestinal smooth muscle is responsible for promoting its diarrheal effect. OC-EtOH also inhibited castor oil-induced diarrhea in a concentration-dependent manner by inhibiting both defecation frequency and liquid feces. The extract was twice more potent and effective in inhibiting watery stools compared with total defecation frequency. This fact is also quite interesting, as it may suggest that OC-EtOH would be interfering in one of the aforementioned ricinoleic acid-activated pathways, acting on liquid stools formation without preventing normal feces formation, which could cause constipation (**Figure 13**).

In conclusion, OC-EtOH showed to be a safe marine product since it was assessed by an animal model of toxicity. For the first time, a biological activity was assigned for *Oceanapia magna* and it was observed that OC-EtOH presented dual effect (spasmogenic due to histamine receptors activation, and spasmolytic by Ca^{2+} influx through Ca_V blockade). Further studies are needed to verify other possible targets for the mechanism of action of OC-EtOH. Its potential benefits by acting in a more balanced way than compounds with a single target and, consequently, would show a satisfactory pharmacological combination effect and/or reduced side effects. Thus, *Oceanapia magna* sponge arises as an oceanic natural product with potential medical use to intestinal diseases, such as diarrhea.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Use (CEUA)/UFPB, certificate no. 146/2015.

AUTHOR CONTRIBUTIONS

JP, IF, FO, SF, and GM were involved in experimental work and preparation of the manuscript. BVS, UP, and TS contributed to obtaining and standardizing the OC-EtOH. BAS and FC elaborated the experimental design and all aspects of this project.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chronic Metformin Therapy is Associated with a Lower Risk of Hemorrhoid in Patients with Type 2 Diabetes Mellitus

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Background: Metformin has anti-inflammatory property and reduces the risk of varicose vein in our previous study.

Aim: To investigate the risk of hemorrhoid, another common disease involving the hemorrhoidal venous plexus, in ever vs. never users of metformin in patients with type 2 diabetes mellitus.

Methods: This is a population-based retrospective cohort study. Patients with new-onset type 2 diabetes mellitus during 1999–2005 were enrolled from Taiwan's National Health Insurance. All patients who were alive on January 1, 2006 were followed up until December 31, 2011. Analyses were conducted in both an unmatched cohort of 152,347 ever users and 19,523 never users and in 19,498 propensity score (PS)-matched pairs of ever and never users. Traditional Cox regression and Cox regression incorporated with the inverse probability of treatment weighting (IPTW) using the PS were used to estimate hazard ratios.

Results: New-onset hemorrhoid was diagnosed in 8,211 ever users and 2025 never users in the unmatched cohort and in 1,089 ever users and 2022 never users in the matched cohort. The hazard ratio for ever vs. never users derived from the traditional Cox regression was 0.464 (95% confidence interval: 0.440–0.488) in the unmatched cohort; and was 0.488 (0.453–0.525) in the matched cohort. In the IPTW models, the hazard ratio was 0.464 (0.442–0.487) in the unmatched cohort and was 0.492 (0.457–0.530) in the matched cohort. A dose-response pattern was observed while comparing the tertiles of cumulative duration, cumulative dose and defined daily dose of metformin therapy to never users in all analyses. A risk reduction of approximately 40–50% was consistently observed in various sensitivity analyses.

Conclusion: Chronic therapy with metformin in patients with type 2 diabetes mellitus is associated with a lower risk of hemorrhoid.

Keywords: diabetes mellitus, hemorrhoid, metformin, National Health Insurance, pharmacoepidemiology, Taiwan

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INTRODUCTION

Hemorrhoid is a very common disease that affects the anorectal area resulting in distal displacement of the anal cushions. Clinical presentations include vascular congestion, inflammation, itching, soiling, pain, bleeding and prolapse. The precise cause remains unknown but conditions that increase intra-abdominal pressure may increase the pressure in the hemorrhoidal venous plexus and precipitate its development. These include straining during constipation, chronic diarrhea, irritable bowel syndrome, pregnancy, delivery, obesity, lack of exercise, low-fiber diets, cigarette smoking, anal intercourse, long-time standing, cirrhosis with ascites, pelvic floor dysfunction and chronic cough (Johannsson et al., 2005; Helvaci et al., 2009; Mott et al., 2018; Yetkin and Ozturk, 2018; Ekici et al., 2019; Nagaraj et al., 2019). Hemorrhoid is age-related with peak prevalence at the age of 45–65 years. Its prevalence was 39% in a routine colorectal cancer screening conducted in Vienna, Austria and among them 55% were asymptomatic (Riss et al., 2012). Medical management, dietary modification and behavioral therapies are initial treatment, but surgical interventions may be necessary in some patients (Mott et al., 2018).

Hemorrhoid and varicose veins share common pathophysiology and may have similar risk factors (Mott et al., 2018; Yetkin and Ozturk, 2018; Ekici et al., 2019). Metformin is now recommended by major treatment guidelines as the first-line therapy for patients with type 2 diabetes mellitus (American Diabetes Association, 2017; Salvatore et al., 2020) because of its multiple benefits beyond glycemic control, including immune modification, anti-inflammation, anti-atherosclerosis, anti-cancer and anti-aging (Wang et al., 2017). Our recent study suggested that use of metformin in patients with type 2 diabetes mellitus is also associated with a lower risk of varicose veins (Tseng, 2020). To our knowledge, there has been no previous study investigating whether metformin use might reduce the risk of hemorrhoid. Because it is reasonable to speculate that the beneficial effect of metformin on varicose veins might also be applied to hemorrhoid, the purpose of the present study was to evaluate whether metformin use in patients with type 2 diabetes mellitus could be associated with a lower risk of hemorrhoid, by using a nationwide reimbursement database of the Taiwan's National Health Insurance (NHI) and comparing the risk of hemorrhoid between ever users and never users of metformin, in both an unmatched cohort and a cohort of 1:1 matched pairs of ever and never users who were well balanced in characteristics based on propensity score (PS).

MATERIALS AND METHODS

Taiwan has implemented a universal and compulsory healthcare system, the NHI, since March 1995. More than 99% of Taiwan's population is covered by the NHI. The Bureau of the NHI has contracts with all hospitals and 93% of all medical settings, and keeps all computer records of disease diagnoses, medication prescriptions and clinical procedures submitted for reimbursement purpose. After ethics review and approval by the Research Ethics Committee of the National Health Research Institutes, the reimbursement database can be used for academic research. Informed consent from the patients was not required according to the local regulations because all personal information has been de-identified for the protection of privacy. The present study was granted an approval number of 99274.

Disease diagnoses during the study period were coded by the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM). Diabetes was coded 250.XX and hemorrhoid was coded 455.

The database has been described in more detail in a previously published paper (Tseng, 2017). The procedures used to enroll an unmatched original cohort and a cohort of 1:1 PS-matched pairs of ever and never users of metformin derived from the original cohort are shown in **Figure 1**. At first, we identified 423,949 patients with a new diagnosis of diabetes mellitus during 1999–2005 in the outpatient clinics and having been prescribed antidiabetic drugs for 2 or more times in the database. The following ineligible patients were then excluded: 1) ever users of metformin who had been prescribed other antidiabetic drugs before the initiation of metformin (n = 183,837); 2) patients with a diagnosis of type 1 diabetes mellitus (n = 2,062), 3) patients with missing data (n = 420), 4) patients with a diagnosis of hemorrhoid before entry or within 6 months of the diagnosis of diabetes mellitus (n = 29,235), 5) patients with a diagnosis of any cancer before entry or within 6 months of the diagnosis of diabetes mellitus (n = 21,206), 6) patients who had been followed up for <180 days (n = 15,319). As a result, 152,347 ever users of metformin and 19,523 never users of metformin were identified and they were considered as the unmatched original cohort. All characteristics shown in **Table 1** plus the date of entry were then used to create the PS by logistic regression. A cohort of 19,498 PS-matched pairs of ever users and never users of metformin (the matched cohort) was then created from the unmatched cohort by using the Greedy $8 \rightarrow 1$ digit match algorithm proposed by Parsons (Parsons, 2020). The following analyses were conducted in both the unmatched cohort and the matched cohort to examine the consistency of the findings.

Potential confounders were categorized into the following subgroups: demographic data, major comorbidities, diabetes-related complications, antidiabetic drugs, commonly encountered comorbidities and potential risk factors and commonly used medications in patients with diabetes mellitus. Demographic data included age, sex, occupation and living region. Major comorbidities included in the analyses were hypertension (401–405), dyslipidemia (272.0–272.4) and obesity (278). Diabetes-related complications included nephropathy (580–589), eye diseases (250.5: diabetes with ophthalmic manifestations, 362.0: diabetic retinopathy, 369: blindness and low vision, 366.41: diabetic cataract, and 365.44: glaucoma associated with systemic syndromes), diabetic polyneuropathy (357.2 and 250.6), stroke (430–438), ischemic heart disease (410–414) and peripheral arterial disease (250.7, 785.4, 443.81 and 440–448). Antidiabetic drugs were categorized as insulin, sulfonylurea, meglitinide, acarbose, rosiglitazone and pioglitazone. Commonly encountered comorbidities and

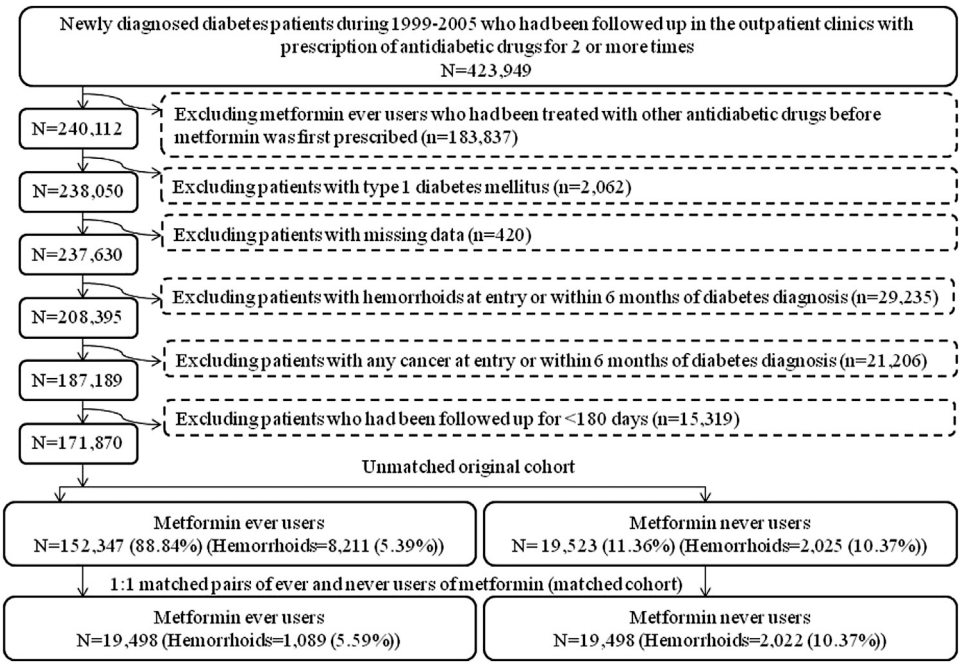

FIGURE 1 | Flowchart showing the procedures in creating an unmatched cohort and a cohort of 1:1 matched-pairs (based on propensity score) of metformin ever users and never users from the reimbursement database of the Taiwan's National Health Insurance.

potential risk factors of hemorrhoid included chronic obstructive pulmonary disease (a surrogate for smoking, 490–496), tobacco abuse (305.1, 649.0 and 989.84), alcohol-related diagnoses (291, 303, 535.3, 571.0–571.3 and 980.0), cancer (140–208), heart failure (398.91, 402.11, 402.91, 404.11, 404.13, 404.91, 404.93 and 428), Parkinson's disease (332), dementia (abridged codes of A210 or A222, or ICD-9-CM codes of 290.0, 290.1, 290.2, 290.4, 294.1, 331.0–331.2 and 331.7–331.9), head injury (959.01) and valvular heart disease (394–396, 424 and 746). Medications commonly used by patients with diabetes mellitus included angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, calcium channel blockers, statins, fibrates and aspirin.

The living region and occupation were described in detail elsewhere (Tseng, 2012). In brief, the living region was classified as Taipei, Northern, Central, Southern, and Kao-Ping/Eastern. Occupation was classified as class I (civil servants, teachers, employees of governmental or private businesses, professionals and technicians), class II (people without a specific employer, self-employed people or seamen), class III (farmers or fishermen) and class IV (low-income families supported by social welfare, or veterans).

Standardized difference for each of the above potential confounders was calculated as a test of balance diagnostic according to Austin and Stuart (2015). A cutoff value of >10% was used as an indication of potential confounding from the variable.

Cumulative duration of metformin therapy in months, cumulative dose of metformin therapy in grams and units of defined daily dose (DDD) of metformin use per day [1 unit of DDD for metformin = 2 g (Chang et al., 2018)] were calculated and their tertiles were used for dose-response analyses. Incidence density of hemorrhoid was calculated for never users of metformin, ever users of metformin, the tertiles of cumulative duration of metformin therapy, the tertiles of cumulative dose of metformin therapy and the tertiles of units of DDD of metformin use per day. Start of follow-up was set on January 1, 2006. The numerator of the incidence was the case number of newly identified hemorrhoid during follow-up. The denominator (expressed in person-years) was the follow-up duration between the start of follow-up and the time of a new diagnosis of hemorrhoid, or the date of death or the date of the last reimbursement record, whichever occurred first up to December 31, 2011.

Kaplan–Meier curves of hemorrhoid-free probability were plotted for never users and ever users of metformin, for never users and tertiles of cumulative duration of metformin therapy, for never users and tertiles of cumulative dose of metformin therapy and for never users and tertiles of units of DDD per day. The significance in different subgroups of metformin exposure was tested by logrank test.

The subgroup of never users of metformin was used as the referent group in the estimation of hazard ratios and their 95% confidence intervals for hemorrhoid for ever users and for each tertile of cumulative duration, cumulative dose and units of DDD. Both traditional Cox regression and Cox regression incorporated with the inverse probability of treatment weighting (IPTW) using the PS were used to examine the consistency of the findings. The IPTW method was proposed by Austin to reduce the potential confounding from the differences in characteristics (Austin, 2013).

TABLE 1 | Characteristics in never and ever users of metformin in the unmatched cohort and the matched cohort.

Variable	Unmatched cohort					Matched cohort				
	Never users (*n* = 19,523)		Ever users (*n* = 152,347)		SD	Never users (*n* = 19,498)		Ever users (*n* = 19,498)		SD
	n	%	*n*	%		*n*	%	*n*	%	
Demographic data										
Age[a] (years)	68.32	13.34	64.11	11.94	−39.37	68.30	13.33	68.09	12.29	−1.09
Sex (men)	10,548	54.03	79,205	51.99	−4.11	10,537	54.04	10,430	53.49	−1.28
Occupation										
I	7,039	36.05	56,816	37.29		7,032	36.07	7,010	35.95	
II	3,249	16.64	32,363	21.24	13.52	3,246	16.65	3,273	16.79	0.25
III	4,814	24.66	35,482	23.29	−3.51	4,809	24.66	4,885	25.05	1.21
IV	4,421	22.65	27,686	18.17	−13.11	4,411	22.62	4,330	22.21	−0.89
Living region										
Taipei	6,518	33.39	47,352	31.08		6,508	33.38	6,404	32.84	
Northern	2,061	10.56	17,604	11.56	3.38	2,061	10.57	2,057	10.55	−0.04
Central	3,359	17.21	27,594	18.11	2.21	3,353	17.20	3,391	17.39	0.49
Southern	3,438	17.61	26,418	17.34	−0.56	3,435	17.62	3,447	17.68	0.40
Kao-Ping and Eastern	4,147	21.24	33,379	21.91	2.61	4,141	21.24	4,199	21.54	0.86
Major comorbidities										
Hypertension	16,513	84.58	126,762	83.21	−5.14	16,489	84.57	16,474	84.49	−0.03
Dyslipidemia	13,461	68.95	123,784	81.25	33.25	13,452	68.99	13,535	69.42	0.92
Obesity	452	2.32	6,212	4.08	10.69	452	2.32	479	2.46	0.90
Diabetes-related complications										
Nephropathy	7,173	36.74	43,358	28.46	−22.29	7,153	36.69	7,083	36.33	−1.15
Eye diseases	3,284	16.82	48,021	31.52	36.51	3,283	16.84	3,140	16.10	−2.60
Diabetic polyneuropathy	3,302	16.91	44,227	29.03	30.82	3,302	16.94	3,272	16.78	−0.78
Stroke	7,710	39.49	49,996	32.82	−18.19	7,689	39.43	7,562	38.78	−1.22
Ischemic heart disease	10,191	52.20	72,530	47.61	−11.73	10,175	52.18	10,001	51.29	−1.62
Peripheral arterial disease	4,864	24.91	40,714	26.72	2.95	4,854	24.89	4,733	24.27	−1.59
Antidiabetic drugs										
Insulin	1,658	8.49	3,487	2.29	−34.88	1,641	8.42	1,489	7.64	−5.15
Sulfonylurea	14,157	72.51	109,708	72.01	8.27	14,155	72.60	14,649	75.13	4.77
Meglitinide	1,695	8.68	6,113	4.01	−21.47	1,689	8.66	1,681	8.62	−0.56
Acarbose	2,138	10.95	8,189	5.38	−19.59	2,131	10.93	2,301	11.80	0.79
Rosiglitazone	558	2.86	7,270	4.77	11.29	558	2.86	613	3.14	0.72
Pioglitazone	444	2.27	3,866	2.54	3.24	443	2.27	473	2.43	0.17
Commonly encountered comorbidities and potential risk factors										
Chronic obstructive pulmonary disease	10,476	53.66	76,095	49.95	−10.34	10,454	53.62	10,478	53.74	0.45
Tobacco abuse	440	2.25	5,437	3.57	8.61	440	2.26	394	2.02	−1.70
Alcohol-related diagnoses	1,188	6.09	9,438	6.20	0.39	1,188	6.09	1,220	6.26	0.55
Cancer	1,822	9.33	11,224	7.37	−7.67	1,817	9.32	1,872	9.60	0.94
Heart failure	5,462	27.98	30,250	19.86	−24.05	5,449	27.95	5,267	27.01	−2.03
Parkinson's Disease	1,120	5.74	5,625	3.69	−12.10	1,112	5.70	1,047	5.37	−1.30
Dementia	2,196	11.25	10,980	7.21	−19.61	2,508	12.86	2,364	12.12	−2.05
Head injury	730	3.74	6,034	3.96	1.18	729	3.74	691	3.54	−1.09
Valvular heart disease	2,791	14.30	15,552	10.21	−16.13	2,785	14.28	2,707	13.88	−1.05
Commonly used medications in diabetes patients										
Angiotensin converting enzyme inhibitors/angiotensin receptor blockers	14,238	72.93	113,939	74.79	3.61	14,216	72.91	14,178	72.72	−0.38
Calcium channel blockers	13,118	67.19	94,952	62.33	−12.16	13,099	67.18	13,036	66.86	−0.43
Statins	10,002	51.23	98,120	64.41	30.28	9,999	51.28	9,958	51.07	−0.66
Fibrates	6,239	31.96	63,635	41.77	23.11	6,237	31.99	6,161	31.60	−0.84
Aspirin	11,985	61.39	96,528	63.36	2.65	11,966	61.37	12,019	61.64	0.70

[a]*Age is expressed as mean and standard deviation.*

Refer to "Materials and Methods" for the classification of occupation.

SD: standardized difference.

Sensitivity analyses were conducted by estimating the overall hazard ratios for ever users vs. never users in more homogeneous subgroups of patients. First, patients with irregular refill of metformin were excluded. This was done by excluding patients who received two consecutive prescriptions of metformin spanning a period of >4 months (Model I). Because the NHI allows drug prescription for chronic diseases for not more than 3 months at each time, these patients represented those who have delayed refill of metformin for more than one month after a previous prescription for 3 months. Second, patients who happened to be treated with incretin-based therapies, either with a dipeptidyl peptidase 4 inhibitor or a

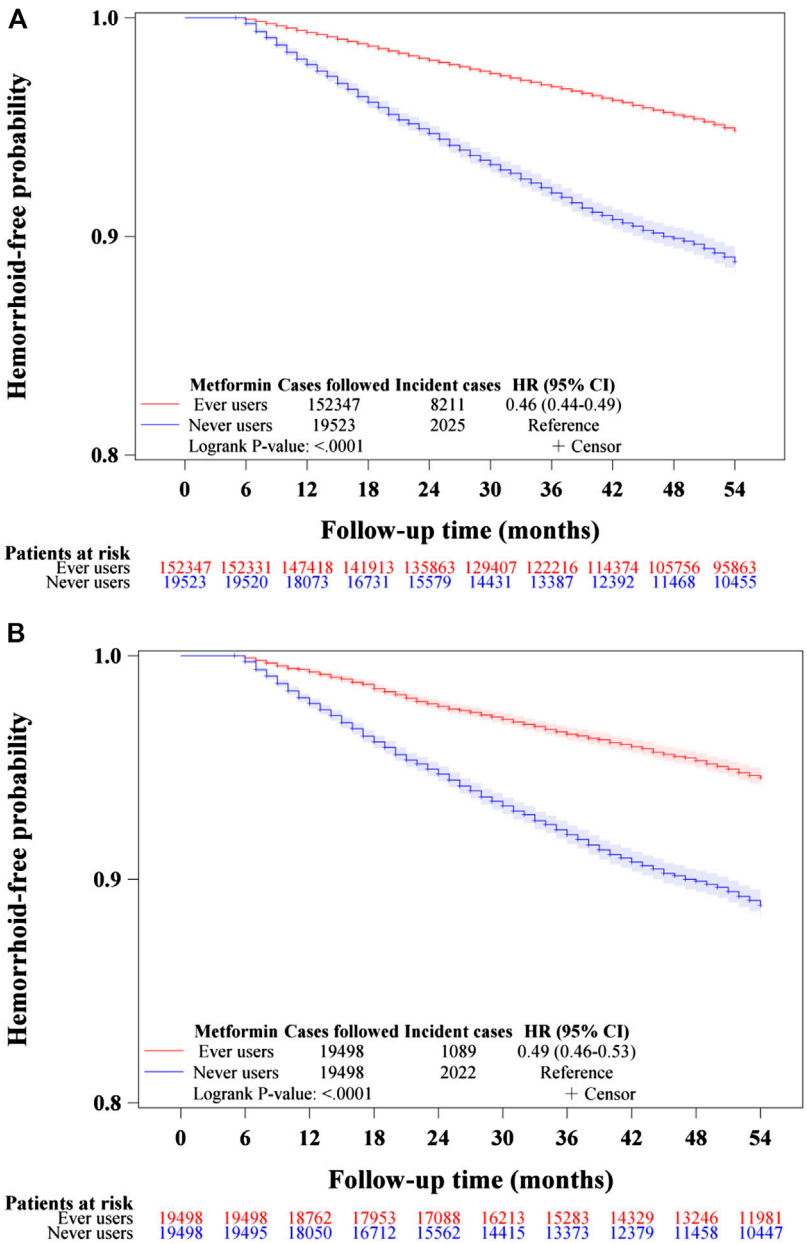

FIGURE 2 | Kaplan–Meier curves comparing hemorrhoid-free probability in never users and ever users of metformin in the unmatched cohort **(A)** and the matched cohort **(B)**. HR: hazard ratio, CI: confidence interval.

glucagon-like peptide 1 receptor agonist, during the follow-up period were excluded (Model II). In Taiwan, the first incretin-based therapy was not reimbursed by the NHI until after 2009. The exclusion of these patients avoided the potential impact of incretin-based therapies during follow-up. Third, patients enrolled during two different periods of 1999–2002 (Model III) and 2003–2005 (Model IV) were analyzed separately. Because more and more antidiabetic drugs have been introduced into clinical use and the guidelines for the use of antidiabetic drugs have evolved over the last 2 decades, these sensitivity analyses examined whether the results could be influenced by these changes. Fourth, to reduce the potential risk of misdiagnosis and misclassification of hemorrhoid at the outpatient clinics, analysis was performed by re-defining the outcome of hemorrhoid by using a more stringent criteria, i.e., as a primary diagnosis at hospitalization (Model V). These hospitalized patients might represent those who had more severe clinical manifestations of hemorrhoid and surgical intervention or more intensive medical care was required. Fifth, subgroup analyses were conducted with regards to the use of aspirin (Model VI: patients receiving aspirin; Model VII: patients not receiving aspirin) and calcium channel blockers (Model VIII: patients

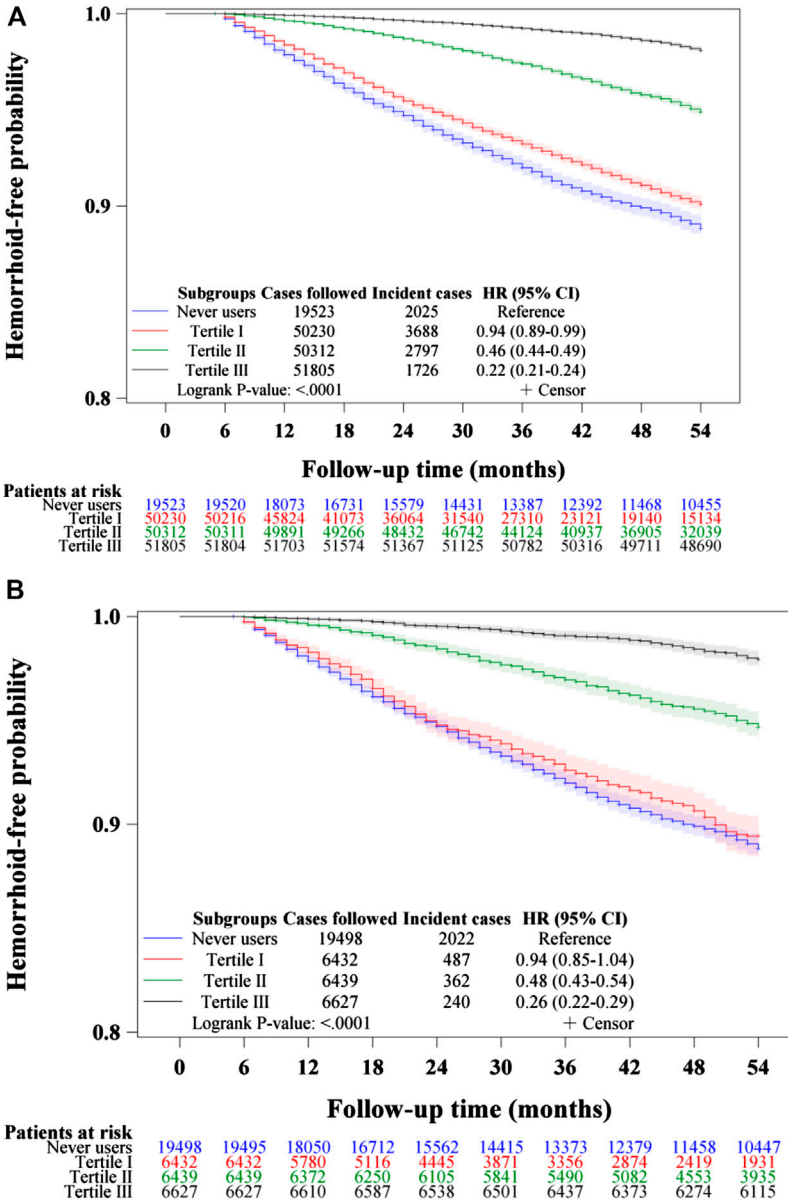

FIGURE 3 | Kaplan–Meier curves comparing hemorrhoid-free probability among never users and tertiles of cumulative duration of metformin therapy in the unmatched cohort **(A)** and the matched cohort **(B)**, respectively. HR: hazard ratio, CI: confidence interval.

receiving calcium channel blockers; Model IX: patients not receiving calcium channel blockers) because these medications may potentially cause bias relating to disease diagnosis. Aspirin can increase the risk of hemorrhoidal bleeding (Davis et al., 2018) but on the other hand it may also be used for hemorrhoidal pain relief (Sun and Migaly, 2016). Calcium channel blockers can reduce resting anal pressure and have been used for the treatment of hemorrhoid (Lohsiriwat, 2012) and anal fissure (Sahebally et al., 2017).

Analyses were conducted using SAS statistical software, version 9.4 (SAS Institute, Cary, NC). $p < 0.05$ was considered statistically significant.

RESULTS

The characteristics in never users and ever users of metformin in the unmatched cohort and the matched cohort, respectively, are shown in **Table 1**. Many of the covariates were not balanced between never and ever users of metformin as indicated by a standardized difference >10% in the unmatched cohort. However, all covariates were well balanced between the two groups in the matched cohort because none of them had a value of standardized difference >10%.

The Kaplan-Meier curves comparing hemorrhoid-free probability with regards to metformin exposure are shown in

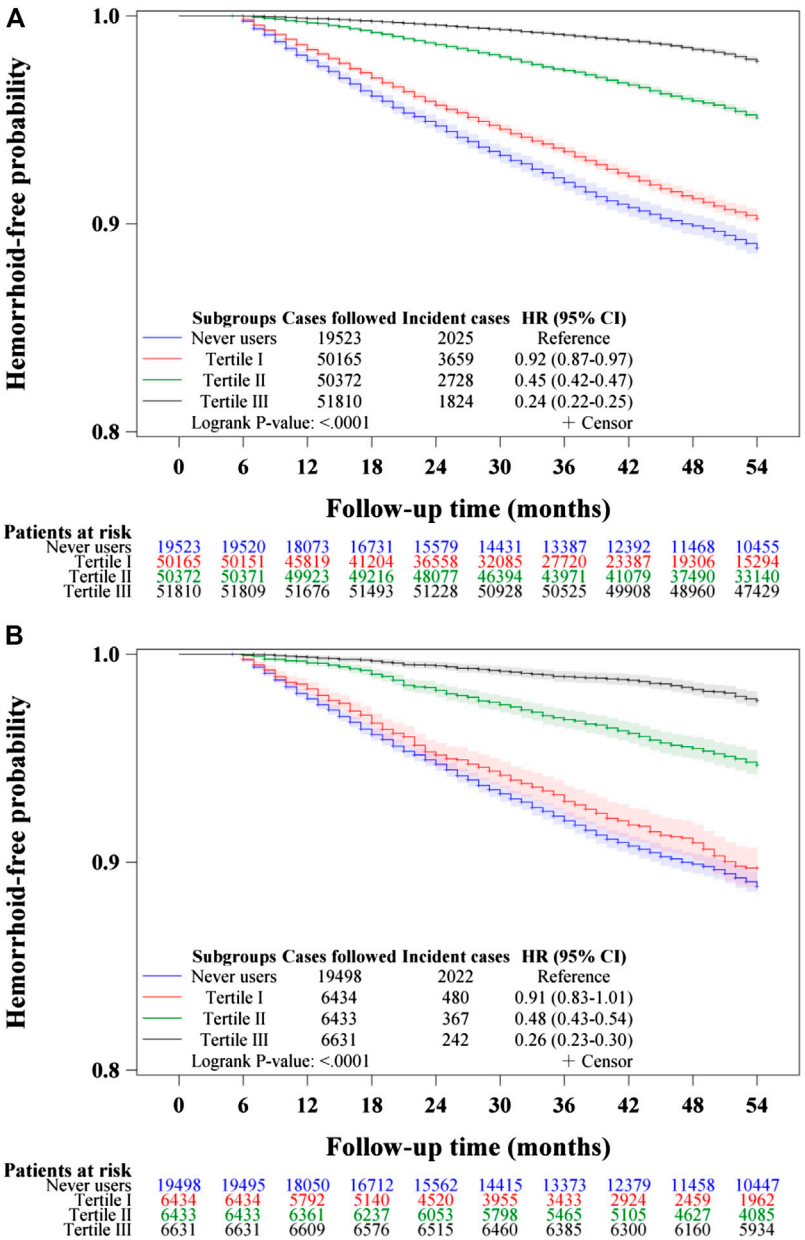

FIGURE 4 | Kaplan–Meier curves comparing hemorrhoid-free probability among never users and tertiles of cumulative dose of metformin therapy in the unmatched cohort **(A)** and the matched cohort **(B)**, respectively.

Figure 2. **Figure 2A** shows the curves for never users and ever users in the unmatched cohort and **Figure 2B** shows the respective curves in the matched cohort. The *p*-values of the logrank test were <0.0001 in both the unmatched cohort and the matched cohort.

Figures 3–5 show the Kaplan-Meier curves comparing hemorrhoid-free probability in never users of metformin and in ever users of metformin categorized according to the tertiles of cumulative duration of metformin therapy (**Figure 3**), the tertiles of cumulative dose of metformin therapy (**Figure 4**) and the tertiles of units of DDD per day (**Figure 5**), respectively. **Figures 3A**, **4A**, and **5A** show the curves in the unmatched cohort; and **Figures 3B**, **4B**, and **5B** show the respective curves in the matched cohort. The logrank test ($p < 0.0001$) supported a significant difference in a dose-response pattern among the various subgroups of metformin exposure in all three parameters.

Table 2 shows the incidence of hemorrhoid and the hazard ratios by metformin exposure in the unmatched cohort and the matched cohort, respectively. A significantly lower risk in ever users could be demonstrated by the overall hazard ratios in both the traditional Cox regression and the Cox regression incorporated with IPTW in either the unmatched cohort or

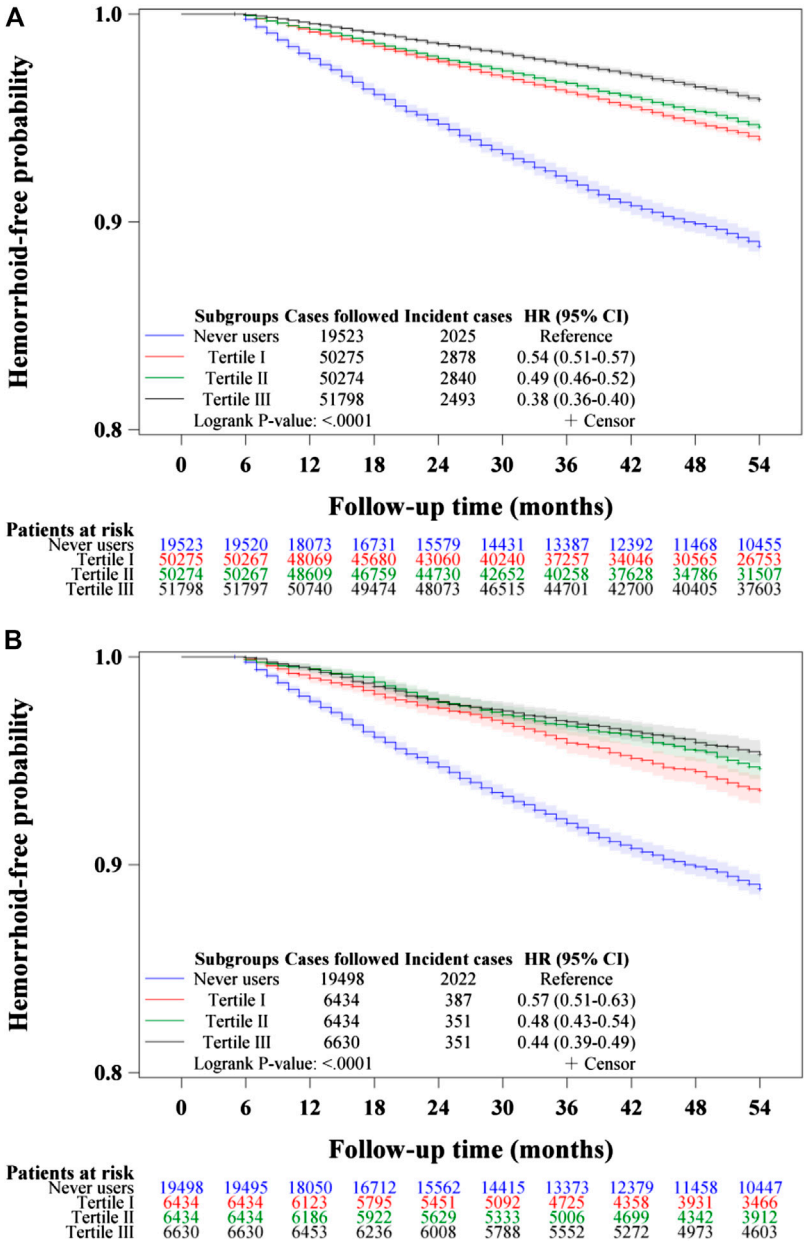

FIGURE 5 | Kaplan–Meier curves comparing hemorrhoid-free probability among never users and tertiles of units of defined daily dose of metformin in the unmatched cohort **(A)** and the matched cohort **(B)**, respectively.

the matched cohort. A dose-response relationship could be seen in the tertile analyses in all models. Significant *p*-values were noted for metformin use for more than approximately 2 years in the cumulative duration analyses (in the second and third tertiles); for more than approximately 750 grams in the cumulative dose analyses (in the second and third tertiles); and for all tertiles in the units of DDD per day analyses. Analyses in the tertiles of units of DDD suggested that the protective effect could be seen across all tertiles with a trend of greater protection in higher daily dose. In the unmatched cohort, the mean (median) values of cumulative duration, cumulative dose and units of DDD of metformin therapy among ever users were 45.7 (40.6) years, 1,692.7 (1,300.0) grams and 0.58 (0.54) units of DDD, respectively. In the matched cohort, the respective values were 45.0 (40.1) years, 1,650.1 (1,265.7) grams and 0.57 (0.54) units of DDD.

The sensitivity analyses shown in **Table 3** consistently supported a 40–50% lower risk of hemorrhoid associated with metformin use in models derived from the unmatched and matched cohorts, respectively; and in either the traditional Cox regression or the Cox regression incorporated with IPTW. The protective effect of metformin was independent of the use of

TABLE 2 | Incidence rates of hemorrhoid and hazard ratios by metformin exposure.

Model/Metformin use	Incident Case number	Cases Followed	Person-years	Incidence rate (per 100,000 person-years)	Traditional Cox model			IPTW model		
					HR	95% CI	p Value	HR	95% CI	p Value
Unmatched cohort										
Never users	2,025	19,523	80,153.12	2,526.41	1.000			1.000		
Ever users	8,211	152,347	692,486.07	1,185.73	0.464	(0.440–0.488)	<0.0001	0.464	(0.442–0.487)	<0.0001
Tertiles of cumulative duration of metformin therapy (months)										
Never users	2,025	19,523	80,153.12	2,526.41	1.000			1.000		
<25.5	3,688	50,230	165,167.79	2,232.88	1.038	(0.978–1.101)	0.2170	0.874	(0.827–0.923)	<0.0001
25.5–56.7	2,797	50,312	236,249.97	1,183.92	0.477	(0.449–0.506)	<0.0001	0.459	(0.434–0.486)	<0.0001
>56.7	1,726	51,805	291,068.31	592.99	0.217	(0.203–0.232)	<0.0001	0.218	(0.205–0.233)	<0.0001
Tertiles of cumulative dose of metformin therapy (grams)										
Never users	2,025	19,523	80,153.12	2,526.41	1.000			1.000		
<756	3,659	50,165	166,490.45	2,197.72	1.019	(0.960–1.081)	0.5398	0.864	(0.818–0.912)	<0.0001
756–1960	2,728	50,372	238,241.26	1,145.06	0.455	(0.429–0.484)	<0.0001	0.444	(0.420–0.471)	<0.0001
>1960	1,824	51,810	287,754.36	633.87	0.231	(0.216–0.246)	<0.0001	0.235	(0.221–0.251)	<0.0001
Tertiles of units of defined daily dose of metformin therapy per day										
Never users	2,025	19,523	80,153.12	2,526.41	1.000			1.000		
<0.49	2,878	50,275	211,323.75	1,361.89	0.531	(0.500–0.564)	<0.0001	0.536	(0.507–0.568)	<0.0001
0.49–0.65	2,840	50,274	227,907.44	1,246.12	0.488	(0.459–0.518)	<0.0001	0.489	(0.462–0.517)	<0.0001
>0.65	2,493	51,798	253,254.89	984.38	0.387	(0.364–0.411)	<0.0001	0.382	(0.360–0.405)	<0.0001
Matched cohort										
Never users	2,022	19,498	80,068.10	2,525.35	1.000			1.000		
Ever users	1,089	19,498	87,196.70	1,248.90	0.488	(0.453–0.525)	<0.0001	0.492	(0.457–0.530)	<0.0001
Tertiles of cumulative duration of metformin therapy (months)										
Never users	2,022	19,498	80,068.10	2,525.35	1.000			1.000		
<24.9	487	6,432	20,745.05	2,347.55	0.985	(0.888–1.092)	0.7740	0.915	(0.828–1.011)	0.0799
24.9–56.0	362	6,439	29,625.10	1,221.94	0.481	(0.430–0.538)	<0.0001	0.478	(0.427–0.534)	<0.0001
>56.0	240	6,627	36,826.55	651.70	0.245	(0.214–0.280)	<0.0001	0.256	(0.224–0.293)	<0.0001
Tertiles of cumulative dose of metformin therapy (grams)										
Never users	2,022	19,498	80,068.10	2,525.35	1.000			1.000		
<736	480	6,434	20,985.04	2,287.34	0.975	(0.879–1.083)	0.6395	0.893	(0.808–0.987)	0.0260
736–1918	367	6,433	29,821.41	1,230.66	0.483	(0.432–0.540)	<0.0001	0.482	(0.431–0.539)	<0.0001
>1918	242	6,631	36,390.26	665.01	0.247	(0.216–0.283)	<0.0001	0.262	(0.229–0.299)	<0.0001
Tertiles of units of defined daily dose of metformin therapy per day										
Never users	2,022	19,498	80,068.10	2,525.35	1.000			1.000		
<0.49	387	6,434	26,985.17	1,434.12	0.574	(0.514–0.642)	<0.0001	0.564	(0.506–0.629)	<0.0001
0.49–0.64	351	6,434	28,633.36	1,225.84	0.476	(0.425–0.533)	<0.0001	0.483	(0.431–0.541)	<0.0001
>0.64	351	6,630	31,578.18	1,111.53	0.427	(0.381–0.479)	<0.0001	0.439	(0.392–0.492)	<0.0001

Hemorrhoid was based on a diagnosis made at the out-patient clinics or during hospitalization.

[a]*Unit of defined daily dose of metformin = 2 grams.*

IPTW: inverse probability of treatment weighting, HR: hazard ratio, CI: confidence interval.

aspirin (models VI and VII) or the use of calcium channel blockers (models VIII and IX).

DISCUSSION

The is the first population-based observational study that showed an overall risk reduction of hemorrhoid associated with metformin use in patients with type 2 diabetes mellitus (**Tables 2** and **3**, **Figures 2**–**5**). A dose-response pattern could be seen in all analyses (**Table 2**, **Figures 3**–**5**).

The mechanisms of a reduced risk of hemorrhoid associated with metformin use require further investigation, but some basic research may provide tentative and reasonable explanations. Results from *in vitro* and *in vivo* studies suggested that metformin may exert cardiac and vascular protective effects via 5′-adenosine monophosphate-activated protein kinase (AMPK)-dependent and AMPK-independent pathways (Nesti and Natali, 2017). Pro-inflammation is a characteristic of insulin resistance (Grandl and Wolfrum, 2018). Metformin increases the expression of insulin receptor and activates tyrosine kinase, and therefore improves insulin resistance (Viollet et al., 2012). Additionally, by changing the composition of the gut microbiota, metformin use is associated with an increase in *Akkermansia* species, which have been shown to improve insulin resistance and reduce tissue inflammation (Hur and Lee, 2015). Metformin may also inhibit the transforming growth factor-beta one signaling pathways (Song et al., 2017), which are activated in cancer cells and several other human diseases involving autoimmunity, fibrosis and cardiovascular system (Serralheiro et al., 2017). Irritable bowel syndrome may cause hemorrhoid (Johannsson et al., 2005; Helvaci et al., 2009)

TABLE 3 | Sensitivity analyses for estimating hazard ratios for hemorrhoid by metformin exposure.

Model/Metformin use	Incident Case number	Cases Followed	Person-years	Incidence rate (per 100,000 person-years)	Traditional Cox model			IPTW model		
					HR	95% CI	p Value	HR	95% CI	p Value
Unmatched cohort										
I. Excluding two consecutive prescriptions of metformin spanning more than 4 months										
Never users	2,025	19,523	80,153.12	2,526.41	1.000			1.000		
Ever users	2,515	52,075	220,925.10	1,138.39	0.437	(0.410–0.466)	<0.0001	0.448	(0.423–0.476)	<0.0001
II. Excluding patients treated with incretin-based therapies during follow-up										
Never users	2,002	18,510	75,573.20	2,649.09	1.000			1.000		
Ever users	7,588	119,286	526,011.63	1,442.55	0.533	(0.506–0.561)	<0.0001	0.540	(0.514–0.567)	<0.0001
III. Patients enrolled during 1999–2002										
Never users	855	8,637	34,586.82	2,472.04	1.000			1.000		
Ever users	5,037	90,236	421,633.37	1,194.64	0.475	(0.440–0.513)	<0.0001	0.473	(0.440–0.508)	<0.0001
IV. Patients enrolled during 2003–2005										
Never users	1,170	10,886	45,566.30	2,567.69	1.000			1.000		
Ever users	3,174	62,111	270,852.70	1,171.85	0.440	(0.409–0.474)	<0.0001	0.455	(0.426–0.487)	<0.0001
V. Defining hemorrhoid as a primary diagnosis at hospitalization										
Never users	349	21,256	90,841.19	384.19	1.000			1.000		
Ever users	1,381	169,495	783,364.48	176.29	0.473	(0.417–0.536)	<0.0001	0.455	(0.405–0.512)	<0.0001
VI. Patients receiving aspirin										
Never users	1,211	11,985	48,552.79	2,494.19	1.000			1.000		
Ever users	5,211	96,528	442,205.39	1,178.41	0.457	(0.428–0.488)	<0.0001	0.464	(0.436–0.494)	<0.0001
VII. Patients not receiving aspirin										
Never users	814	7,538	31,600.33	2,575.92	1.000			1.000		
Ever users	3,000	55,819	250,280.68	1,198.65	0.477	(0.439–0.518)	<0.0001	0.462	(0.428–0.499)	<0.0001
VIII. Patients receiving calcium channel blockers										
Never users	1,315	13,118	53,210.21	2,471.33	1.000			1.000		
Ever users	5,168	94,952	432,049.30	1,196.16	0.469	(0.440–0.501)	<0.0001	0.477	(0.449–0.507)	<0.0001
IX. Patients not receiving calcium channel blockers										
Never users	710	6,405	26,942.91	2,635.20	1.000			1.000		
Ever users	3,043	57,395	260,436.76	1,168.42	0.455	(0.417–0.496)	<0.0001	0.439	(0.405–0.477)	<0.0001
Matched cohort										
I. Excluding two consecutive prescriptions of metformin spanning more than 4 months										
Never users	2,022	19,498	80,068.10	2,525.35	1.000			1.000		
Ever users	349	7,170	30,027.67	1,162.26	0.456	(0.407–0.511)	<0.0001	0.459	(0.410–0.514)	<0.0001
II. Excluding patients treated with incretin-based therapies during follow-up										
Never users	1,999	18,486	75,489.58	2,648.05	1.000			1.000		
Ever users	1,034	15,806	68,477.61	1,509.98	0.567	(0.525–0.611)	<0.0001	0.567	(0.526–0.612)	<0.0001
III. Patients enrolled during 1999–2002										
Never users	854	8,627	34,557.82	2,471.22	1.000			1.000		
Ever users	676	11,583	53,212.25	1,270.38	0.496	(0.448–0.550)	<0.0001	0.509	(0.460–0.563)	<0.0001
IV. Patients enrolled during 2003–2005										
Never users	1,168	10,871	45,510.28	2,566.45	1.000			1.000		
Ever users	413	7,915	33,984.45	1,215.26	0.464	(0.414–0.519)	<0.0001	0.471	(0.421–0.527)	<0.0001
V. Defining hemorrhoid as a primary diagnosis at hospitalization										
Never users	347	21,199	90,667.01	382.72	1.000			1.000		
Ever users	179	21,199	96,460.75	185.57	0.481	(0.401–0.577)	<0.0001	0.484	(0.404–0.579)	<0.0001
VI. Patients receiving aspirin										
Never users	1,208	11,966	48,487.73	2,491.35	1.000			1.000		
Ever users	647	12,019	53,843.20	1,201.64	0.473	(0.429–0.520)	<0.0001	0.479	(0.436–0.527)	<0.0001
VII. Patients not receiving aspirin										
Never users	814	7,532	31,580.38	2,577.55	1.000			1.000		
Ever users	442	7,479	33,353.50	1,325.20	0.510	(0.454–0.573)	<0.0001	0.512	(0.456–0.574)	<0.0001
VIII. Patients receiving calcium channel blockers										
Never users	1,312	13,099	53,141.72	2,468.87	1.000			1.000		
Ever users	721	13,036	58,168.46	1,239.50	0.493	(0.450–0.540)	<0.0001	0.500	(0.456–0.547)	<0.0001
IX. Patients not receiving calcium channel blockers										
Never users	710	6,399	26,926.38	2,636.82	1.000			1.000		
Ever users	368	6,462	29,028.24	1,267.73	0.481	(0.423–0.546)	<0.0001	0.478	(0.422–0.542)	<0.0001

Hemorrhoid was based on a diagnosis made at the out-patient clinics or during hospitalization in all models except Model V.
IPTW, inverse probability of treatment weighting; HR, hazard ratio; CI, confidence interval.

and peptide YY plays an important role in the pathophysiology of irritable bowel syndrome (El-Salhy et al., 2020). Metformin has profound effects on gut hormone signaling including glucagon-like peptide 1 and peptide YY (Glossmann and Lutz, 2019). Whether metformin may prevent the development of hemorrhoid through its actions on gut hormones is an interesting research topic awaiting more in-depth investigation. Metformin may cause increased levels of growth differentiation factor 15; and this increase mediates its effect of body weight loss (Coll et al., 2020). Therefore, metformin may also protect against hemorrhoid by weight reduction following its use. Taken together, metformin may reduce the risk of hemorrhoid via multiple mechanisms by improving insulin resistance, reducing inflammation and fibrosis, affecting gut hormone signaling and weight reduction.

The findings of the present study extended the beneficial effects beyond glycemic control of metformin to the prevention of a very common clinical disease of hemorrhoid. There are some clinical implications. First, metformin has many beneficial effects beyond its glucose lowering effect. These include insulin sensitization, anti-inflammation, cardiovascular protection, anti-aging, anti-cancer and even anti-microbial effects (Maniar et al., 2017; Tseng, 2018a; Tseng, 2018b; Malik et al., 2018). Together with our recent studies, metformin may also exert protection against the development of venous diseases like varicose veins (Tseng, 2020) and hemorrhoid (findings of the present study). These provide good rationales for the use of metformin as the first-line therapeutic drug in the treatment of type 2 diabetes mellitus as recommended by major treatment guidelines (American Diabetes Association, 2017; Salvatore et al., 2020). Second, because all metformin-treated patients seemed to benefit from such a protective effect disregarding the units of DDD taken per day and the protective effect was mainly observed after a cumulative duration of 2 years or a cumulative dose of 750 grams, the use of metformin should be maintained to reach these thresholds when other antidiabetic drugs are added for better glycemic control. Third, the saving of the total healthcare expenditures for the management of other clinical diseases that can be prevented by the continuous use of metformin in the diabetes patients is expected to surpass the drug cost of metformin, an inexpensive drug that does not cause hypoglycemia by itself. Fourth, this observational study gives good rationale for initiating clinical trials to investigate the preventive and therapeutic effects of metformin on hemorrhoid, in either the diabetes patients or the non-diabetes people. However, at this moment, it is still not realistic or justified to recommend metformin for the prevention of hemorrhoid and hemorrhoid-associated complications until the findings are further confirmed by additional observation studies or by clinical trials. If such a protective effect can be confirmed, the clinical usefulness of metformin will be expanded.

In recent years, administrative databases have been popularly used to evaluate long-term safety or beneficial or side effects of medications in pharmacoepidemiological studies. These big data analyses are especially useful for outcomes with low incidence or when randomized trials are not practical. However, some methodological limitations should be carefully addressed. These may include selection bias, prevalent user bias, immortal time bias and confounding by indication.

The present study was designed and conducted to address these potential methodological limitations. First, selection bias can be avoided by using the nationwide NHI database that covers >99% of the population. Second, the potential risk of prevalent user bias was prevented by enrolling patients with new-onset diabetes mellitus and new users of metformin. Additionally, the exclusion of ever users of metformin who had ever been treated with other antidiabetic drugs before metformin was initiated (**Figure 1**) might have reduced the impacts of other antidiabetic drugs that could occur and be carried over to the period when metformin was initiated.

Third, we tried our best to reduce the immortal time bias. Immortal time refers to the follow-up period during which the outcome cannot happen, and immortal time bias can be introduced when treatment status or follow-up time is inappropriately assigned (Lévesque et al., 2010). We tried to exclude patients with uncertain diagnosis of diabetes mellitus by enrolling only patients who had been prescribed antidiabetic drugs for 2 or more times (**Figure 1**). Misclassification of treatment status with metformin was not likely because all prescription information was available in the NHI reimbursement database during the long follow-up period. The immortal time between the diagnosis of diabetes mellitus and the initiation of antidiabetic drugs and the immortal time during the short follow-up period of <180 days had been purposely excluded in the calculation of person-years. It is worthy to note that the immortal time pointed out by Lévesque et al. (2010) during the waiting period between drug prescription and dispense at hospital discharge would not happen in Taiwan because all patients can get their discharge drugs from the hospital at the time they are discharged.

Fourth, we aimed at reducing the confounding by indication by using the PS-matched cohort and the Cox regression incorporated with IPTW (**Tables 2** and **3**). Because all values of standardized difference were <10% in the matched cohort (**Table 1**), the possibility of residual confounding from the covariates was small in the models created from the matched cohort. Additionally, the consistency of the findings in the unmatched cohort, in the analyses by using the traditional Cox regression and in the sensitivity analyses all supported that the results are robust and not liable to changes in different cohorts or by using different statistical methods.

Study limitations may include a lack of measurement data of confounders like biochemical and humoral profiles, anthropometric factors, lifestyle, physical activity, history of constipation and diarrhea, history of sexual intercourse, daily standing time, numbers of births and pelvic disease in women, cigarette smoking, alcohol drinking, dietary pattern, family history and genetic parameters. Visceral neuropathy or pudendal neuralgia may cause chronic constipation and hemorrhoid (Bharucha et al., 2013). However, we do not have such information in the database for analyses. Because the diagnosis of hemorrhoid was based on ICD-9-CM code without supportive laboratory data, it is possible that misclassification of hemorrhoid could not be entirely excluded.

However, the hazard ratios would only be underestimated if the misclassifications were not differential in ever users and never users of metformin (Kesmodel, 2018). To further confirm the preventive role of metformin on hemorrhoid, sensitivity analyses were conducted by re-defining the outcome with a more stringent criterion of a primary diagnosis of hemorrhoid during hospitalization (Models V, **Table 3**). The estimated hazard ratios were very similar to those derived from the main analyses in **Table 2**. Finally, there is a possibility that some patients might not report their symptoms or signs related to hemorrhoid to their attending doctors and might have bought medications to treat their hemorrhoids by themselves. Because the coverage rate of NHI is very high and the patients always do not need to give extra payment if they get their medications for hemorrhoid at the same time when they receive their antidiabetic prescriptions, it is believed that the diabetes patients would rather report related symptoms to their doctors and requested medications for their hemorrhoids than buy over-the-counter medications by themselves that would cost extra expenses out of their pockets. Furthermore, if such misclassification was not differential between ever users and never users of metformin, the estimated hazard ratios would only be biased toward the null (Kesmodel, 2018).

There are some additional strengths. First, self-reporting bias could be much reduced by using medical records. Second, although detection bias related to different socioeconomic status can be a problem in some countries, this was less likely a problem here because the drug cost-sharing in the Taiwan's NHI healthcare system is low. Furthermore, much expense can be waived in veterans and in patients with low-income or when the patients receive prescription refills for chronic disease.

CONCLUSION

The findings of this study support a lower risk of hemorrhoid associated with chronic therapy of metformin in patients with type 2 diabetes mellitus in Taiwan when the cumulative duration is >2 years or the cumulative dose is >750 grams. However, confirmation in other populations is necessary. Because metformin does not cause hypoglycemia when used as a monotherapy and it is inexpensive and safe for long-term use, its protective effect on hemorrhoid is worthy of more investigation, not only in patients with diabetes mellitus but also in non-diabetes people.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available because public availability of the dataset is restricted by local regulations to protect privacy.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of the National Health Research Institutes. Written informed consent from the participants was not required in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Pharmacological Therapies and Their Clinical Targets in Irritable Bowel Syndrome With Diarrhea

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Irritable bowel syndrome (IBS) is one of the most common disorders of the gut-brain axis, which affects approximately 4% of the global population. The Rome IV criteria define IBS as chronic or recurrent abdominal pain associated with altered bowel habits. Patients can be categorized in four subtypes: IBS with predominant constipation (IBS-C), predominant diarrhea (IBS-D), mixed bowel habits (IBS-M), and unclassified (IBS-U). IBS is associated with a lower quality of life, reduced work productivity, and high healthcare costs. When comparing subtypes, patients with IBS-D report lower disease related quality of life. Due to the scope of this review, we have solely focused on patients with IBS-D. Choosing the right pharmacological treatment in these patients remains challenging due to the heterogeneous patient population, patients' expectation of the treatment outcome, unavailability of efficacious drugs, and the multifactorial and incompletely understood underlying pathophysiology. Currently, pharmacological treatment options target individual symptoms, such as abdominal pain, altered bowel habits, and bloating. In this review, we aimed to summarize the current and recent pharmacological treatment options in IBS-D, targeting the predominant gastrointestinal symptoms. Additionally, we proposed a pharmacological treatment algorithm which healthcare professionals could use when treating individual patients with IBS-D.

Keywords: diarrhea—therapy, pharmacology, irritable bowel syndrome with diarrhea, clinical management algorithms, abdominal pain, loose stools, bloating, irritable bowel syndrome (IBS)

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INTRODUCTION

With a worldwide prevalence of approximately 4%, irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal disorders (FGIDs) (Sperber et al., 2021). These FGIDs have recently been renamed to disorders of the gut-brain axis (DGBIs). IBS is ranked as one of the most common reasons for a consultation in primary care and a referral to secondary or tertiary care in gastroenterology (Drossman, 2016). The prevalence of 4% is obtained using the current Rome IV diagnostic criteria. They define IBS as recurrent abdominal pain associated to at least two of the following items; defecation, a change in stool frequency or a change in stool form. On average, this recurrent abdominal pain should have occurred at least one day per week in the last three months and symptoms must have been present for at least six months (Lacy et al., 2016). Apart from the observed abdominal pain and abnormal bowel habits, frequently reported symptoms include

abdominal distention, bloating, and flatulence (Lacy et al., 2016). Based on the predominant stool type, assessed with the Bristol stool form scale (BSFS) (Lewis and Heaton, 1997), IBS patients can be categorized into four subgroups: IBS with predominant constipation (IBS-C), IBS with predominant diarrhea (IBS-D), IBS with mixed bowel habits (IBS-M), and unclassified IBS (IBS-U) (Lacy et al., 2016). However, over time, symptoms can differ and patients can move from one entity to another.

Currently, there are no objective biomarkers available for IBS to guide treatment. Routine clinical investigations do not indicate organic abnormalities that can explain the symptoms. The well-established pathophysiological factors include visceral hypersensitivity (Simrén et al., 2018), altered gastrointestinal (GI) motility (Manabe et al., 2010; Törnblom et al., 2012), disturbances in gut-brain interaction, and psychological distress. More recently, research is demonstrating and further investigating alterations in the microbiome, intestinal immune activation, increased intestinal permeability, and food hypersensitivity with a focus on gut luminal factors (Simrén et al., 2001; Öhman et al., 2015; Barbara et al., 2016; Bednarska et al., 2017; Tap et al., 2017). However, not all these factors are seen in every patient. Therefore, one should consider heterogeneity when assessing the clinical characteristics in IBS.

Due to the incompletely elucidated pathophysiology and the heterogeneity among the symptom profiles of IBS patients, the pharmacological treatment options target the most common individual IBS symptoms; i.e., abdominal pain, altered bowel habits, and bloating. Therefore, treating patients is often described as a process of trial-and-error. No treatment option fits all patients, making the management of these patients very versatile and complex. Due to the more recent focus on the gut luminal factors, such as food and the microbiome, dietary interventions also became more important (Algera et al., 2019).

In this review, we will focus on the pharmacological treatment options for patients with IBS-D, targeting GI motility, visceral hypersensitivity, and altered gut luminal factors. Research shows that patients with IBS-D have a lower disease-related quality of life compared to patients with IBS-C, which impacts work productivity and daily life activities (Singh et al., 2015; Buono et al., 2017). Fecal urgency is considered the most troublesome symptom. In general, most IBS-D patients regularly use multiple treatments that are unsatisfactory, and report substantial psychological burden (Törnblom et al., 2018). Higher severity scores are also associated with increased medication use and a worse attitude toward the condition itself (Emmanuel et al., 2020). This should all be considered in the management of these patients. Healthcare professionals understand the high symptom burden IBS-D patients experience, but still find the condition difficult to treat (Törnblom et al., 2018).

Therefore, we have aimed to summarize the first- and second-line pharmacological treatment options in patients with IBS-D. Due to their good accessibility, popularity, and applicability in patients with severe comorbidities or contraindications to pharmacological treatment, we also aimed to summarize 'probiotics and plant-derived product'. In addition, we proposed a pharmacological treatment algorithm for healthcare professionals, which could provide guidance in working toward an individualized pharmacological management of patients with IBS-D.

METHODS

This review is based on literature searches performed in the PubMed database in October 2020 using the search terms "irritable bowel syndrome", "functional bowel disorder", "diarrhea", "therapy", "treatment", "randomized clinical trial", "probiotics", "antibiotics", "antispasmodics", "peppermint oil", "herbal", "plant-derived", "antidepressants", "loperamide", "cholestyramine", "alosetron", "ramosetron", "ondansetron", "rifaximin", "eluxadoline", "xyloglucan", "crofelemer". In the identified articles, reference lists were used to add additional papers. Both clinical research and review articles in English were considered, without restrictions regarding publication year. Papers about IBS in children, case reports, case–controlled studies were excluded.

First-Line Pharmacological Treatments

Antispasmodics Targeting Abdominal Pain

For patients who experience abdominal pain, antispasmodics have been the first-line treatment option in primary care for decades. Depending on the agent, the mechanism of action is related to their anticholinergic and calcium channel blocking properties leading to smooth muscle relaxation in the gut (Annaházi et al., 2014). A subgroup of IBS patients, and especially IBS-D patients, have an exaggerated gastro-colonic reflex that is partially mediated by a cholinergic pathway (Chey et al., 2001). Therefore, antispasmodics might be best suited for patients with abdominal cramps and altered bowel habits. Frequently used examples are: alverine citrate (+simethicone), mebeverine, otionium bromide, pinaverium bromide, and phloroglucinol. **Table 1** gives an overview of the most-described randomized controlled trials (RCTs) assessing the effect of different antispasmodics in approximately 2900 IBS patients. In general, placebo-controlled trials show only low-quality efficacy evidence and most often only short-term symptom relief (Tack et al., 2006b; Ford et al., 2008; Annaházi et al., 2014). Antispasmodics are relatively safe, but one should be aware of the potential anticholinergic side effects and contraindications. The anticholinergic side effects include constipation, dry mouth, visual disturbances, and urinary retention. Most common contraindications include glaucoma, GI obstruction, autonomic neuropathy, obstructive uropathy, and patients with an allergy for barbiturates, Belladonna Alkaloids or antiepileptics with arene oxide metabolites. Due to the anticholinergic side effects, their usage in elderly, patients with benign prostate hyperplasia, glaucoma, urinary bladder neck obstruction, myasthenia gravis, and Alzheimer's disease is most often problematic.

Good candidates for antispasmodic treatment (first-line)
Mild or moderate IBS patients presenting cramps and/or intermittent abdominal pain as the predominant symptom.

TABLE 1 | Randomized controlled trials evaluating the efficacy of antispasmodics in IBS patients with diarrhea.

Study	Population (*n*), Rome criteria	Period (weeks)	Dose	Main outcome, significant difference compared to placebo	Adverse events
Cimetropium bromide					
Centonze et al. (1988), Italy	IBS (*n* = 48), no Rome criteria	24	50 mg t.i.d	Decrease in pain scores (RR: 87% vs. 16%)	Dry mouth and sleepiness
Dobrilla et al. (1990), Italy	IBS (*n* = 70, 35 cimetropium bromide), no Rome criteria	12	50 mg t.i.d	Decreased severity pain scores (RR: 85% vs. 52%)	Dry mouth
Drotaverine hydrochloride					
Rai et al. (2014), India	IBS (*n* = 170, 85 drotaverine HCl), Rome II	4	80 mg t.i.d	Decreased pain frequency and severity scores (RR: 78% vs. 31%)	Headache, heartburn, flatulence
Phloroglucinol (+ Trimethylphloroglucinol)					
Chassany et al. (2007), France	IBS with acute abdominal pain (*n* = 300, 151 P + TMP), Rome II	1	62.2 mg P + 80 mg TMP t.i.d	Decrease in pain intensity (RR: 60% vs. 32%)	Constipation, flatulence, and abdominal pain
Shin et al. (2020), Korea	IBS-D (*n* = 72, 36 P), Rome III	3	160 mg t.i.d	Global symptom improvement (RR: 62% vs. 31%)	Nausea
Otilonium bromide					
Glende et al. (2002), Italy	IBS (*n* = 317, 160 OB), Rome I	15	40 mg t.i.d	More frequent improvement of GI symptoms (RR: 37% vs. 23%)	None related to the study medication
Clavé et al. (2011), Spain	IBS (*n* = 356, 179 OB), Rome II	25	40 mg t.i.d	Reduction in the number of abdominal pain episodes. (–0.90 ± 0.88 vs. –0.65 ± 0.91)	Dry mouth and nausea
Battaglia et al. (1998), Italy	IBS (*n* = 325, 160 OB), no Rome criteria	15	40 mg t.i.d	Reduced frequency of abdominal pain episodes (RR: 55% vs. 40%)	Not reported
Mebeverine					
Kruis et al. (1986), Germany	IBS (*n* = 120, 40 mebeverine), no Rome criteria, mixed	16	400 mg o.d	No significant symptomatic improvement	Not reported
Everitt et al. (2010), United Kingdom	IBS (*n* = 135, 44 mebeverine), Rome III	6	135 mg t.i.d. vs. b.i.d	No significant differences in IBS-SSS score	None related to the study medication
Pinaverium bromide					
Zheng et al. (2015), China	IBS-D (*n* = 427, 218 pinaverium bromide), Rome III	4	50 mg t.i.d	Improved abdominal pain (RR: 62% vs 30%)	Nausea, dizziness, increased BP, abdominal discomfort
Alverine citrate					
Ducrotte et al. (2014), France	IBS (*n* = 436, 222 alverine citrate/ simethicone), Rome III	24	60 mg + 300 mg t.i.d	Decreased total score IBS-Severity Scoring System (170 vs. 111)	Not reported
Wittmann et al. (2010), Hungary	IBS (*n* = 409, alverine citrate-simethicone), Rome III	4	60 mg + 300 mg t.i.d	50% decrease in abdominal pain/ discomfort VAS scores (RR: 47% vs. 34%)	Not reported

b.i.d., twice daily; BP, blood pressure; IBS, irritable bowel syndrome; IBS-D, IBS with predominant diarrhea; o. d., once daily; P, phloroglucinol; RR, response rate; t. i.d., thrice daily; TMP, trimethylphloroglucinol; VAS, visual analogue scale.

Peppermint Oil Targeting Abdominal Pain

One of the safer, more "natural", agents with antispasmodic properties is peppermint oil with the active ingredient, L-menthol. Apart from its antispasmodic effect, research remains rather unclear about the additional beneficial properties of L-menthol. This over-the-counter relaxant induces a blockade of L-type calcium channels without activating transient receptor potential cation channel subfamily M member 8 (TRPM8) channel or nitrous oxide (Hills and Aaronson, 1991). Its analgesic characteristics might also be related to the effect on the transient receptor potential cation channel, subfamily A, member 1 (TRPA1), in the cells of Cajal, inducing concentration-dependent membrane potential depolarization (Chumpitazi et al., 2018) (**Figure 1**). Furthermore, it has been shown that peppermint oil is antimicrobial, antifungal, and anti-viral, most often targeting obligate and facultative anaerobes and enteric pathogens. The agents also appear to be anti-inflammatory by suppressing the production of inflammatory mediators originating from monocytes (Chumpitazi et al., 2018). **Table 2** describes RCTs that investigated the efficacy of peppermint oil in approximately 500 IBS patients. A number of RCTs suggested that enteric peppermint oil has a positive effect on IBS patients by relieving abdominal pain and discomfort, and global IBS symptoms after 4 weeks of treatment (Cappello et al., 2007; Merat et al., 2010; Alammar et al., 2019; Black et al., 2020b). Earlier studies do not provide high-quality data regarding the long-term efficacy. Only the more recent formulation of peppermint oil with small-intestinal-release demonstrated to significantly reduce IBS severity, abdominal pain and discomfort, bloating, and urgency (Cash et al., 2016; Weerts et al., 2020). The one with ileocolonic-release failed to demonstrate any efficacy compared to placebo (Weerts et al., 2020). Heartburn, urine and/or feces that smell like menthol, and nausea are common side effects of peppermint oil. The usage of peppermint oil is contraindicated in patients with severe liver, gallbladder or bile ducts disease, and in patients who are hypersensitive or allergic to menthol.

Good candidates for the treatment with peppermint oil (first-line)
Moderate IBS-D patients with permanent (or intermittent) abdominal pain and discomfort as predominant symptom.

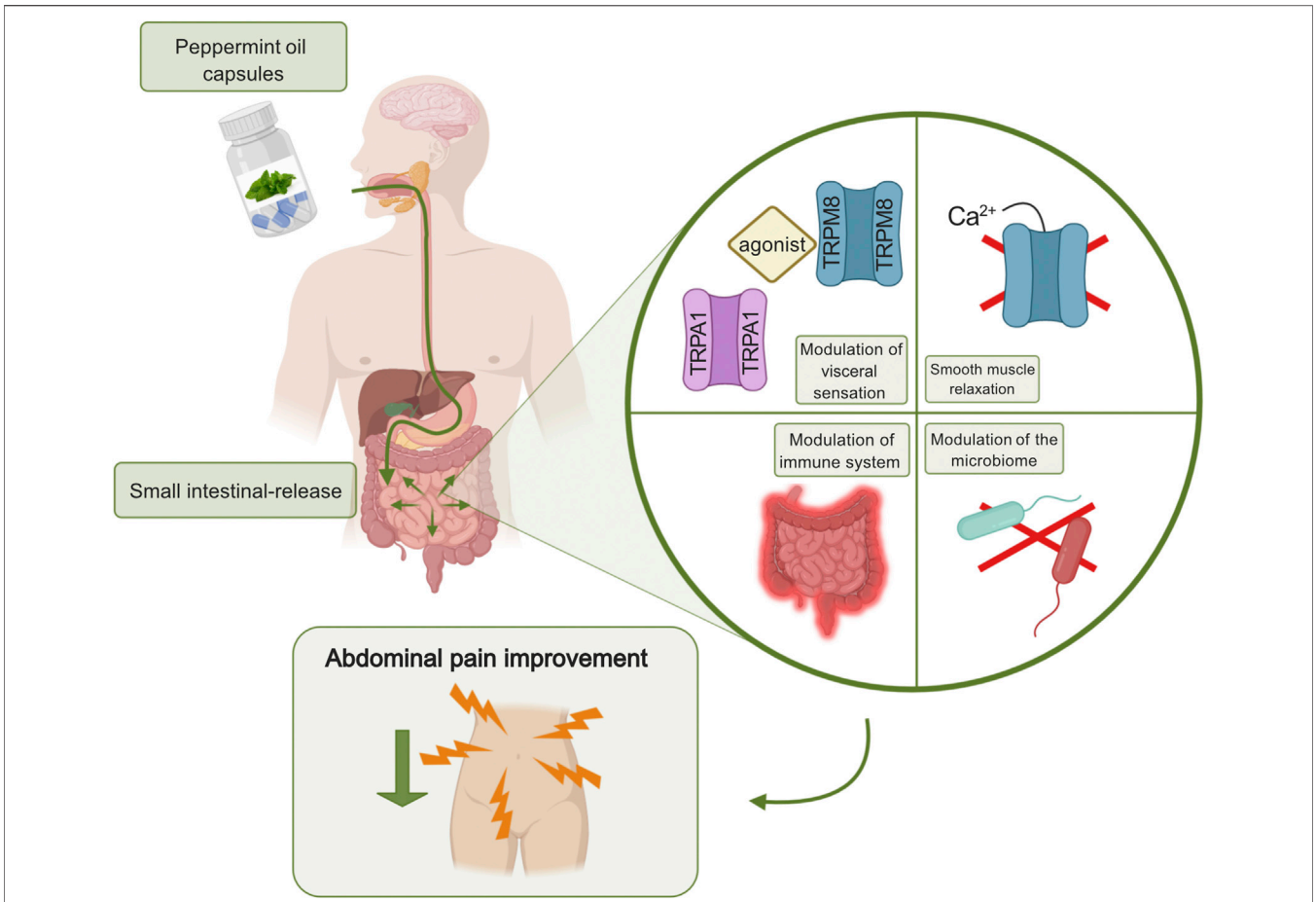

FIGURE 1 | Peppermint oil targeting abdominal pain in IBS. Enteric-coated peppermint oil capsules are released in the small intestine causing a modulation of visceral sensation, smooth muscle relaxation, a modulation of the immune system, and a modulation of the microbiome. The agent is anti-viral and anti-fungal. All proposed mechanisms of action can lead to a decrease in abdominal pain perception. Created with BioRender.com.

TABLE 2 | Randomized controlled trials evaluating the efficacy peppermint oil in IBS patients with diarrhea.

Study	Population (*n*), Rome criteria	Period (weeks)	Dose	Main outcome, significant difference compared to placebo	Adverse events
Liu et al. (1997), Taiwan	IBS (*n* = 101, 52 PO), No Rome criteria	4	187 mg t.i.d. or b.i.d	Alleviation of the severity in abdominal pain (RR: 79% vs. 43%)	Heartburn and mild transit skin rash
Cappello et al. (2007), Italy	IBS (*n* = 57, 28 PO), Rome II	4	450 mg b.i.d	Decrease >50% in total IBS symptom scores (RR: 75% vs. 38%)	Prolonged heartburn
Merat et al. (2010), Iran	IBS (*n* = 90, 33 PO), Rome II	8	187 mg t.i.d	Significant difference in the number of abdominal pain free patients (RR: 42% vs. 22%)	Heartburn, headache, and dizziness
Cash et al. (2016), United States	IBS-M and IBS-D (*n* = 72, 35 PO), Rome III	4	180 mg t.i.d	Reduction of the total IBS symptom scores (RR: 40% vs. 24%)	Dyspepsia, gastroesophageal reflux, flatulence
Weerts et al. (2020), The Netherlands	IBS (*n* = 189, 62 SBR-PO 63 ICR-PO Rome IV	8	182 mg t.i.d	No significant differences in abdominal pain	SBR-PO: heartburn, GERD, belching, headache; ICR-PO: Altered anal sensation or sensitive urethra, headache, abdominal cramps

b.i.d., twice daily; GERD, gastro-esophageal reflux disease; ICR-PO, ileo-colonic release-peppermint oil; IBS, irritable bowel syndrome; IBS-D, IBS with predominant diarrhea IBS-M, IBS with mixed bowel habits; o. d., once daily; PO: peppermint oil; RR, response rate; SBR-PO: small-bowel release-peppermint oil; t. i.d., thrice daily.

Loperamide Targeting Altered Bowel Habits

This μ-opioid-receptor agonist, decreasing the contractions of the smooth muscles in the intestinal wall, is specifically used to improve stool frequency and consistency in IBS-D patients (Ford et al., 2018b). By binding to its receptor and inhibiting acetylcholine and prostaglandins release, loperamide hydrochloride also affects the water and electrolyte movement through the intestinal wall (Ford et al., 2019). Furthermore, this first-line treatment for IBS-D also increases the anal sphincter tonus which improves symptoms such as, urgency and incontinence (Ford et al., 2018b). However, the evidence regarding this long-standing treatment option is limited and is more based on clinical practice than on high-quality RCTs. Outdated RCTs don't provide significant differences in the improvement of overall IBS symptoms or abdominal pain when comparing placebo and loperamide for 3–13 weeks (Hovdenak, 1987; Lävo et al., 1987). Nevertheless, these findings did support an improvement in urgency, stool consistency, and frequency. Currently, due to the unfavorable outcomes, the long-term use of loperamide is often not authorized. Despite the rather low-quality evidence, the drug is frequently used to reduce acute diarrhea symptoms with a dose of approximately 2 mg o. d. or b. i.d. IBS-D patients use loperamide preventively to avoid the onset of diarrhea in certain situations that could exacerbate symptoms (Moayyedi et al., 2019). One should be aware of common side effects and contraindications. Side effects include abdominal pain, constipation, nausea, vomiting, and bloating. Torsade de pointes, prolonged QT intervals are the most important contraindications.

Good candidates for loperamide (first-line)

Mild to severe IBS-D patients with diarrhea (*i.e.* high stool frequency, loose consistency, with or without urgency, fecal incontinence) as predominant symptom. Loperamide can also be used prophylactically in stressful situations.

Second-Line Pharmacological Treatments

Antidepressants Targeting Persistent Abdominal Pain

Antidepressants are beneficial for IBS patients potentially due to their central effects, but most importantly due to their peripheral effects targeting underlying mechanisms, such as visceral hypersensitivity, pain perception, and motility (Ford et al., 2019). Antidepressants may alter the patient's pain perception by modulating the visceral afferents via anticholinergic effects and by blocking incoming pain impulses (Ford et al., 2019). However, the precise mechanism of these agents in IBS and other abdominal pain related DGBIs is incompletely understood. Their beneficial influence on GI motility could be originating from their effects on the levels of neurotransmitters serotonin and norepinephrine and of the brain-gut peptides, including motilin, ghrelin, and neuropeptide-Y, which can regulate the secretory and motor functions of the GI tract (Huang et al., 2013). The classes of antidepressants that are most frequently used in IBS management are the tricyclic antidepressants (TCA), slowing down GI transit, and the selective serotonin reuptake inhibitors (SSRIs), accelerating transit (Ford et al., 2019). This explains why TCAs are mostly prescribed to IBS-D patients and SSRIs either to IBS-C patients or to IBS patients with predominant psychological comorbidities. In **Table 3**, you can find an overview of RCTs providing evidence for and against the use of TCAs and SSRIs in approximately 500 IBS patients in total.

The dose of TCAs prescribed to improve IBS symptoms, is a dose below the prescribed concentration to treat psychiatric disorders (Clouse, 2003). Benefits of taking antidepressants can be seen after taking these agents for at least 1–3 months and effects can be long-lasting without tachyphylaxis. This difference in this dose, the treatment target, and delayed effect is something that needs to be clearly explained to the patient. The patient needs to be aware that antidepressants are prescribed to target severe and persistent chronic abdominal pain and not depression. Examples that are prescribed to IBS patients include imipramine, desipramine, amitriptyline, and its equivalent nortriptyline. However, when starting with these second-line medications, patients risk experiencing anticholinergic side effects, such as dry mouth and eyes, constipation, drowsiness, weight gain, and QT-interval prolongation (Ford et al., 2019). Usually, a few days or weeks after these side effects appear, they fade away. However, keeping a low and steady dose (especially during the first week to the first 3 months), is necessary to monitor these potential side effects. Contraindications include heart or liver disease, glaucoma, and epilepsy.

The dose of SSRIs prescribed to IBS patients represent the full psychiatric dose, used to reduce anxiety and depression (Tack et al., 2006a). The most often prescribed agents include citalopram, fluoxetine, paroxetine, escitalopram, sertraline, and venlafaxine. Findings support that the effect of SSRIs mostly originates from a decrease in psychiatric comorbidities that indirectly affect IBS symptoms (Creed, 2006; Creed et al., 2008). However, literature also indicates that there is an analgesic effect, supporting the fact that SSRIs do improve general IBS symptoms (mostly abdominal pain) independent of improved depression scores (Kuiken et al., 2005; Creed, 2006), in spite of one study assessing the efficacy of one specific SSRI (fluoxetine). They showed that fluoxetine did not improve rectal sensitivity (Kuiken et al., 2003). Furthermore, these second-line agents seem to decrease multiple bodily symptoms or somatization, and improve health-related quality of life. Side effects are less common for SSRIs compared to TCAs, but include dry mouth, nausea, drowsiness, insomnia, and hyperhidrosis. The use of SSRIs in patients with bleeding disorders, type 1 and 2 diabetes, kidney disease or epilepsy is contraindicated.

Good candidates for antidepressants (second-line)

TCAs

Moderate to severe IBS-D patients (with potential overlap of other pain-related DGBIs or with somatization) with persistent and/or severe abdominal pain as predominant symptom.

SSRIs

Moderate to severe IBS-D patients with psychological comorbidities. However, SSRIs are not frequently used in clinical practice, and most often considered when the usage of TCAs is contraindicated.

TABLE 3 | Randomized controlled trials evaluating the efficacy of TCA and SSRI in IBS patients with diarrhea.

Study	Population (*n*), Rome criteria	Period, weeks	Dose	Main outcome, significant difference compared to placebo	Adverse events
Imipramine (TCA)					
Abdul-Baki et al. (2009), Lebanon	IBS (n = 107, 31 imipramine), Rome II	12	25–50 mg o.d	Significant difference in global symptom relief (RR: 81% vs. 48%)	Sleep disturbance, dizziness, urologic symptoms, anxiety, palpitations, dry mouth, flushing, constipation
Amitriptyline (TCA)					
Vahedi et al. (2008), Iran	IBS-D (n = 50, 25 amitriptyline), Rome II	8	10 mg o.d	Complete loss of all symptoms (RR: 68% vs. 28%)	Sleepiness, tachycardia, constipation, and blurred vision and dry mouth
Imipramine (TCA) vs citalopram (SSRI)					
Talley et al. (2008), Australia	IBS (n = 51, 17 citalopram, 18 imipramine), Rome II	12	25–50 mg o.d. vs. 20–40 mg o.d. (increase w3)	No significant difference in adequate IBS symptom relief	Abdominal pain, diarrhea, constipation, bloating, headache, and nausea
Citalopram (SSRI)					
Tack et al. (2006a), Belgium	Non-depressed IBS (n = 23, 11 citalopram), Rome II	6	20–40 mg o.d. (increase w4)	≥50% reduction of abdominal pain days (RR: 100% vs. 33%)	Nausea
Ladabaum et al. (2010), United States	IBS (n = 54, 27 citalopram), Rome II	8	20–40 mg o.d. (increase w5)	No significant differences in adequate symptom relief	Not reported
Fluoxetine (SSRI)					
Kuiken et al. (2003), Netherlands	IBS (n = 40, 19 fluoxetine), Rome I	6	20 mg o.d	No significant differences in rectal sensitivity or abdominal pain scores	Dizziness and drowsiness
Paroxetine (SSRI)					
Creed et al. (2003), United Kingdom	IBS (n = 171, 86 paroxetine), Rome I	64	20 mg o.d	Both improved the physical aspect of health-related quality of life	Sedation, light-headedness, sexual or sleep problems, nausea, and diarrhea

b.i.d., twice daily; IBS, irritable bowel syndrome; IBS-D, IBS with predominant diarrhea; o. d., once daily; RR, response rate; SSRI, selective serotonin re-uptake inhibitors; TCA, tricyclic antidepressants; t. i.d., thrice daily; w, week.

Cholestyramine Targeting Altered Bowel Habits

Twenty-five to 50% of the patients with IBS-D show signs of excess bile acids entering the colon or bile acid malabsorption (BAM) (Camilleri, 2015). BAM leads to the stimulation of secretion and motility and in turn to symptoms, such as loose or watery stools, urgency, and fecal incontinence, defined as bile acid diarrhea (Mekjian et al., 1971; Bampton et al., 2002; Mottacki et al., 2016). BAM leading to bile acid diarrhea often occurs after cholecystectomy. Usually, symptoms improve approximately 6 months after the intervention, but sometimes patients end up suffering from chronic diarrhea. Research show that 96% of the patients with chronic diarrhea suffer from BAM (Sciarretta et al., 1992). Ninety-two percent of BAM patients experience symptom improvement after being treated with bile acid binding agent, cholestyramine. Recent findings show that when the sequestrant is compared to hydroxypropyl cellulose, cholestyramine has a significantly greater effect in the number of watery stools (Fernández-Bañares et al., 2015). BAM can be recognized by decreased levels of fibroblast growth factor 19 in the serum (Camilleri, 2015). The diagnosis can also be made with the help of a fecal bile acid test, quantifying individual and total bile acids in 2-days stool collections or with the serum 7αC4 test, assessing serum C4 levels, which are elevated in patients with BAM (Mottacki et al., 2016). The test used most of the time in Europe is the 75selenium homotaurocholic acid 7-days retention test (SeHCAT), including a capsule with a synthetic analogue of the natural conjugated bile acid tauroselcholic acid and 75Se (a gamma-emitter). A gamma camera can be used to trace the radionuclide and therefore measure if the radionuclide is lost or retained in the feces. However, the SeHCAT test is only available at tertiary care centers in a limited number of European counties (and in Canada) and is relatively expensive. Often, clinicians test the efficacy of cholestyramine to diagnose, and sometimes simultaneously manage BAM without the result of a fecal bile acid or SeHCAT test.

Good candidates for cholestyramine (second-line)
Mild to severe IBS-D patients with BAM or patients with diarrhea as predominant symptom (after loperamide failure or worsening of the symptoms after cholecystectomy).

Serotonin Receptor Antagonists Targeting Altered Bowel Habits and Abdominal Pain

5-Hydroxytryptamine (5-HT), i.e., serotonin, is of importance in signaling pathways in the gut-brain interaction. Secretory and peristaltic reflexes in the gut are activated by 5-HT via primary afferent neurons. The 5-HT_3 receptor is one of seven subtypes of the 5-HT receptors, and its main function is to stimulate release of neurotransmitters. Serotonin stimulates the 5-HT_3 receptor to release acetylcholine in the nerve ends, which causes smooth muscle contraction and enhanced

intestinal secretion (Marciani et al., 2010). Ondansetron, alosetron, and ramosetron are 5-HT_3 receptor antagonists which inhibit the 5-HT_3 receptor activation on the mucosal processes of the primary afferent neurons, and reduce activity of the secretory and motor reflex, by inhibiting the submucosal plexus and myenteric plexus respectively (**Figure 2**). 5-HT_3 receptor antagonists also reduce depolarization of sensory neurons, which causes reduced sensory signals, which affects GI pain signals to the brain, and intestinal secretion. It has been demonstrated that 5-HT_3 antagonists reduce abdominal pain, stool frequency, urgency, and increase stool consistency in patients with IBS-D (Zheng et al., 2017; Black et al., 2020a).

Multiple RCTs, in more than 3,700 IBS-D or IBS-M patients, have demonstrated that alosetron significantly reduces abdominal pain and improves stool consistency compared to placebo (**Table 4**). The severe complication ischemic colitis was reported incidentally, which first led to withdrawal of alosetron in the USA. However, trials in women have been done and suggested that alosetron is effective and

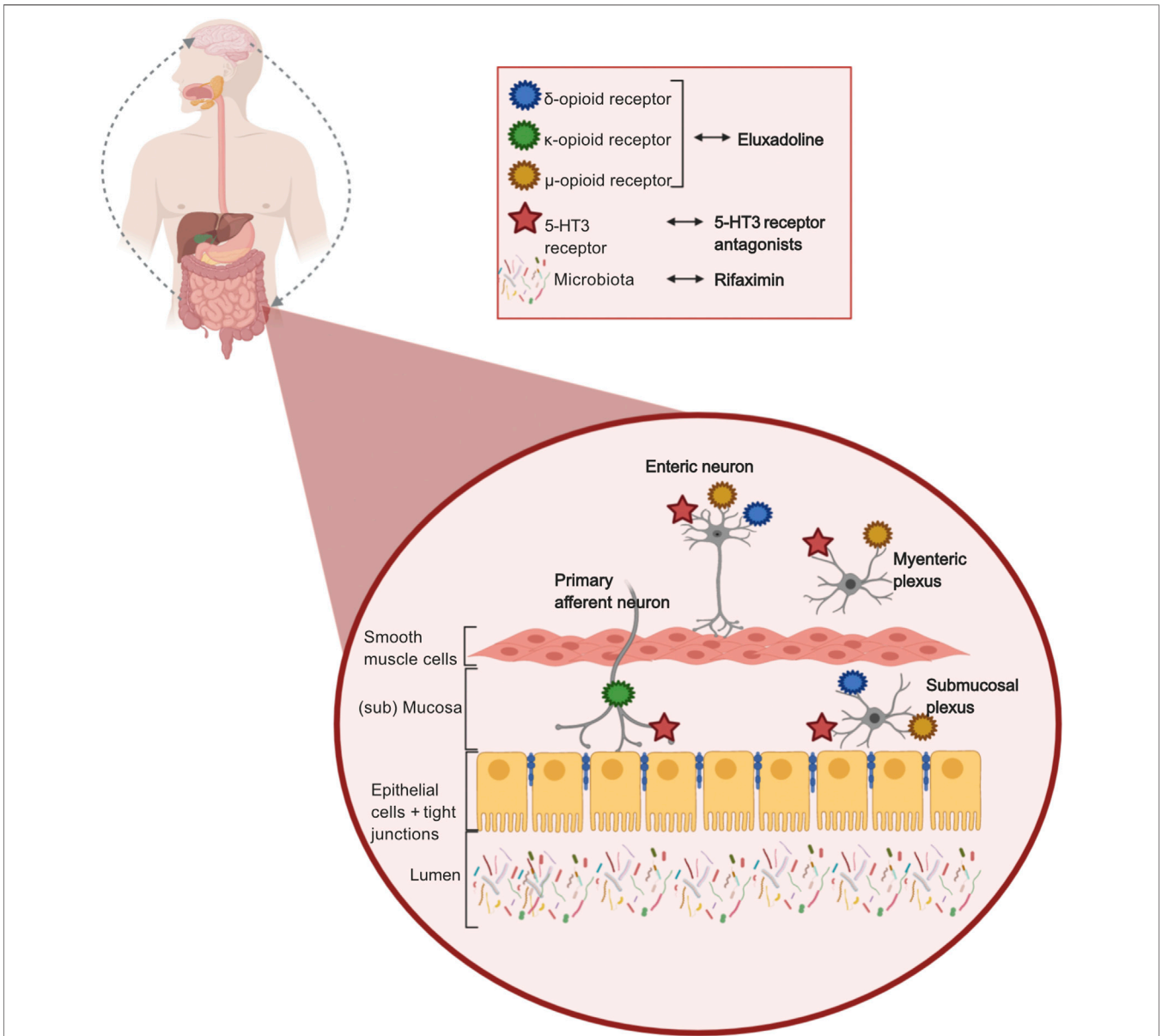

FIGURE 2 | Mechanisms of action of pharmacological treatments in IBS-D. 5-HT_3 receptor antagonists, targeting 5-HT_3 receptors located on enteric neurons, myenteric plexus, submucosal plexus, and primary afferent neurons, reducing sensory signals, secrotory and motor reflex in the gut. Eluxadoline (opioid receptors agonist), targeting δ-,μ-, and κ-opioid receptors located on enteric neurons, myenteric plexus, submucosal plexus, and primary afferent neurons delaying transit by reducing secretory and sensory signals. Rifaximin, targeting the luminal gut microbiome due to its non-absorbable and non-systemic properties. Created with BioRender.com.

TABLE 4 | Randomized controlled trials evaluating the efficacy of 5-HT_3 receptor antagonists, rifaximin, and eluxadoline in IBS patients with diarrhea.

Study	Population (*n*), Rome, women/men/mixed	Period (weeks)	Dose	Main outcome, significant difference compared to placebo	Adverse events
Alosetron (5-HT_3 receptor antagonist)					
Krause et al. (2007), United States	IBS-D (*n* = 353, 177 alosetron), Rome II, women	12	0.5 o.d. or 0.5 or 1 mg b.i.d	Improved global symptoms in all groups (RR: 51% 0.5 mg o.d., 48% 1 mg o.d., 43% 1 mg b.i.d., and 31% placebo)	Constipation
Chang et al. (2005), United States	IBS-D (*n* = 386, 258 alosetron), Rome I, men	12	0.5 or 1 mg b.i.d	Adequate relief of global symptoms (RR: 53% vs. 40%)	Constipation ischemic colitis
Chey et al. (2004), United States	IBS-D (*n* = 569, 279 alosetron), Rome I, women	48	1 mg b.i.d	Adequate relief of global symptoms (RR: 52% vs. 44%)	Constipation
Camilleri et al. (2001), United States	IBS-D (*n* = 626, 309 alosetron), Rome I, men	12	1 mg b.i.d	Adequate relief of global symptoms (RR: 43% vs. 26%)	Constipation
Lembo et al. (2001), United States	IBS-D or IBS-M (*n* = 801, 532 alosetron), Rome II, women	12	1 mg b.i.d	Improved global symptoms (RR: 76% vs 44%)	Constipation
Camilleri et al. (2000), United States	IBS-D or IBS-M (*n* = 647, 324 alosetron), Rome I, women	12	1 mg b.i.d	Adequate relief of global symptoms (RR: 41% vs. 29%)	Constipation
Camilleri et al. (1999), United States	IBS-D or IBS-M (*n* = 152, 72 alosetron), Rome I, mixed	12	1, 2, 4, 8 mg b.i.d	Adequate relief of global symptoms in women (RR: 60% 1 mg, 59% 2 mg, 51% 4 mg, 52% 8 mg, and 33% placebo)	Constipation
Ramosetron (5-HT_3 receptor antagonist)					
Fukudo et al. (2017), Japan	IBS-D (*n* = 305, 203 ramosetron), Rome III, women	12	1.25, 2.5 or 5 μg o.d	Improved abdominal discomfort and pain (RR: 64% vs. 41%)	Constipation
Fukudo et al. (2016), Japan	IBS-D (*n* = 576, 292 ramosetron), Rome III, women	12	2.5 μg o.d	Improved global symptoms (RR: 51% vs. 32%)	Constipation
Fukudo et al. (2014), Japan	IBS-D (*n* = 296, 147 ramosetron), Rome III, men	12	5 μg o.d	Improved stool consistency (RR: 50% vs. 20%)	Hard stools
Matsueda et al. (2008a), Japan	IBS-D (*n* = 212, 103 ramosetron), Rome II, mixed	12	5 or 10 μg o.d	Adequate relief of symptoms (5 μg 43%, 10 μg 43%, and placebo 27%)	Hard stools, constipation
Matsueda et al. (2008b), Japan	IBS-D (*n* = 539, 270 ramosetron), Rome II, mixed	12	5 μg o.d	Adequate relief of symptoms (RR: 47% vs. 27%)	Hard stools, constipation
Ondansetron (5-HT_3 receptor antagonist)					
Plasse et al. (2020), United States	IBS-D (*n* = 126, 75 ondansetron), Rome III, mixed	8	12 mg o.d. (bimodal release)	Improved stool consistency (RR: 56% vs. 35%)	Constipation, flatulence
Garsed et al. (2014), United Kingdom	IBS-D (*n* = 120), Rome III, mixed	5 + 5	4 mg o.d	Cross-over study. Improved stool consistency, mean difference stool form (–0.9, 95% CI –1.1–-0.6)	Constipation
Rifaximin (Antibiotics)					
Pimentel et al. (2011), United States	IBS-D or IBS-M (*n* = 623, 309 rifaximin), Rome II, mixed	2	550 mg t.i.d	Improved global symptoms (RR: 41% vs. 32%)	No differences with placebo
Pimentel et al. (2011), United States	IBS-D or IBS-M (*n* = 637, 316 rifaximin), Rome II, mixed	2	550 mg t.i.d	Improved global symptoms (RR: 41% vs. 32%)	No differences with placebo
Lembo et al. (2016b), United States	IBS-D (*n* = 692[a], 328 rifaximin), Rome III, mixed (repeat treatment)	2	550 mg t.i.d	More responders with improved global symptoms (RR: 38% vs. 32%)	Nausea
Eluxadoline (opioid receptors agonist)					
Brenner et al. (2019), Canada, United States	IBS- D (*n* = 346, 172 eluxadoline), Rome III, mixed	12	100 mg b.i.d	Improved global symptoms (RR: 23% vs 10%)	Nausea, pain, constipation, vomiting
Lembo et al. (2016a), United States, Europe	IBS-D (*n* = 1,282, 855 eluxadoline), Rome III, mixed	52	75 or 100 mg b.i.d	Improved stool consistency and abdominal pain, composite score (RR: 24% 75 mg, 25% 100 mg, and 17% placebo)	Nausea, pain, constipation, pancreatitis
Lembo et al. (2016a), United States, Europe	IBS-D (*n* = 1,146, 764 eluxadoline), Rome III, mixed	26	75 or 100 mg b.i.d	Improved stool consistency and abdominal pain, composite score (RR: 29% 75 mg, 30% 100 mg, and 16% placebo)	Nausea, pain, constipation, pancreatitis
Dove et al. (2013), United States	IBS-D (*n* = 348, 176 eluxadoline), Rome III, mixed	12	5, 25, 100, 200 mg b.i.d	Improved clinical response (RR: 12% 25 mg, 14% 200 mg, and 6% placebo)	Nausea, pain, constipation, pancreatitis

[a]*Responders to rifaximin 550 mg t. i.d. 2 weeks with relapse of symptoms within 18 weeks, were randomized in repeat treatment or placebo.*

5-HT, 5-hydroxytryptamin; b. i.d., twice daily; CI, confidence interval; GI, gastrointestinal; IBS, irritable bowel syndrome; IBS-D, IBS with predominant diarrhea; IBS-M, IBS with mixed bowel habits; o. d, once daily; RR, response rate; t. i.d., thrice daily.

safe to use (Olden et al., 2019). Therefore, alosetron is only available in a selected population: women with severe IBS in the USA, but still unavailable anywhere else in the world. In order to identify possible predictors of response, a pilot study investigated psychological distress and neural activity in IBS patients. They found that less activity in the orbitofrontal cortex (bilateral) and medial temporal gyrus predicted greater symptom improvement (Jarcho et al., 2008).

Ramosetron has also been studied extensively in close to 2,000 IBS-D patients (**Table 4**). So far, there are no indications that ramosetron causes serious adverse events such as ischemic colitis. Ramosetron especially improves abdominal pain in IBS-D and IBS-M, but is for now only available in Japan (and a few other Asian countries). In both alosetron and ramosetron, constipation is the most frequent reported adverse event, contraindications are severe constipation and other GI diseases e.g., inflammatory bowel diseases and colon carcinoma.

In the same class, ondansetron is an older treatment, commonly used in patients undergoing chemotherapy to reduce nausea and vomiting. It has not been used extensively in IBS-D, but recent research showed that ondansetron effectively reduced GI symptoms in this population (Garsed et al., 2014) (**Table 4**). A follow-up study, assessing rectal biopsies of the patients, found that patients with the lowest 5-HT concentration in the rectum, responded the greatest to ondansetron (Gunn et al., 2019). Moreover, a recent RCT indicated that bimodal release ondansetron, i.e., RHB-102, is a promising treatment in IBS-D (**Table 4**), with indications of C-reactive protein (CRP) as a predictor of response. Comparing subgroups, patients with higher levels of CRP (still in the normal range) seemed to have a better response to the treatment compared to patients with lower levels of CRP (Plasse et al., 2020), but this needs to be confirmed in larger trials. The studies did not report any contraindications. Thus, large clinical trials are needed to confirm and determine the effects of ondansetron and bimodal release ondansetron in IBS-D.

5-HT_3 receptor antagonists seem to be effective in patients with IBS-D, a frequent adverse event is constipation, demonstrating that GI transit is effectively delayed. Unfortunately, alosetron and ramosetron are not widely available despite their potential side-effects. However, ondansetron which is available worldwide, seems to be a promising safe alternative for IBS-D patients, but large clinical trials are still needed.

Good candidates for 5-HT_3 receptor antagonists (second-line).

Alosetron
Women with severe IBS-D (in the USA) with diarrhea or abdominal pain as predominant symptoms.

Ramosetron
Moderate to severe IBS-D patients (in Japan, and a few other Asian countries) with diarrhea or abdominal pain as predominant symptoms.

Ondansetron
Moderate to severe IBS-D patient with diarrhea and/or bloating as predominant symptoms.

Rifaximin Targeting Bloating

Rifaximin, which is a broad-spectrum, non-systemic oral antibiotic, specifically targets the gut microbiome. Thus, it is likely that the possible mechanism of action is gut microbiota modulation (**Figure 2**). However, data from a study in rats indicates that rifaximin prevents putative pathophysiological mechanisms (i.e., impaired gut permeability, visceral hyperalgesia, and low-grade inflammation) induced by stress as well (Xu et al., 2014).

Three large RCTs (in total almost 2,000 IBS-D or IBS-M patients) have investigated the effects of rifaximin in patients with IBS-D (**Table 4**). They concluded that rifaximin 550 mg t. i.d. improved global IBS symptoms, especially bloating in IBS patients compared to placebo. However, the differences in response between rifaximin and placebo were modest. No effects were seen on stool consistency, and adverse events were not different from placebo (i.e., constipation), except for nausea (Lembo et al., 2016b). One trial found that rifaximin was effective and safe to repeat in IBS-D patients that relapsed after an initial effective treatment (Lembo et al., 2016b). However, a recent systematic review and meta-analysis concluded that rifaximin failed to achieve a response in global IBS symptoms and abdominal pain (Black et al., 2020a). On the contrary, all trials suggest that rifaximin effectively relieves bloating in IBS-D patients (Pimentel et al., 2011; Lembo et al., 2016b). Additionally, one study investigated the possibility of lactulose breath testing as a predictor of response to rifaximin. They found that IBS-D patients with a positive baseline lactulose breath test, had a higher likelihood of responding to rifaximin (Rezaie et al., 2019), but this needs to be further investigated in larger studies.

Unlike 5-HT_3 receptor antagonists, constipation was not reported as an adverse event in the trials assessing rifaximin. It has been demonstrated that rifaximin actually increases colonic transit time in non-constipated IBS patients (Acosta et al., 2016). Therefore, the only contraindication is a history of obstruction in the GI tract. The exact mechanism why a subgroup of IBS patients respond to rifaximin is not known. Due to its safety and absence of adverse events, rifaximin is a suitable first- or second-line treatment option for patients with IBS-D with predominant bloating, comorbidities or contraindications to other treatments.

Good candidates for rifaximin (first- or second-line)
Moderate to severe IBS-D patients (with or without SIBO) with bloating as predominant symptom.

Eluxadoline Targeting Stool Consistency and Abdominal Pain

Like loperamide, eluxadoline activates μ-opioid receptors in the gut, causing delayed transit and treating diarrhea. Eluxadoline activates not only the μ-opioid receptors, but also the κ-opioid receptors and the δ-opioid receptors (**Figure 2**), involving secretion and sensation (Bitar and Makhlouf, 1982).

So far, eluxadoline has been investigated by four large RCTs, assessing more than 3,100 IBS-D patients (**Table 4**), with promising results. In three trials, eluxadoline improved stool

consistency at 12 weeks, but the differences compared to placebo were modest (Dove et al., 2013; Lembo et al., 2016a). A post-hoc analysis was done assessing two phase III studies, from Lembo *et al.*, they found that the response in the early phase of the trial, could predict the therapeutic benefit after a follow-up of six months (Chey et al., 2017). One trial also investigated patients that did not respond to initial loperamide treatment, and found also that IBS-D patients refractory to loperamide had improved stool consistency and abdominal pain with eluxadoline (Brenner et al., 2019). Frequent adverse events were constipation, nausea, and vomiting. The dose of 100 mg b. i.d. was effective for all endpoints in all the studies. However, there are concerns regarding safety. Pancreatitis and sphincter of Oddi spasms were observed in multiple individuals, especially in patients that underwent cholecystectomy prior to the study. Therefore, eluxadoline is contraindicated in patients with biliary duct obstruction, history of cholecystectomy, alcoholism, pancreatitis, and hepatic impairment. Thus, eluxadoline is a suitable option for non-constipated IBS patients with prior failure of loperamide.

Good candidates for eluxadoline (second-line)
Women and men with moderate IBS-D, and both abdominal pain and diarrhea as predominant symptoms, after loperamide failure and without any contraindication (cholecystectomy, biliary duct obstruction, pancreatitis, hepatic impairment, alcohol abuse, and chronic or severe constipation).

Probiotics and Plant-Derived Products

Probiotics are not considered as pharmacological treatments, but due to their good accessibility, their popularity among patients, and applicability in IBS-D patients with contraindications, we included probiotics in this review. Recently, plant-derived products have also emerged as treatment options in IBS-D. Note that these are also not pharmacological treatments, but they are worth mentioning as options for patients with comorbidities or contraindications for other pharmacological treatments.

Probiotics Targeting the Dysbiosis Between the Host and the Microbiota

Probiotics are living bacteria that confer a health benefit to the host when consumed in sufficient quantities. For decades, IBS patients have been using probiotics on an empirical basis, due to their suggested beneficial effect on dysbiosis. Researchers observed differences in the microbial compositions of IBS patients compared to healthy controls (Tap et al., 2017). When alterations in the homeostatic state appear, the tolerance among commensal microbes that maintain symbiotic functions as well as the barrier integrity cannot be persevered. Due to a potential alteration in the immune response, pathogens can easily provoke inflammation which will in turn affect the gut luminal environment and its microbial composition. Thus, dysbiosis which is a microbial imbalance with a reduced microbial diversity can arise (Pédron and Sansonetti, 2008; Chong et al., 2019). However, a comprised epithelial barrier and an altered immune activation are not the only factors implicated in the IBS pathophysiology and the microbial alterations seen in IBS patients. Post-infectious alterations, dietary changes, altered stress levels, low-grade mucosal inflammation with visceral hypersensitivity, and motility disturbances are all factors influencing each other and the onset of IBS symptoms that may or may not be linked to dysbiosis individually (Chong et al., 2019; Pimentel and Lembo, 2020).

Probiotics are a rather old treatment option for IBS, but recently more and more research groups are interested in their specific targets (Didari et al., 2015; Zhang et al., 2016). Proposed targets include the dysbiotic microbial composition, small intestinal bacterial overgrowth (SIBO) that is often associated with IBS, and post-infectious alterations (Simreń et al., 2013; Chong et al., 2019). Interpreting the results of these probiotic studies, remains a continuous challenge due to the variety of the available species, strains, doses, duration, repetition, preparations and targeted patient populations. Furthermore, due to their short lifespan, patients need repeated doses to experienced adequate benefit. Meta-analyses suggested a superior role for *Lactobacillus* and Bifidobacterium compared to placebo, when it comes to reducing global IBS symptom scores and abdominal pain (Simreń et al., 2013). However, a trend seen in these meta-analyses is that higher-quality studies tend to demonstrate less of a treatment effect. Consequently, recommendations regarding individual species, preparations, or strains are difficult to make at this moment. **Figure 3** describes examples of different probiotics that have been tested in RCTs and which GI symptom(s) they are targeting.

Plant-Derived Products Targeting Stool Consistency and Abdominal Pain

A recent double-blind, placebo-controlled cross-over trial (n = 30, duration 4 + 4 weeks) investigated the efficacy of a recent plant-based medical device, which is intended to protect the intestinal mucosa, and thereby relieve GI symptoms and prevent diarrhea (Trifan et al., 2019). The components are a combination of pea protein, tannins (derived from a grape seed extract), xylo-oligosaccharides, and xyloglucan (*i.e.* tamarind seeds). The proposed mechanism of xyloglucan is that it forms a physical barrier, due to its mucin-like structure, that can protect the gut mucosa against proinflammatory components (e.g. food components), microorganisms or allergens (Piqué et al., 2018). IBS-D patients reported more BSFS type 3 and 4 stool types compared to placebo (response rate 90 vs. 12%), and that abdominal pain and bloating were more acceptable. No adverse events or contraindications were reported (Trifan et al., 2019).

Another promising plant-derived product is Crofelemer, which is extracted from stem bark latex of the *Croton* lechleri tree (Cottreau et al., 2012). Crofelemer was initially used in primary secretory diarrheal disorders, e.g., travelers' diarrhea, cholera, and acute GI infections. A large RCT in IBS-D

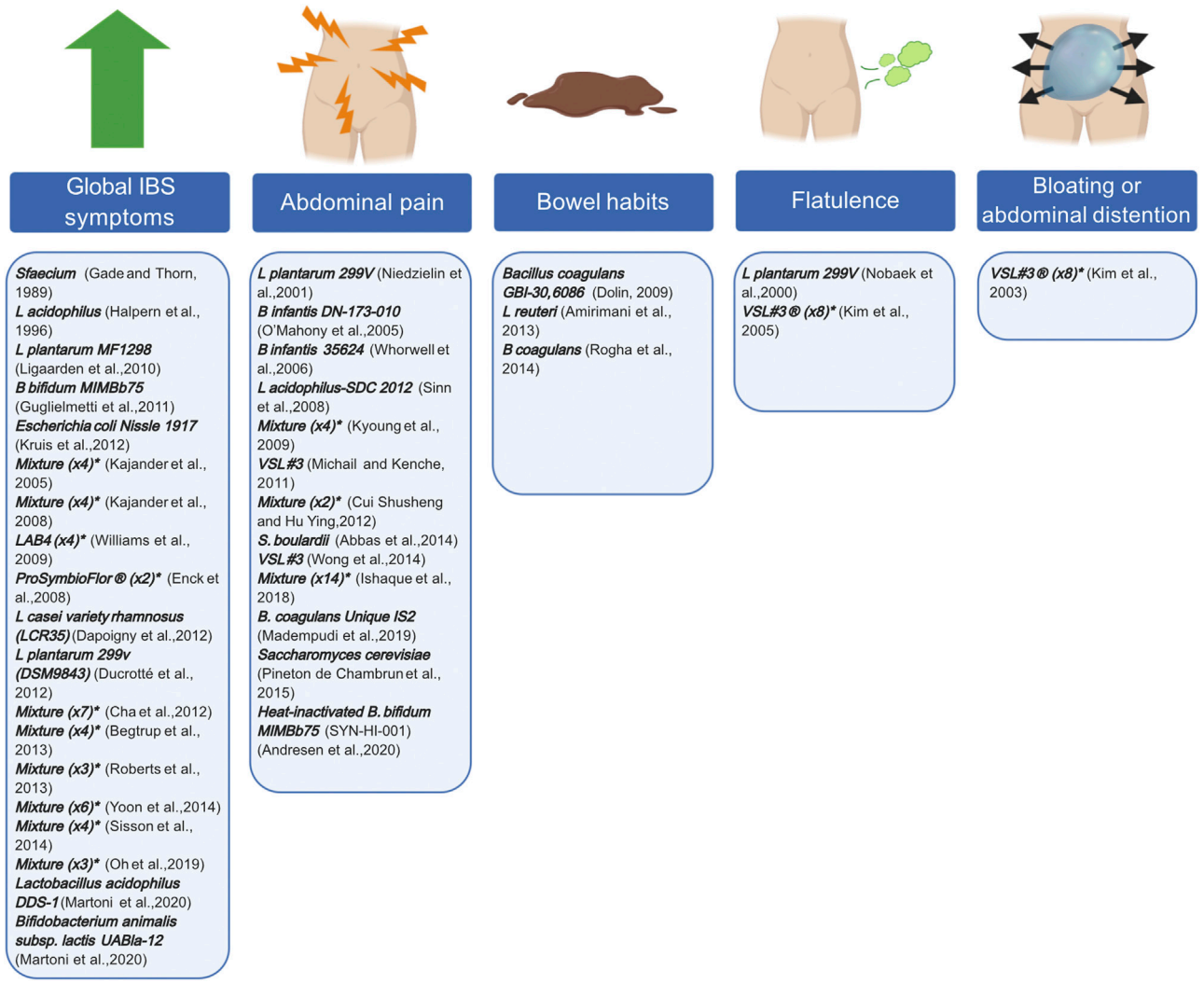

FIGURE 3 | Probiotics and their symptom target in patients with IBS. Different probiotics, assessed in former and more recent RCTs, can target individual IBS symptoms. RCTs either demonstrated a significant effect on global IBS symptoms, abdominal pain, bowel habits, flatulence, bloating or abdominal distention or a combination thereof. * indicated the number of bacterial strains included in the mixture. Created with BioRender.com.

investigated the efficacy of Crofelemer in 240 patients, and found no differences in the primary outcome of stool consistency. However, they did find that women had improvement of pain- and discomfort-free days compared to placebo, no differences were seen in men (Mangel and Chaturvedi, 2009). Another large RCT (also in 240 IBS-D patients) aimed to investigate the analgesic properties of Crofelemer in women. They found that there was no significant difference in abdominal pain improvement (primary endpoint) between Crofelemer and placebo. However, they did find that Crofelemer significantly improved abdominal pain on the FDA monthly responder endpoint (Nee et al., 2019). Hence, large clinical trials are needed to investigate these plant-derived products further. Currently, we can not strongly recommend these options due to the absence of high-quality evidence.

Good candidates for probiotics or plant-derived products (first- and second-line)

Probiotics
Mild IBS patients with abdominal pain or bloating/abdominal distension as predominant symptom. Due to their safety profile, probiotics are a good option for pregnant or lactating women or patients with long term usage of antibiotics.

Xyloglucan + Pea protein = Tannins
Women and men with IBS-D with mainly liquid stools (BSFS 6–7) with bloating and/or flatulence. Patients with comorbidities could safely use this treatment.

Crofelemer
Women with IBS-D with abdominal pain as predominant symptom. Patients with comorbidities could safely use this treatment.

CONCLUSION

As described previously, multiple incompletely elucidated pathophysiological mechanisms are involved in IBS. This has resulted in a wide range of pharmacological treatments, with heterogeneous treatment responses in IBS-D patients. Besides existing treatments, many recent treatments are still being discovered. We have reviewed the first- and second-line pharmacology targeting predominant IBS symptoms, as well as probiotics and plant-derived products. Choosing the right treatment for the right patients, remains a challenge for clinicians. A fundamental cause for this challenge is the heterogeneity in the IBS-D population and therefore, the inability of finding the specific mechanism that is causing the symptoms that need to be targeted in individual patients. Furthermore, the differences between pharmacological treatments and placebo (**Tables 1–4**) are often modest. This is most probably again caused by the heterogeneity between the patients in the disorder. Moreover, a placebo response is common in clinical trials in patients with IBS (Ford and Moayyedi, 2010).

Another challenge for clinicians is the low efficacy level of the available pharmacological treatments shown in clinical practice, and outdated RCTs in patients with IBS-D (Ford et al., 2008; Ford et al., 2014). First-line therapy is often chosen by healthcare professionals because of its wide availability instead of its specific target. Moreover, the availability of the more recent drugs is scarce in different geographical areas. At the moment, authorization of pharmacological treatments is different between countries. For example, in some countries in Europe high-quality evidence is required, where the more recent treatment is compared to an older pharmacological alternative, but this not the case in all countries (Authorisation of medicines, 2020). The data of the systematic review and meta-analysis show that both alosetron and ramosetron are most efficacious in IBS-D compared to placebo, but they are solely available with a restricted prescription (women with severe IBS-D) in the USA and Japan (and a few other Asian countries) respectively (Black et al., 2020a). For alosetron, incidental cases of ischemic colitis, with a low-prevalence risk (Ford et al., 2009), has caused unavailability for men with IBS-D. An explanation for the unavailability is that treatment-related adverse events are not well accepted in IBS, due to less morbidity and no increased risk of mortality compared to other disorders or diseases (Staller et al., 2020). As previously reviewed, the more effective treatments seem to have more adverse events. Currently, there are more possibilities available in safe supplements (*e.g.* probiotics and plant-derived products), but high-quality evidence is scarce (Ford et al., 2018a). These supplements are (usually) inexpensive and assessed for safety but, they are not assessed as pharmacological treatments by government institutes (e.g., Food and Drug Administration, USA), where also strict efficacy is needed to get approval. Thus, healthcare professionals should be reserved when it comes to recommending these supplements even though they are often highly preferred by patients. Currently, high-quality evidence regarding which supplement is targeting which IBS symptom is lacking. Therefore, further research is needed.

A more personalized approach in the management of patients with IBS is desirable, and for now, targeting the most bothersome predominant symptoms in IBS-D (i.e., loose stools, abdominal pain, and/or bloating) seems to be the only practical and suitable treatment strategy. Not only preferences and history of the patient needs to be taken into consideration, but also healthcare associated costs, which are substantial (Nellesen

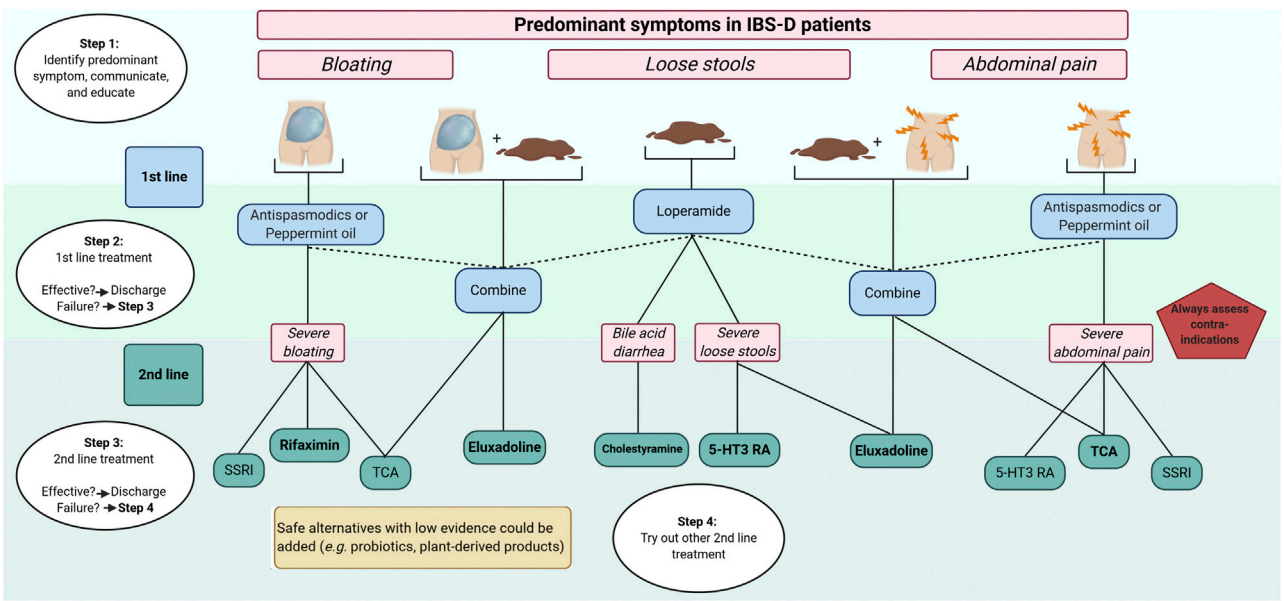

FIGURE 4 | Predominant symptom-based algorithm for pharmacological treatments in IBS-D 5-HT3-RA, 5-hydroxytryptamine-3 receptor antagonists; IBS, irritable bowel syndrome; IBS-D, IBS with predominant diarrhea; TCA, tricyclic antidepressants; SSRI, serotonin re-uptake inhibitors. Created with BioRender.com.

et al., 2013). Successful pharmacological management usually starts with a good relationship between the healthcare professional and the patient. Treatment options should be discussed, as well as side effects. Key factors in the management of IBS-D patients are proper education about the disorder and treatments (Ringström et al., 2012; Lindfors et al., 2020), as well as explaining the reasons to do (or not do) investigations (e.g., colonoscopy) (Black and Ford, 2020). Moreover, besides pharmacology, initial simple dietary- and life-style advice is required (Algera et al., 2019), and effective psychological treatments (e.g., hypnosis and cognitive behavioral therapy) are available as well (Ford et al., 2014). Due to the scope of this review and the current challenges in identifying individual pathophysiological factors in IBS patients, we propose a predominant symptom-based approach solely focused on pharmacological management (algorithm, **Figure 4**). First-line pharmacology includes loperamide, peppermint-oil, and antispasmodics. Even if the level of proof is low, they are safe and could be used for a long time. With failure to improve symptoms, second-line treatment should be initiated. It remains important that clinicians check treatment failures. Obvious reasons (i.e., inadequate treatment period or practical usage) should be excluded. Second-line treatment includes 5-HT_3 receptor antagonists, central neuromodulators (including TCA and SSRI) as well as recent opioid receptors agonists (eluxadoline). Bile-acid sequestrants can be chosen for patients with suspected (or confirmed by ^{75}SeHCAT retention) BAM. Due to the unavailability of recent 5-HT_3 receptor antagonists in some countries, ondansetron might be a suitable and safe alternative. Supplements, including probiotics and plant-derived products, are safe to use in clinical practice but, high-quality evidence of efficacy is currently lacking.

FUTURE PERSPECTIVES

Pharmacological treatments targeting the gut-brain interaction seem to be effective in patients with IBS-D (Ford et al., 2019), but available literature is not always of high quality. Moreover, there is a lack of clinical trials comparing pharmacological treatments. Therefore, large clinical trials are needed to assess and compare the efficacy of these treatments, ideally in a double-blinded, randomized, parallel design. Furthermore, future studies should focus on identifying predictors for treatment responsiveness, including comorbidities (e.g., anxiety and depression) and possible biological markers. As previously discussed, some studies demonstrate promising results, assessing predictors of response (Jarcho et al., 2008; Plasse et al., 2020). Future studies should reinvestigate these predictors of response in larger studies, and also focus on potential others. This will possibly enable the individual tailoring of pharmacological treatment options in patients with IBS-D.

AUTHOR CONTRIBUTIONS

EC and JA, Conceptualization, original draft preparation; CM, Supervision and contents curation; EC, JA, and CM, Writing and reviewing.

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REVIEW
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Management of Ineffective Esophageal Hypomotility

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Esophageal hypomotility in general and especially ineffective esophageal motility according to the Chicago criteria of primary motility disorders of the esophagus, is one of the most frequently diagnosed motility disorders on high resolution manometry and results in a large number of patients visiting gastroenterologists. Most patients with esophageal hypomotility present with gastroesophageal reflux symptoms or dysphagia. The clinical relevance of the motility pattern, however, is not well established but seems to be correlated with disease severity in reflux patients. The correlation with dysphagia is less clear. Prokinetic agents are commonly prescribed as first line pharmacologic intervention to target esophageal smooth muscle contractility and improve esophageal motor functions. However, the beneficial effects of these medications are limited and only confined to some specific drugs. Serotonergic agents, including buspirone, mosapride and prucalopride have been shown to improve parameters of esophageal motility although the effect on symptoms is less clear. Understanding on the complex correlation between esophageal hypomotility and esophageal symptoms as well as the limited evidence of prokinetic agents is necessary for physicians to appropriately manage patients with Ineffective Esophageal Motility (IEM).

Keywords: esophageal hypomotility, ineffective esophageal motility, high resolution manometry, prokinetic, gastroesophageal reflux, dysphagia

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INTRODUCTION

High resolution manometry (HRM) is widely applied to evaluate esophageal motor function, resulting in a better recognition of esophageal motility disorders (Sweis et al., 2018). The most recently updated classification for esophageal motility disorders, the Chicago Classification version 3.0, was proposed in 2015 after two previous versions in 2008 and 2012. This classification was developed based on the analysis of clinical studies in healthy volunteers and patients, and categorized esophageal body motility disorders into major and minor disorders of peristalsis (Boland et al., 2016). Achalasia and major disorders of peristalsis, including distal esophageal spasm, jackhammer esophagus and absent contractility, reveal clinically relevant conditions for which evidence-based treatments are available—with the exception of absent contractility. This is in strong contrast with minor esophageal motility disorders, particularly ineffective esophageal motility (IEM), which still have unclear clinical implications and of which the management is not well established (Boland et al., 2016).

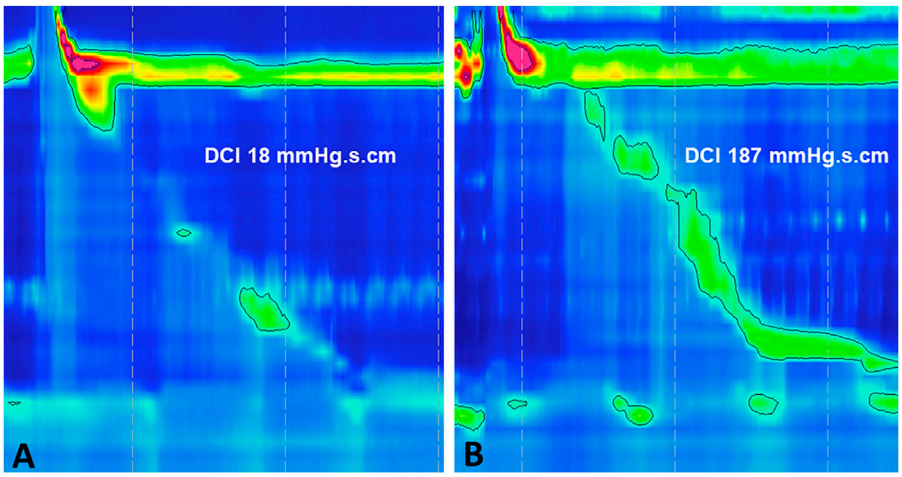

FIGURE 1 | Esophageal high-resolution manometry demonstrating Ineffective Esophageal Motility (IEM) **(A)** failed peristalsis (DCI <100 mmHg s cm) and **(B)** weak peristalsis (DCI 100–450 mmHg s cm). IEM, ineffective esophageal motility; DCI, distal contractile integral.

IEM is reported in as many as 30% of patients undergoing HRM. It is defined by the Chicago classification as over 50% of swallows being either weak or failed [Distal Contractile Integral (DCI) ≤450 mmHg s cm], while normal lower esophageal sphincter relaxation is preserved [normal Integrated Relaxation Pressure (IRP)] (**Figure 1**) (Kahrilas et al., 2015).

In this narrative review, we summarize the available literature on the clinical associations of a manometric diagnosis of IEM and its treatment with prokinetics. A PubMed literature search was performed that included published articles in English through October 31, 2020 with combinations of the terms "ineffective esophageal motility," "high resolution manometry," "clinical relevance," "pharmacological treatment," and "prokinetic." Reference lists of the retrieved articles were also searched for additional articles.

ESOPHAGEAL HYPOMOTILITY AND ESOPHAGEAL SYMPTOMS

IEM is one of the most frequent findings on esophageal HRM. However, the association of esophageal hypomotility with symptoms is still controversial which makes this a confusing diagnostic entity. A prospective study of Hollenstein et al. in healthy volunteers revealed that as many as 17% of asymptomatic subjects demonstrated a pattern of IEM on routine esophageal manometry (Hollenstein et al., 2017). Moreover, IEM is detected in patients with a variety of esophageal symptoms, particularly gastroesophageal reflux symptoms and dysphagia, but these symptoms are not discriminative of IEM. A retrospective study from China evaluated 256 dysphagia patients who had unremarkable findings on esophagogastroscopy and underwent HRM. In this population IEM was the most common feature, in 38.6% of patients (Wang et al., 2019). However, several studies failed to demonstrate a correlation between IEM and esophageal symptoms (Xiao et al., 2014; Shetler et al., 2017). Indeed, proportions of heartburn, regurgitation, dysphagia, chest pain, and belching were similar in patients with and without IEM in observational studies (Min et al., 2015; Shetler et al., 2017). In addition, the correlation of the perception of dysphagia with abnormal bolus transit resulting from weak or absent peristalsis is also limited (Lazarescu et al., 2010; Roman et al., 2011).

IEM is more prevalent in smooth muscle disorders, such as scleroderma and other connective tissue disorders (Carlson et al., 2016). Moreover, phosphodiesterase inhibitors, calcium channel blockers and non-benzodiazepine antispasmodic agents can reduce esophageal contraction vigor and should be avoided in patients with esophageal hypomotility (Simren et al., 2003; Rangan et al., 2018).

ESOPHAGEAL MOTOR DYSFUNCTION AND GASTROESOPHAGEAL REFLUX DISEASE

Available data indicate that Gastroesophageal Reflux Disease (GERD) results from multiple predisposing factors in upper gastrointestinal motility, especially transient lower esophageal sphincter relaxations (TLESRs) (Schneider et al., 2010) which are more likely to be associated with reflux in GERD patients (Sifrim et al., 2001). In addition, esophageal body hypomotility is also more frequent in pH-monitoring proven GERD (Chan et al., 2011; Savarino et al., 2017).

Impaired esophageal clearance of the refluxate caused by ineffective primary and secondary peristalsis has also been illustrated in a higher proportion of patients with erosive esophagitis compared to a non-erosive reflux disease group (29 vs. 15%; $p = 0.030$) (Wu et al., 2007; Chen et al., 2014). Furthermore, the study of Wang et al. also demonstrated that erosive esophagitis and increasing GERD symptom severity are consistently associated with a greater likelihood of IEM, while the prevalence of IEM in non-erosive reflux disease and physiologic acid exposure is low (Wang et al., 2009). Additionally, severe IEM, defined as over 70% ineffective peristalsis, provides supportive evidence for a more severe GERD phenotype with

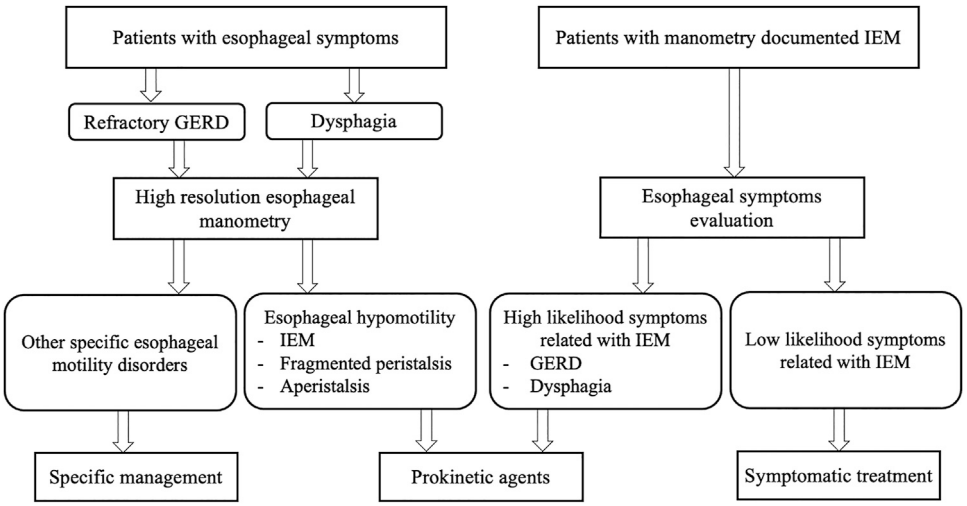

FIGURE 2 | Algorithm for decision making on prokinetic prescription in patients with esophageal hypomotility. IEM, ineffective esophageal motility; GERD, gastroesophageal reflux disease.

an increased acid exposure in supine position (Shetler et al., 2017; Rengarajan et al., 2018). These data suggest a role for IEM in the pathophysiology of GERD. However, a case could also be made that esophageal hypomotility may result from increased reflux exposure. Longitudinal data, studying the sequence between GERD and esophageal hypomotility, are not available, but improvement of hypocontractility after anti-reflux surgery has been reported, suggesting that successful anti-reflux treatment may correct esophageal dysmotility (Munitiz et al., 2004). However, prolonged proton pump inhibitor (PPI) treatment in 23 patients with severe erosive esophagitis did not correct esophageal hypomotility (Xu et al., 2007). In a recent study on the reproducibility of esophageal manometric diagnoses, PPI treatment was not clearly associated with improved motility in esophageal hypomotility (Sandhu et al., 2020).

NATURAL HISTORY AND PROGNOSIS

There is limited understanding of the natural history of IEM. However, IEM does not progress over time, and quality of life does not seem to be much impacted (Ravi et al., 2015). Patients with this minor esophageal motor abnormality reported minimal symptoms and needed few medical interventions during long-term follow-up over 6 years. Interestingly, the presence of peristaltic reserve by provocative maneuvers including multiple rapid swallowing (MRS) predicted a better prognosis and efficacy of prokinetics (Min et al., 2015; Gyawali et al., 2019).

GENERAL PHARMACOLOGICAL MANAGEMENT

Pharmacologic interventions that are able to improve esophageal smooth muscle contractility or associated symptoms, are still limited and poorly effective (Smout and Fox, 2012). There is also no clear directive on when IEM needs management, as symptoms (e.g., dysphagia) and even GERD is not consistently identified with IEM. Therefore—unless GERD is identified—the decision if and how symptomatic patients with IEM should be treated, is challenging.

Diet, lifestyle modification and medical GERD management remain the cornerstone of therapy (Triadafilopoulos et al., 2016). Basically, acid-suppressive medication will treat reflux and reflux-related symptoms but it may not improve esophageal motor function (Xu et al., 2007; Sandhu et al., 2020). Besides PPI therapy, prokinetic agents are advised in GERD patients with esophageal motility disorders and PPI-refractory symptoms to enhance clearance of the refluxed contents (Scarpellini et al., 2016; Lin et al., 2019).

Taking into account the limitations of IEM in terms of correlation to symptoms and GERD, we propose an algorithm to guide clinical decision making on prokinetic prescription in patients with esophageal hypomotility (**Figure 2**).

PROKINETIC TREATMENT

Esophageal peristalsis results from a concerted contraction and relaxation of circular and longitudinal musculature to propel the ingested food bolus toward the stomach. Peristalsis in the proximal esophagus, which is composed entirely of striated muscle, is dependent on central mechanisms, involving sequential activation of vagal lower motor neurons originating from the nucleus ambiguous (Kahrilas and Boeckxstaens, 2013). In contrast, distal esophageal peristalsis, which is mainly composed of smooth muscle fibers, is controlled by both central input, but mainly orchestrated by the ganglia of the enteric nervous system in the esophageal wall (**Figure 3**). There are two types of postganglionic myenteric motor

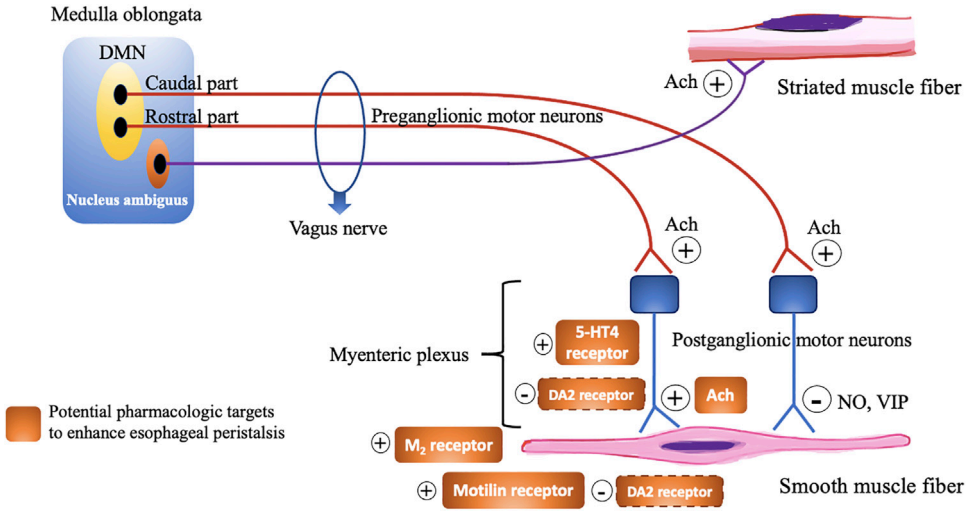

FIGURE 3 | Schematic overview demonstrating the motor innervation of the esophagus and pharmacological targets to enhance esophageal peristalsis. Dashed line signify uncertainty of the exact receptor localization. DMN, Dorsal motor nucleus; Ach, Acetylcholine; NO, Nitric oxide; VIP, Vasoactive intestinal polypeptide; 5-HT, 5-Hydroxytryptamine; M, Muscarinic; DA, Dopamine.

neurons: excitatory neurons releasing the neurotransmitter acetylcholine (ACh), and inhibitory neurons that contain nitric oxide (NO) and vasoactive intestinal polypeptide (VIP). The balanced activation and interaction between these neurons and neurotransmitters are critical for the normal peristaltic function of the esophagus (Park and Conklin, 1999) and can be targeted by pharmacologic interventions, using prokinetics, in patients with severe esophageal hypomotility or absent contractility to restore esophageal motor function (**Table 1**; **Figure 4**). In patients with mild esophageal hypomotility we recommend a conservative approach taking into account the limited available clinical evidence and benign natural history. We also propose a hierarchy in the use of the available prokinetics based on the efficacy data and adverse events (**Figure 4**). In this overview we did not focus on cisapride and tegaserod because these drugs have been withdrawn from the market because of cardiovascular adverse events. Although tegaserod has been re-approved in 2019, the specific indication was limited to female patients with constipation predominant irritable bowel syndrome.

Dopamine 2 Receptor Blockers

Metoclopramide augmented esophageal contraction in healthy volunteers (Mikami et al., 2016) but in IEM, this conventional prokinetic agent is not beneficial (Gyawali et al., 2019). The acute effects of oral metoclopramide (40 mg/day) and domperidone (80 mg/day) on esophageal motor activity and acid reflux has been assessed in a randomized, double-blind, placebo-controlled study in 20 patients with erosive esophagitis. Both drugs caused a significant increase in lower esophageal sphincter (LES) pressure. However, neither esophageal body motility nor duration of esophageal acid exposure were affected by the prokinetics in comparison to placebo (Grande et al., 1992). Both domperidone (QT prolongation) and metoclopramide (extrapyramidal manifestations) have been associated with relevant adverse events, which are potentially serious (Leelakanok et al., 2016; Svendsen et al., 2018). In view of the lack of substantial efficacy they should probably not be used when attempting to treat esophageal hypomotility.

Serotonin Modulating Agents

Buspirone

Buspirone, an anxiolytic drug, is a partial agonist for 5-HT (hydroxytryptamine) 1A receptors, as well as an antagonist for dopamine D2 autoreceptors, with some evidence of a weak agonistic effect on 5-HT2 receptors (Loane and Politis, 2012). In the enteric nervous system, 5-HT1A receptors activation can release ACh from the nerve terminals and then stimulate esophageal motor function by muscarinic receptors on smooth muscle cells. (Eduard et al., 2017). Buspirone has been shown to augment esophageal peristaltic amplitude in healthy volunteers. Blonski et al. and Di Stefano et al. administered 20 mg of buspirone to healthy adults and measured esophageal motility by conventional manometry within 60 min of administration in a blinded, placebo-controlled trial. The mean distal esophageal amplitude and duration were increased in both studies after a single dose of buspirone (Blonski et al., 2009; Di Stefano et al., 2012).

In systemic sclerosis (SSc), Karamanolis et al. evaluated 21 consecutive symptomatic patients with esophageal hypomotility, using a one-time dose of 10 mg buspirone compared to 10 mg domperidone. They found an increased resting lower esophageal sphincter (LES) pressure after buspirone, but no other significant change in esophageal peristalsis (Karamanolis et al., 2015). Another study of the same group, showed the same results in a non-randomized open-label trial of 4 weeks of 20 mg buspirone dosage in 22 SSc patients (Karamanolis et al., 2016). However, patients with SSc are a specific group within the spectrum of esophageal hypomotility and in many patients, esophageal manometry will show absent contractility rather than IEM.

TABLE 1 | Potentially beneficial prokinetic medications for esophageal hypomotility that have been studied in patients.

Prokinetic groups	Mechanism of action	Study design	Patients	Dose and duration	Outcome
Serotonergic agents					
- Buspirone Aggarwal et al. (2018)	5-HT1A agonist	Prospective, double-blind, placebo-controlled, crossover study	10 IEM/FD patients	10 mg before meals three times daily for 2 weeks	No difference in esophageal HRM parameters
- Mosapride Ruth et al. (1998)	5-HT4 agonist and weak 5-HT3 antagonist	Double-blind crossover trial	21 GERD patients	40 mg for 2 days	Decrease in total number of reflux episodes
Ruth et al. (2003)		Double-blind, randomized, double-dummy, three-way crossover study	41 GERD patients	30 mg three times daily for 7 days	- Small effects on peristaltic durations and amplitudes - No significant effect on the total number of esophageal contractions
Chen et al. (2013)		Prospective, double-blind, placebo-controlled, crossover study	18 IEM patients	40 mg single dose	Improved esophageal sensitivity of secondary peristalsis
- Prucalopride Lei et al. (2018)	High affinity and specificity for 5-HT4 agonist	Randomized placebo-controlled, crossover trial	15 GERD patients with IEM	4 mg single dose	- Increased peristaltic wave amplitude - Decreased threshold for triggering secondary peristalsis
- Sumatriptan Grossi et al. (2003)	5-HT1 agonist	Prospective, double-blind, placebo-controlled, crossover study[40]	10 IEM patients with chest pain and dysphagia	6 mg subcutaneous in the morning and afternoon (two doses)	- Increased number of swallows - Increased number of primary esophageal motor waves
Motilin receptor agonists					
- Erythromycin Chrysos et al. (2001)		Randomized single-blind study	15 GERD patients	200 mg IV single dose	Increased amplitude, duration, velocity and strength of esophageal peristalsis
Chang et al. (2003)		Single arm study	45 DM patients	Oral 250 mg three times daily for 2 weeks	Shorter esophageal transit time
Tsai et al. (1995)		Single arm study	15 DM patients	- Oral form for 2 weeks - Dose not specified	- Shorter esophageal transit time - Less esophageal residue
Muscarinic receptor agonists					
- Bethanechol Agrawal et al. (2007)		Single arm, interpreter blinded study	Seven severe IEM patients	50 mg orally	- Improved contraction pressures - Improved distal esophageal amplitude - Enhanced complete bolus transit of the esophagus
Grevenitis et al. (2012)		Retrospective chart review	26 IEM patients	25 mg three times daily for an average of 7 months	50% improvement in dysphagia

5-HT, 5-hydroxytryptamine; IEM, ineffective esophageal motility; FD, functional dysphagia; HRM, high resolution manometry; GERD, gastroesophageal reflux disease; IV, intravenous; DM, diabetes mellitus.

Even if the data are limited, the pathophysiological mechanisms underlying esophageal dysmotility in SSc are probably due to a complex interplay of vascular, immune, and neural abnormalities. Pharmacological therapy may provide some benefit in neuropathic and myopathic dysfunction, while it will most likely not be efficient in later stages of fibrosis (Suto and Czirjak, 2009; Scheerens et al., 2015).

Recently, buspirone was studied in IEM and functional dysphagia patients, but there was no statistically significant difference in the high resolution esophageal parameters measured, as well as symptom outcomes compared to placebo (**Table 1**) (Aggarwal et al., 2018).

Mosapride

Mosapride is a 5-HT4 receptor agonist, and its metabolites also have a weak 5-HT3 antagonistic effect. Mosapride has no affinity for 5-HT1, 5-HT2 or dopamine D2 receptors. In 20 asymptomatic volunteers, mosapride 3 mg t.i.d. for 3 days increased the rate of complete bolus transit and accelerated esophageal bolus transit in a randomized double-blind crossover design (Cho et al., 2006). It has also been demonstrated that a single 40 mg dose of mosapride increased the likelihood of secondary peristaltic responses to abrupt intra-esophageal air distension (Chen et al., 2011). Another study in nine healthy volunteers by Fukazawa et al. (2014) revealed that mosapride 40 mg augmented peristaltic contractions, especially in the distal esophageal segments (Fukazawa et al., 2014).

In GERD patients, mosapride was significantly more effective than placebo in decreasing the total number of reflux episodes (Ruth et al., 1998). Moreover, a high dose of mosapride (90 mg/day) has been reported to improve esophageal motor function and acid reflux parameters (Ruth et al., 2003).

In IEM patients, a single high-dose of mosapride (40 mg) decreased the threshold volume of secondary peristalsis during

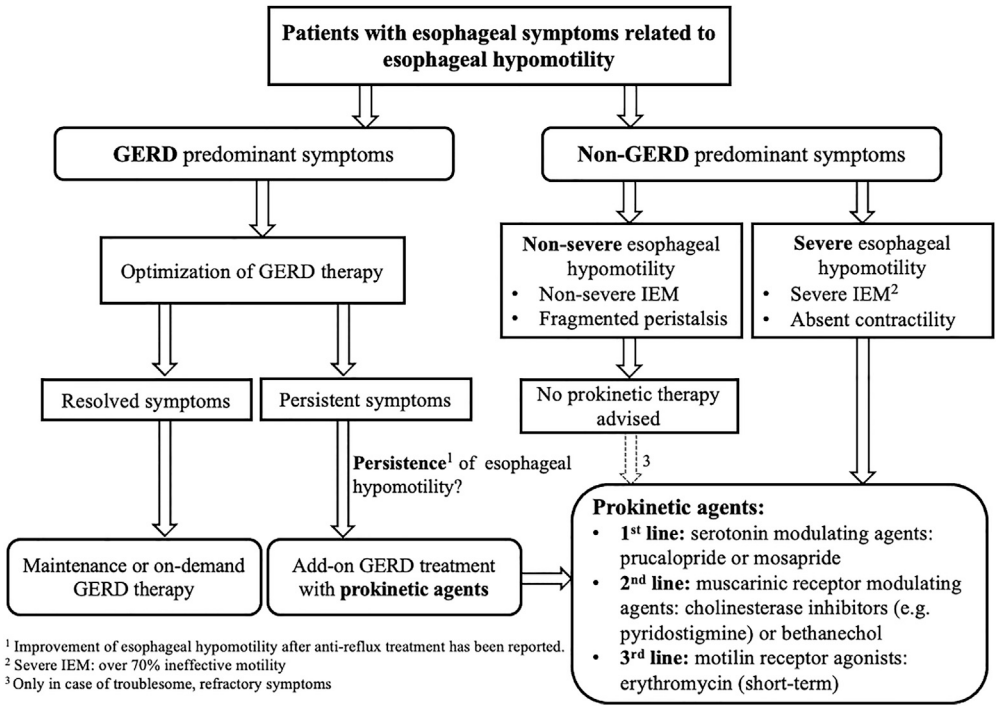

FIGURE 4 | Guided algorithm illustrating prokinetic drugs application in setting of esophageal hypomotility GERD, Gastroesophageal reflux disease; IEM, Ineffective esophageal motility; 5-HT, 5-Hydroxytrptamine.

rapid air distension compared with placebo, but had limited effect on the motor properties (Chen et al., 2013). A partial effect of mosapride on esophageal motility has only been shown with high dose, in contrast to low or standard dose (15 mg/day) which did not change the esophageal motility parameters (Koshino et al., 2010).

Prucalopride

Prucalopride is an enterokinetic agent which acts by facilitating the release of ACh from neurons of the myenteric plexus *via* a high affinity and specificity for 5-HT4 receptors (Briejer et al., 2001). Because of its highly specific effect on the 5-HT4 receptor in the absence of affinity for the hERG cardiac potassium channel, no cardiac toxicity has been reported in contrast to older 5-HT4 agonists, including cisapride (De Maeyer et at., 2008; Tack et al., 2012). Acute administration of 4 mg prucalopride enhanced mechanosensitivity of distension-induced secondary peristalsis and promoted esophageal contractility in 11 healthy adults (Yi et al., 2016). Kessing et al. demonstrated that 4 mg-prucalopride for 6 days in 21 healthy subjects reduced esophageal acid exposure and accelerated gastric emptying without significant effects on esophageal motility (Kessing et al., 2014).

In GERD patients with IEM, a single-dose of prucalopride enhanced primary and secondary peristalsis. The threshold volume for triggering secondary peristalsis during slow and rapid injection of air into esophagus was decreased, with limited impact on secondary peristaltic amplitude (Lei et al., 2018).

Sumatriptan

Sumatriptan is a 5-HT1D receptor agonist used in the treatment of migraine. In 3–5% of the patients this medication triggered chest symptoms which were hypothesized to originate from esophageal hypercontractility (Brown et al., 1991). In 16 healthy volunteers, one subcutaneous injection of 6 mg of sumatriptan significantly altered esophageal motor function with higher amplitude of esophageal contractions (Foster et al., 1999).

In patients with dysphagia and chest pain with IEM on manometry, a subcutaneous injection of sumatriptan increased the number of swallows and primary peristaltic waves, but not the amplitude or propagation velocity of esophageal motility (Grossi et al., 2003).

Motilin Receptor Agonists

Erythromycin

Erythromycin is an old macrolide antibiotic with prokinetic properties. The prokinetic action of erythromycin has been mainly attributed to its property of activating motilin receptors on smooth muscle fibers (Sanger et al., 2013). Its prokinetic efficacy was studied in GERD patients with a significant increase of the amplitude, duration, velocity and strength of esophageal peristalsis after a single intravenously administered dose (Chrysos et al., 2001).

Furthermore, oral erythromycin improved esophageal and gastric motility in diabetic patients and also resulted in a

better control of blood sugar. The esophageal transit time, evaluated by radionuclide labeled liquid and solid meals, was significantly shorter (Chang et al., 2003). Another study from Taiwan reported an improvement of esophageal hypomotility in 15 diabetic patients, as evaluated by a non-invasive radionuclide esophageal transit test (Tsai et al., 1995). Despite a possible benefit on esophageal motility, disadvantages, including the risk of inducing microbial resistance, tachyphylaxis and cardiac dysrhythmia (QTc prolongation), should be taken into consideration (Goossens et al., 2005; Ray, et al., 2004).

Muscarinic Receptor Modulating Agents

Bethanechol

Bethanechol, a direct-acting muscarinic receptor agonist, has been used in the past as a promotility agent for treating GERD. This drug acts by mimicking the effect of ACh directly at the postganglionic cholinergic receptors, and has been shown to increase the LES pressure and improve esophageal peristaltic pressures in healthy volunteers (Ramirez and Richter, 1993).

In patients with severe IEM, oral bethanechol has been shown to significantly improve contraction pressures, distal esophageal amplitude and complete bolus transit of the esophagus (Agrawal et al., 2007). A retrospective chart review of 26 patients with a known diagnosis of IEM who were treated with bethanechol at the esophageal disorders clinic, also reported a positive response, defined as improvement of dysphagia, in 50% of patients (Grevenitis et al., 2012). However, more than a quarter of patients discontinued the treatment due to intolerable cholinergic side effects, including nausea, somnolence and increased urinary frequency.

CONCLUSION AND FUTURE DIRECTION

It is important to emphasize that esophageal hypomotility is a manometric diagnosis that can be seen in healthy asymptomatic individuals and does not necessarily have a clear relevance in esophageal symptoms. Prokinetic agents can be considered in patients with esophageal symptoms thought to originate from IEM. However, currently available conventional prokinetic agents (mainly dopamine-2 antagonists) have not shown ability to restore the esophageal motor function in IEM. The potentially beneficial pharmacological agents are confined to specific serotonergic agents and motilin receptor agonists, but the scientific evidence is limited and larger future studies with a double-blind, randomized controlled design potentially including simultaneously impedance monitoring for bolus flow are needed to clearly identify its efficacy and clinical implication in patients with esophageal hypomotility.

AUTHOR CONTRIBUTIONS

SJ performed the literature review. SJ and TV drafted the first manuscript. AG, HG, NR, and JT revised the manuscript and provided substantial comments.

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MINI REVIEW
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Rikkunshito as a Therapeautic Agent for Functional Dyspepsia and its Prokinetic and Non-Prokinetic Effects

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Prokinetics is one of the therapeutic agents for functional and motility disorders of the stomach. However, its efficacy is limited. Kampo medicine is a unique medical system that was developed in Japan. In Kampo medicine, herbal medicine is prescribed based on the patient's condition. Therefore, even for functional and motility disorders of the stomach, some herbal medicines are considered as a therapeutic option. Recently, there has been an increase in evidence for the efficacy or the mechanism of herbal medicine for functional and motility disorders of the stomach. Among these, rikkunshito is a well-studied herbal medicine that could be used as an alternative to prokinetics. In this review, we discuss the possibilities of rikkunshito for functional dyspepsia with its prokinetic and non-prokinetic effects and provide an overview of their current use with a focus on their therapeutic mechanism.

Keywords: functional and motility disorders of the stomach, non-prokinetic treatment, rikkunshito, Kampo, functional dyspepsia

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INTRODUCTION

Kampo is a traditional herbal medicine that was introduced from China to Japan. After that, Kampo medicine has been uniquely developed in Japan. Research on Kampo medicine has thus been studied mainly in East Asia and has not yet garnered worldwide study. This way of evaluating patients is different from Western medicine. The most characteristic aspect of Kampo medicine lies in how it is used to evaluate patients. It evaluates the whole body condition, called the "pattern" and "qi, blood, and fluid", not each organ. Based on the patient's condition, herbal medicine is prescribed in Kampo medicine. Therefore, its application is broad and can include patients with Functional Dyspepsia (FD). Some herbal medicines are prescribed to patients with FD, and, of these, rikkunshito is a well-studied herbal medicine. This review focuses on rikkunshito and discusses its potential as an alternative therapeutic option for FD besides traditional treatments.

PATHOPHYSIOLOGY AND TREATMENT OF FD

Functional gastrointestinal disorders (FGIDs) are characterized by subjective abdominal symptoms and lack of organic disease. FGIDs are defined by the Rome IV criteria, which were revised in 2016 (Drossman and Hasler, 2016). Functional dyspepsia (FD) is one of the FGIDs of gastroduodenal lesions. In the Rome IV criteria, symptoms of FD are bothersome postprandial fullness, early satiation, epigastric pain, and epigastric burning occurring more than 6 months prior to diagnosis and persisting for more than 3 months. FD is sub-classified as post-prandial distress syndrome (PDS) or epigastric pain syndrome (EPS), and these sub-classifications can overlap.

The pathophysiology of FD is heterogeneous, and it involves various pathological conditions, such as dysmotility, visceral hypersensitivity, low-grade inflammation, dysregulation of the gut-brain axis, and psychological factors. Recently, the involvement of impaired duodenal mucosal integrity has been suggested (Vanheel et al., 2014; Wauters et al., 2020), although the details of the mechanism have not been fully elucidated.

Because of the heterogeneity in pathophysiology, the basic treatment of FD has not yet been established. When FD is diagnosed, the presence of a *Helicobacter pylori* infection should be checked for and, if present, treated. If an infection is absent or symptoms do not improve after treatment, proton pump inhibitors (PPI) and H_2 receptor blockers, or prokinetics such as acotiamide, domperidone, mosapride, and itopride, should be used. If no improvement is observed, additional antidepressants are recommended (Moayyedi et al., 2017).

The usefulness of PPI is constantly discussed. Some reports indicated that a 2–8-week dosing is more useful than placebo (Pinto-Sanchez et al., 2017), while others suggested that it is useful when complicated by gastroesophageal reflux disease (GERD) and is ineffective on FD itself (Wong et al., 2002; Pinto-Sanchez et al., 2017; Tack and Camilleri, 2018), and the efficacy of PPI is thus controversial. Although PPI are generally considered safe and well tolerated, they are associated with a risk of developing a *Clostridium difficile* infection, pneumonia, fractures, and acute interstitial nephritis with long-term administration (Wilhelm et al., 2013).

Although the effectiveness of prokinetics for FD was analyzed in the meta-analysis, there is a variation in the report (Moayyedi et al., 2017), and the evidence level is not high. Thus, a high-quality randomized controlled trial (RCT) is desirable (Pittayanon et al., 2018).

FD is not a life-threatening disease; however, it remarkably burdens the quality of life. Therefore, establishing effective therapy is urgently needed.

WHAT IS RIKKUNSHITO?

Rikkunshito is an herbal medicine that is widely used for improving gastrointestinal symptoms, such as FD, mainly in Asian countries. In China, it is called Liu-Jun-Zi Tang, and it is a Chinese herbal formula originally described in the Chinese classic medical book.

In Kampo medicine, when prescribing herbal medicine, the condition of patients is often assessed using the labels "pattern" and "qi, blood, and fluid." This concept markedly differs from that of Western medicine. In brief, the "pattern" is the evaluation of physical fitness and resistance to illness, and "qi, blood, and fluid" considers the causes of the disorder in these three parts. One way of dividing the "pattern" is "excess or deficiency." Individuals who are thin, delicate, and prone to diarrhea and chilling and those with weak gastrointestinal function a low stamina are classified as having a deficiency. Conversely, those who have sufficient energy and muscular strength are classified as having an excess pattern. Rikkunshito is prescribed mainly for patients with a deficiency pattern. When translating rikkunshito directly to English, it would be translated to "soup of six nobles." Rikkunshito is composed of the following eight herbal medicines: extracts of *Atractylodes lancea* Rhizome, Ginseng, *Pinellia tuber*, *Poria sclerotium*, *Jujube*, *Citrus unshiu* Peel, *Glycyrrhiza*, and *Ginger*. Among these, the extracts of the *Atractylodes lancea* Rhizome, *Ginseng*, *Poria sclerotium*, *Pinellia tuber*, *Citrus unshiu* Peel, and *Glycyrrhiza* were imagined as the six nobles (Yagi et al., 2004). It has been clarified that the ingredients of rikkunshito include β-eudesmol derived from the *Atractylodes lancea* Rhizome, ginsenosides derived from the Ginseng, hesperidin derived from the *Citrus unshiu* Peel, glycyrrhizin derived from the *Glycyrrhiza*, and shogaol derived from the ginger. Rikkunshito is widely used for upper gastrointestinal disorders in Japan. As side effects, hepatic dysfunction or pseudoaldosteronism owing to glycyrrhizin contained in *Glycyrrhiza* are known.

Clinical Effect of Rikkunshito on FD

In 1993, Tatsuta et al. (Tatsuta and Iishi, 1993) divided 42 patients with FD into the rikkunshito group ($N = 22$) and the placebo group ($N = 20$) and compared gastric emptying using the acetaminophen absorption method. They reported that gastrointestinal symptoms significantly improved and gastric emptying was enhanced in the rikkunshito group after 7 days of medication. In 2014, Suzuki et al. performed a double-blind RCT, dividing FD patients into a rikkunshito group and a placebo group where patients received 8 weeks of medication. Improvement of epigastric pain ($p = 0.04$) and postprandial fullness ($p = 0.06$) were observed in the group treated with rikkunshito, and this suggested that the efficacy may be lower in *H. pylori*-uninfected individuals (Suzuki et al., 2014). In a post hoc analysis (Togawa et al., 2016), it has been reported that a lower level of ghrelin in blood was an independent factor supporting the effectiveness of rikkunshito in FD patients without *H. pylori* infection and that it was more therapeutically effective in patients with *H. pylori* infection in the absence of alcohol consumption. Another double-blind, RCT was conducted in Japan in which 192 FD patients who met the Rome III criteria were enrolled (Tominaga et al., 2018). After a 2-week single-blind placebo period, patients were divided into the rikkunshito group ($N = 64$) and the placebo group ($N = 61$) for 8 weeks. The authors showed significant improvement in psychiatric symptoms and upper gastrointestinal symptoms in the rikkunshito group (Tominaga et al., 2018). However, a systematic review and a meta-analysis review have suggested that further studies are needed for supporting the usefulness of rikkunshito (Hoshino et al., 2019). Mausy et al. examined the effect of rikkunshito on symptoms and gastric motility in European FD-PDS patients using a randomized, placebo-controlled, crossover study. Rikkunshito did not alter gastric motility. Treatment with rikkunshito improved upper gastrointestinal symptoms in FD patients, but similarly, high placebo effects were observed (Masuy et al., 2020). A meta-analysis has reported that the combination of Western medication and rikkunshito was more helpful than the use of Western medication alone (Ko

et al., 2020). Further accumulation of evidence for the clinical effect of rikkunshito on FD is demanded.

MECHANISM OF ACTION OF RIKKUNSHITO ON SENSORY-MOTOR FUNCTION IN ANIMALS *VIA* THE NON-GHRELIN MEDIATED PATHWAY

When the effects of metoclopramide, trimebutine, cisapride, and rikkunshito were examined on the gastric adaptive relaxation in guinea pig stomachs, only rikkunshito induced adaptive relaxation of the stomach and improvement of the gastric volume. Oral administration of N(G)-nitro-L-arginine (L-NNA), a nitic oxide (NO) synthetic inhibitor to the stomach, inhibited gastric adaptive relaxation. Rikkunshito, but not gastroprokinetics, overcame the effect of the NG-nitro-L-arginine. This result suggests the action of rikkunshito *via* the NO-mediated pathway (Hayakawa et al., 1999).

Serotonin (5-hydroxytryptamine (5-HT)) is synthesized from tryptophan, and 90% of 5-HT exists in the gastrointestinal tract. 5-HT regulates the sensitization, motility, and secretion of the gastrointestinal tract. 5-HT receptors are classified into seven families and 14 subtypes. Among these, 5-HT_3 and 5-HT_4 receptors are involved in the regulation of sensation and motor function in the gastrointestinal tract (Grider et al., 1998; Taniyama et al., 2000), and 5-HT_{2C} is involved in eating behavior.

The efficacy of rikkunshito for cisplatin-induced anorexia in rats has been reported. Administration of 5-HT and dopamine-induced delayed gastric emptying in rats. Administration of a 5-HT_3 receptor antagonist, ondansetron, and rikkunshito did not improve the delayed gastric emptying induced by dopamine but ameliorated the delayed gastric emptying induced by 5-HT. Furthermore, their effect was abolished by atropine administration. Therefore, the 5-HT_3 receptor-mediated pathway has been suggested as a mechanism of action of rikkunshito (Tominaga et al., 2011). Additionally, several flavonoids contained in rikkunshito cross the blood-brain barrier. The action of rikkunshito for anorexia may be mediated via 5-HT_{2C} receptors in the central nervous system (Arai et al., 2013). Moreover, association between plasma ACTH and cortisol levels under stress and rikkunshito is also reported (Naito et al., 2003).

Regarding gastric emptying, the effect of rikkunshito is mainly studied with rats. Improvement of delayed gastric emptying *via* the NO-mediated pathway is reported. In this study, as ingredients in rikkunshito, roles of Hesperidin in *Citrus unshu* Peel and L-Arginine in Ginseng, *Pinella ternate* and *Atractylodes lancea* Rhizome are suggested (Kido et al., 2005). Improvement of delayed gastric emptying *via* antagonistic action of the 5-HT(3) receptor is suggested (Sadakane et al., 2011). Moreover, potentiation of phase III-like contractions by rikkunshito is reported. In this study, as ingredients in rikkunshito, roles of Hesperidin in *Citrus unshu* Peel, and L-Arginine in Ginseng, *Pinella ternate* and Atractylodin in *Atractylodes lancea* Rhizome are suggested (Nahata et al., 2018). Regarding gastric adaptive relaxation or gastric accommodation, the effect of rikkunshito is mainly studied with guinea pigs. Potentiation of gastric adaptive relaxation is reported (Hayakawa et al., 1999). Moreover, suppression of impairment of gastric accommodation *via* the NO- and 5-HT(2B) receptor-mediated pathway is reported (Miwa et al., 2016). These findings are summarized in **Table 1**.

In 2020, Zhao et al. reported that rikkunshito significantly attenuated visceral hypersensitivity in FD model rats, and the overexpression of EC cells, 5HT, TPH1, PAX4, and 5HT3R in the duodenum of FD model rats was also reduced by rikkunshito decoction (Zhao et al., 2020).

MECHANISM OF ACTION OF RIKKUNSHITO *VIA* THE GHRELIN-MEDIATED PATHWAY

Ghrelin is a 28-amino-acid peptide identified as a ligand for the growth hormone secretagogue receptor (GHS-R) in 1999. Ghrelin is mainly secreted by gastric endocrine X/A-like cells (Kojima et al., 1999). Secreted ghrelin enhances food intake through the activation of food intake stimulating neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons and the inhibition of anorexigenic pro-opiomelanocortin (POMC) neurons. These neurons exist in the arcuate nucleus of the hypothalamus (Kageyama et al., 2010; Andrews, 2011). Ghrelin regulates growth hormone secretion and feeding, and the blood level of ghrelin is negatively correlated with the body mass index in healthy individuals and uncomplicated type 2 diabetes mellitus patients; it has been reported to be lower in obese patients and higher in lean patients with cancer, chronic heart failure, and anorexia nervosa (Ueno et al., 2007). Administration of a selective serotonin uptake inhibitor containing fenfluramine reduced blood ghrelin levels and gastrointestinal peristalsis in rats. Oral administration of rikkunshito improved blood ghrelin levels and dietary intake (Fujitsuka et al., 2009). In clinical studies in humans, the administration of rikkunshito increases blood ghrelin levels in healthy participants and in patients after gastrectomy (Fujitsuka and Uezono, 2014). Rikkunshito may ameliorate anorexia by promoting the secretion of ghrelin. In 2008, Takeda et al. firstly reported the relationship between rikkunshito and ghrelin. They reported oral administration of rikkunshito suppressed the cisplatin-induced decrease in plasma acylated-ghrelin levels. (Takeda et al., 2008). Since then, a variety of rikkunshito's ghrelin-mediated mechanisms have become apparent. Thirty-two ingredients were considered as typical in rikkunshito. In total, 18 or 21 ingredients from plasma or urine were detected after administration of rikkunshito in healthy volunteer study (Kitagawa et al., 2015). Among these, 9 ingredients were elucidated their mechanism as ghrelin mediated therapeutic effect of rikkunshito by *in vivo* and *in vitro* study. Pachymic acid in *Poria sclerotium* and 10-gingerol in *Ginger* inhibit

TABLE 1 | List of *in vivo* studies that examine the efficacy of rikkunshito on motor patterns.

Effect	Ingredient	Mechanism	Utilized animals	References
Improvement of delayed gastric emptying	Hesperidin, L-Arginine	NO mediated action	Rats	Kido et al. (2005)
Improvement of delayed gastric emptying		Antagonistic action of the 5-HT(3) R	Rats	Tominaga et al. (2011)
Potentiate phase III-like contractions	Atractylodin		Rats	Nahata et al. (2018)
Potentiate gastric adaptive relaxation			Guinea pigs	Hayakawa et al. (1999)
Suppress impairment of gastric accommodation		NO and 5-HT(2B)R mediated pathway	Guinea pigs	Miwa et al. (2016)

NO, nitric oxide; 5-hydroxytryptamine 3 receptor, 5-HT(3) R; 5-hydroxytryptamine 2B receptor, 5-HT(2B)R.

TABLE 2 | Rikkunshito ingredients with a ghrelin-related effect.

Herb	Ingredient	Target	Role	References
Poria sclerotium	Pachymic acid		Inhibit ghrelin degration	Sadakane et al. (2011)
Citrus unshu Peel	Heptamethoxyflavone	PDEIII	Improve ghrelin resistance	Takeda et al. (2010)
	Nobiletin	PDEIII	Improve ghrelin resistance	Takeda et al. (2010)
	Isoliquiritigenin	PDEIII	Improve ghrelin resistance	Takeda et al. (2010)
	Hesperidin	5-HT(2C)R	Promote ghrelin secretion	Nahata et al. (2013)
Ginger	8-Gingerol	5-HT(2C)R	Promote ghrelin sectertion	Nahata et al. (2013)
	10-Gingerol		Inhibit ghrelin degration	Sadakane et al. (2011)
Glycyrrhiza	Isoliquiritigenin	5-HT(2B)R	Promote ghrelin secretion	Yamada et al. (2013)
Atractylodes lancea Rhizome	Atractylodin		Promote ghrelin secretion	Fujitsuka et al. (2011)

PDE III, phosphodiesterase III; 5-hydroxytryptamine 2C receptor, 5-HT(2C)R, 5-hydroxytryptamine 2B receptor, 5-HT(2B)R.

ghrelin diacylation by inhibiting ghrelin-deacylating enzymes (Sadakane et al., 2011). Heptamethoxyflavone, Nobiletin, and Isoliquiritigenin in *Citrus unshu* Peel improve ghrelin resistance via central phosphodiesterase III inhibition (Takeda et al., 2010). Atractylodin in *Atractylodes lancea* Rhizome, Hesperidin in *Citrus unshu* Peel, 8-gingerol in *Ginger*, and Isoliquiritigenin in *Glycyrrhiza* promote ghrelin secretion (Fujitsuka et al., 2011; Nahata et al., 2013; Yamada et al., 2013). Particularly, Hesperidin and 8-gingerol promote ghrelin secretion by central 5-HT2C receptor antagonism, and Isoliquiritigenin promotes ghrelin secretion *via* peripheral 5-HT2B receptor antagonism. The effects of the above ingredients of rikkunshito are summarized in **Table 2**.

Most of the circulating ghrelin is synthesized and secreted by X/A-like cells in the stomach. Morevoer, neurons in the hypothalamus also express ghrelin (Castañeda et al., 2010). As mentioned above, rikkunshito has both of peripheral and central effect for promoting ghrelin secretion. However, whether the central or peripheral effect of rikkunshito is more important for promoting ghrelin secretion is not clarified yet.

The plasma levels of ghrelin decrease with increasing gastric mucosal atrophy (Suzuki et al., 2004). In the randomized clinical trial of rikkunshito for the treatment of functional dyspepsia, rikkunshito was relatively more effective among *Helicobacter pylori*-infected participants (rikkunshito: 40.0% vs. placebo: 20.5%, $p = 0.07$) and seemed less effective among *H. pylori*-uninfected participants (rikkunshito: 29.3% vs. placebo: 25.6%, $p = 0.72$). Among *H. pylori-positive* individuals, acyl ghrelin levels were improved just in rikkunshito group (Suzuki et al., 2014). From these findings, there is a possibility that rikkunshito returns the ghrelin level to normal when the ghrelin secretion is below the normal level.

A randomised double-blind cross-over study of healthy volunteers showed an increase in the appetite and energy consumption in the free-choice buffet (Wren et al., 2001). Therefore, long term administration of ghrelin may have a risk for evoking obesity. However, to the best of our knowledge, a risk of obesity has not yet been linked to rikkunshito administration.

DISCUSSION

As mentioned above, in an *in vivo* study, the mechanisms of action of rikkunshito, such as promoting ghrelin secretion, mediating gastric adaptive relaxation, and stimulating gastric emptying, have been elucidated (Kido et al., 2005; Arai et al., 2013; Fujitsuka and Uezono, 2014). The effect of rikkunshito on gastric motility in humans has been studied with various modalities. Kusunoki et al. measured the cross-sectional area of the proximal part of the stomach using abdominal ultrasound and calculated the gastric emptying rate, motility index, and reflux index before and after administration of rikkunshito in patients with FD. However, there was no improvement in symptoms; all measurements except the reflux index significantly increased after the administration of rikkunshito (Kusunoki et al., 2010). Shiratori et al. used a barostat for measuring the volume of the stomach before and after the administration of rikkunshito in nine healthy volunteers and reported that the

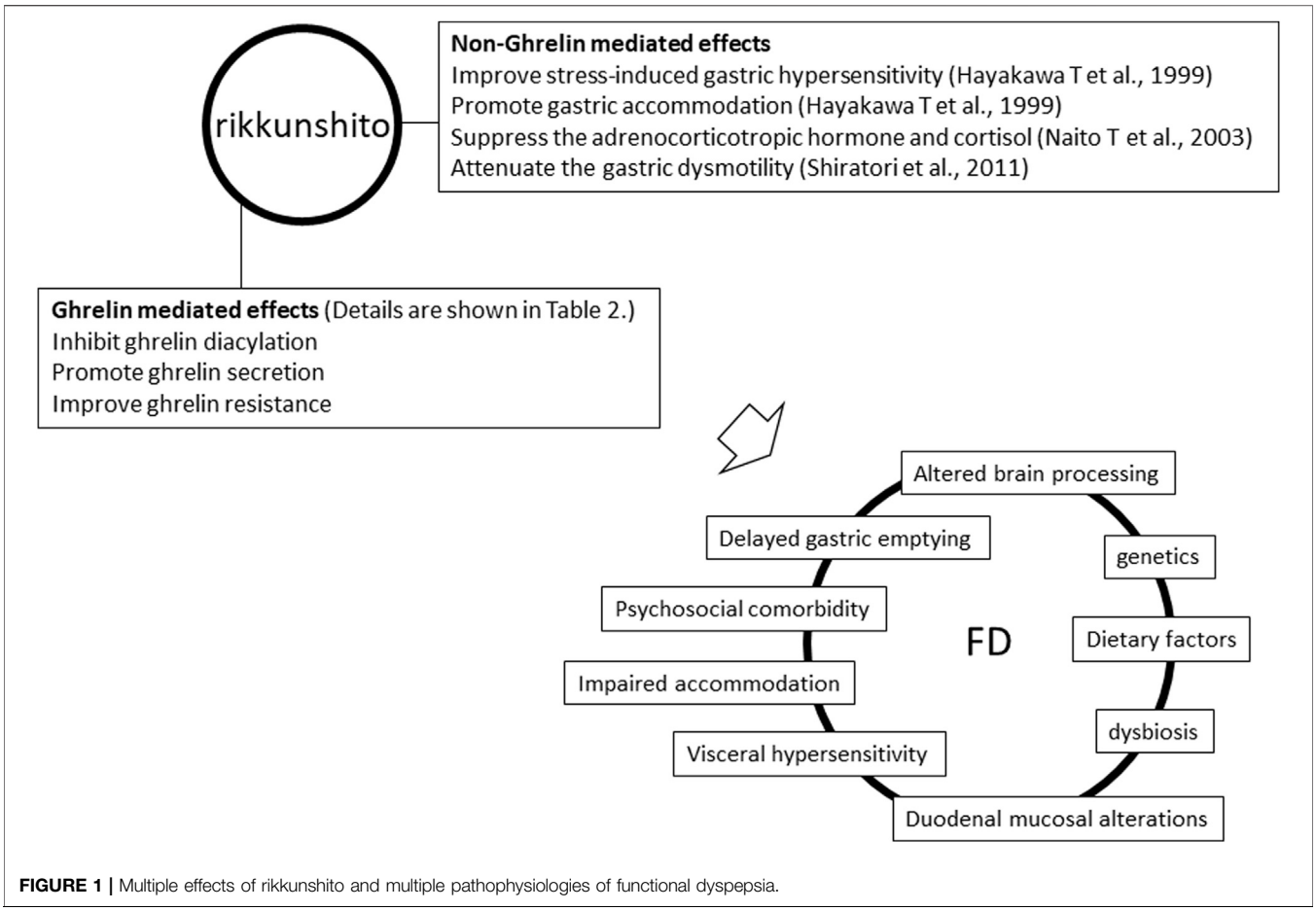

FIGURE 1 | Multiple effects of rikkunshito and multiple pathophysiologies of functional dyspepsia.

volume of the stomach increased significantly after the administration, with improvement in perceived stress (Shiratori et al., 2011). Mausy et al. examined the effect of rikkunshito on intra-gastric pressure with high-resolution manometry before and after treatment with rikkunshito; however, rikkunshito did not alter gastric motility (Masuy et al., 2020). These differences might be caused by the characteristics of each modality. Regarding the effect of rikkunshito, *in vivo* studies were ahead of clinical studies. Compared with the results of the aforementioned *in vivo* studies, human studies did not entirely reproduce results of *in vivo* studies. Further research on the effects of rikkunshito on the human stomach and its association with symptomatic improvement is needed.

As described in the Introduction section, Kampo medicine, in contrast to Western medicine, a suitable drug for the patient is prescribed based on the assessment of the patient's pattern in addition to the symptoms. Furthermore, herbal medicine contains a large number of active ingredients; the effects of herbal medicine come from intertwined ingredients and have a complex mechanism of action. They can act on the whole body rather than locally. Kampo medicine may not be very popular in Western countries because of the differences in the way of choosing herbal medicine. Herbal medicine used in Kampo medicine includes multiple herbals, and each herb includes many chemicals. Therefore, herbal medicine has various mechanisms, whereas a western drug is generally composed of a single chemical with a single mechanism of action (Yu et al., 2006).

Moreover, because many of the previous studies on Kampo medicine conducted in the 1980s–1990s have been published in Japanese, these pioneering studies have not been referred to in English literature; they have not been indexed in PubMed. This inaccessibility of studies may be one of the reasons for the difficulty in sharing the results globally (Oka et al., 2014). There is also a possibility that unexpected drug interactions may emerge in combination with Western medicines. Some side effects and components of herbal medicine, such as pseudoaldosteronism and glycyrrhizin in *Glycyrrhiza*, have been identified. Though it is not so for all cases, some drug interaction between herbal medicine and Western drugs could be solved through cautious use; therefore, the possibility of safe usage of Kampo in Europe is suggested (Homma, 2016).

As mentioned above, Rikkunshito can exert prokinetic activity and other effects, such as visceral hypersensitivity alleviation. There is no high-quality and sufficient evidence on the effectiveness of rikkunshito on FD. In contrast, it may be of

sufficient value to attempt approaches applying Kampo medicine for complex and poorly understood pathologies, such as FD (**Figure 1**). In the future, we hope that Kampo, including rikkunshito, will become popular worldwide, and studies to elucidate the disease state of FD and the efficacy of Kampo will be actively performed.

AUTHOR CONTRIBUTIONS

KI wrote the initial draft of the manuscript and revised the manuscript. TM designed this mini review and revised the manuscript. TK critically reviewed the manuscript, and all authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work by ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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Action Mode of Gut Motility, Fluid and Electrolyte Transport in Chronic Constipation

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Chronic constipation is a common gastrointestinal disorder, with a worldwide incidence of 14–30%. It negatively affects quality of life and is associated with a considerable economic burden. As a disease with multiple etiologies and risk factors, it is important to understand the pathophysiology of chronic constipation. The purpose of this review is to discuss latest findings on the roles of gut motility, fluid, and electrolyte transport that contribute to chronic constipation, and the main drugs available for treating patients. We conducted searches on PubMed and Google Scholar up to 9 February 2021. MeSH keywords "constipation", "gastrointestinal motility", "peristalsis", "electrolytes", "fluid", "aquaporins", and "medicine" were included. The reference lists of searched articles were reviewed to identify further eligible articles. Studies focusing on opioid-induced constipation, evaluation, and clinic management of constipation were excluded. The occurrence of constipation is inherently connected to disorders of gut motility as well as fluid and electrolyte transport, which involve the nervous system, endocrine signaling, the gastrointestinal microbiota, ion channels, and aquaporins. The mechanisms of action and application of the main drugs are summarized; a better understanding of ion channels and aquaporins may be helpful for new drug development. This review aims to provide a scientific basis that can guide future research on the etiology and treatment of constipation.

Keywords: pharmacological treatment, pathophysiology, gut motility, fluid and electrolyte transport, chronic constipation

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INTRODUCTION

Chronic constipation is a common gastrointestinal condition that is characterized by a high incidence rate, complex etiology, and difficult treatment. It can manifest as abdominal pain or bloating, or more commonly as chronic symptoms such as difficulty passing stools, infrequent stools, or incomplete defecation. Sometimes it can last for weeks, months, or years (Lacy et al., 2016). The prevalence of chronic constipation is increasing due to changes in diet composition, accelerated pace of life, and the influence of complex social and psychological factors. Epidemiological surveys have shown that the incidence of chronic constipation is between 14 and 30% worldwide, affecting individuals of all ages, races, socioeconomic status, and nationalities (Camilleri et al., 2017). However, it is perceived as being less frequent and not serious, and is often overlooked. Chronic constipation negatively impacts on quality of life, causing abdominal pain, bloating, loss of appetite and/or nausea, headaches, bad breath, restlessness, anxiety and/or depression, and is associated with

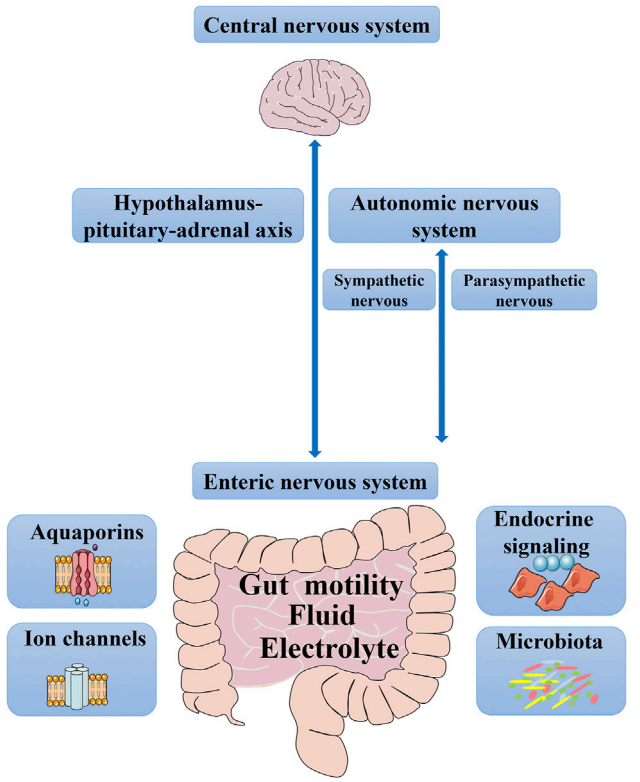

FIGURE 1 | Factors that control gut motility, fluid and electrolyte. From top to bottom, it encompasses the central nervous system, the sympathetic and parasympathetic branches of the autonomic nervous system, hypothalamus-pituitary-adrenal axis, the enteric nervous system, endocrine signaling, microbiota, aquaporins, and ion channels.

a substantial economic burden to individuals and society (Peery et al., 2019). The annual healthcare cost for patients with chronic constipation was reported to be $11,991 in the United States. The direct costs are substantial and include outpatient services (44.8%), inpatient hospitalizations (33.9%), prescriptions (17.8%), and emergency department visits (3.5%) (Cai et al., 2014). The economic burden of chronic constipation includes the costs of medical treatment or hospitalization, as well as those associated with treatment failure, which increase both healthcare resource utilization and medical costs (Guerin et al., 2014).

The mechanisms, evaluation, and management of chronic constipation were recently reviewed. The main mechanisms discussed were colonic sensorimotor dysfunction and alteration of the microbiome (Bharucha and Lacy, 2020). However, the pathogenesis of chronic constipation is multifactorial and includes colonic motility and fluid transport, anorectal movement and sensory functions, as well as dietary and behavioral factors. The purpose of this review is to introduce the latest findings on the role of gut motility and fluid and electrolyte absorption and secretion in the development of chronic constipation.

Chronic constipation is the earliest manifestation of gastrointestinal hypomotility, which is characterized by significantly prolonged intestinal transit time (Wang, 2015). Slower colonic transport prolongs the retention of intestinal contents and increases the reabsorption of water and electrolytes, resulting in a reduced volume and hardening of stools. Several factors jointly regulate gut motility, fluid, and electrolytes: the enteric nervous system (ENS), autonomic nervous system (ANS), central nervous system (CNS), endocrine signaling, microbiota, ion channels, and aquaporins (AQPs) (**Figure 1**). An imbalance or dysfunction in any of these components may cause abnormal intestinal function, which can lead to symptoms of constipation. First, gut motility and its influencing factors have been summarized, and then water and electrolyte transport will be discussed, focusing on the complexity of AQPs and ion channels. In terms of intestinal movement, one mechanism is neural networks, which control ENS independently, and the other involves the ANS and CNS. When it comes to fluid and electrolyte, we have mainly focused on channels, such as ion channels and AQPs. Finally, medication for treating chronic constipation has been discussed. Increasing motility and water secretion are currently the main goals of treatment for constipation; therefore, laxatives, which affect gut motility and water absorption/secretion, will be highlighted. Understanding the regulatory mechanism of gut motility and fluid and electrolytes transport in chronic constipation is helpful for further pathophysiological exploration and to provide a necessary theoretical basis for the further use of safe and effective drugs.

GUT MOTILITY PATTERN

In most cases, chronic constipation is considered to be closely related to disorders of gut motility (Wang, 2015). Two main types of intestinal movement enable feces to enter the rectum: peristalsis and colonic propulsion. These movements ensure the regular contraction of the colon wall and enable the contents to advance, thus promoting the normal excretion of feces.

Peristalsis

Peristalsis is the basis of most gastrointestinal propulsion movements, mainly in the colon, and includes the coordinated contraction and relaxation of the intestinal muscle layer (Camilleri et al., 2017). Relaxation/inhibition and contraction/excitement are thought to spread along the intestinal tract following stimulation. The neural control hierarchy for peristalsis is as follow: the primary regulator is the ENS, followed by the ANS and then the CNS. Additionally, neurotransmitters, gastrointestinal hormones, and microbiota work together to modulate peristalsis. Factors that modulate peristalsis are shown in **Figure 2**.

Regulation by ENS

The exertion of intestinal motor function is largely achieved by regulation of the ENS, which acts independently from the CNS and is a highly autonomous gastrointestinal neural network mainly composed of enteric neurons and enteric glial cells (EGCs) (Avetisyan et al., 2015). Enteric neurons include

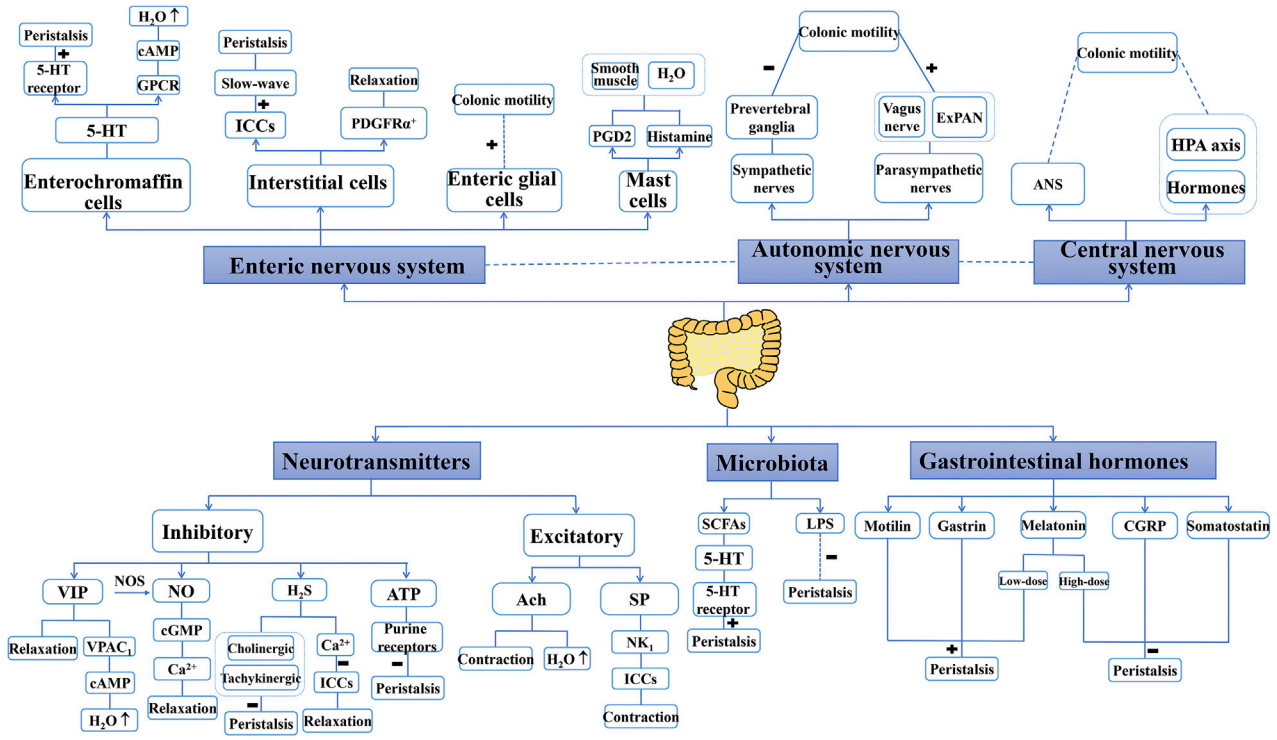

FIGURE 2 | Factors that modulate peristalsis. "+" indicate a promoting role, "–" indicate an inhibition role. Enteric nervous system, autonomic nervous system, central nervous system, neurotransmitters, gastrointestinal hormones and microbiota are the main factors to control peristalsis. Some of these factors also involve the secretion of water. 5-HT: 5-hydroxytryptamine; Ach, acetylcholine; ANS, autonomic nervous system; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CGRP, calcitonin gene-related peptide; ExPANs, extrinsic primary afferent neurons; GPCR, G-protein coupled receptors; HAP axis, hypothalamus–pituitary–adrenal axis; H_2S, hydrogen sulfide; ICCs, interstitial cells of Cajal; LPS, lipopolysaccharide; NK_1, neurokinin 1; NO, nitric oxide; NOs, nitric oxide synthase; PDGFRα$^+$, platelet-derived growth factor receptor α-positive; PGD2, prostaglandin D2; SCFAs, short-chain fatty acids; SP, substance P; VIP, vasoactive intestinal peptide.

afferent neurons, interneurons, motor neurons, mechanosensitive neurons, etc., which are connected in peristalsis reflex circuitries (De et al., 2004). They extend process to communicate with diverse cells types including other enteric neurons, enterochromaffin cells (ECs), interstitial cells, and mast cells (Furness, 2000). EGCs are distributed inside and outside the nerve plexus (between the submucosal nerve plexus and the lamina propria), which can interact with intestinal neurons or directly participate in the regulation of gut motility (Aubé et al., 2006). EGCs help to promote the normal regulation of intestinal electric ion transport and secretory motor function (Grubišić and Gulbransen, 2017).

Peristalsis can be activated by chemical and/or mechanical stimuli that are sensed by ECs (Gershon and Tack, 2007). Most 5-hydroxytryptamine (5-HT) in the human body is secreted by ECs. 5-HT is a mediator involved in regulating a variety of physiological functions in the gastrointestinal tract and plays an important role in regulating gut motility and intestinal secretion (Reynaud et al., 2016). 5-HT can activate the peristaltic reflex by activating the 5-HT receptor on the mucosal end of the intrinsic primary afferent neurons, including 5-HT_{2B}, 5-HT_3, 5-HT_4 and 5-HT_7 receptors (Wouters et al., 2007; Smith et al., 2014). These receptors signals through the myenteric plexus, which induces a wave of intestinal smooth muscle contraction. And the propulsion requires activation of both ascending excitatory neurons and descending inhibitory neurons. 5-HT also binds to G-protein coupled receptors in enterocytes (except for 5-HT_3 receptors), increasing the levels of cyclic adenosine monophosphate (cAMP) in target cells (Smith et al., 2014). An increase in cAMP causes the cystic fibrosis transmembrane conductance regulator (CFTR) to open, leading to Cl^- and water outflow (Dawson, 1991). Accordingly, the regulation of 5-HT can have profound effects on gut motility and intestinal secretion.

Interstitial cells produce and transmit electrical signals that regulate the excitement and inhibition of smooth muscle. Two types of interstitial cells are involved: interstitial cells of Cajal (ICCs) and platelet-derived growth factor receptor α positive (PDGFRα$^+$) cells. Constipation may be related to changes in the structure and shape of ICCs (Cohen et al., 2017), which play a significant physiological role in gut motility. They form a network of cells around the myenteric plexus between the circular and longitudinal muscle layers of the entire intestine and are responsible for regulating the contraction of gastrointestinal smooth muscle (Komuro, 2006). The pacing activity induced by ICCs causes a rhythmic slow-wave of smooth muscle cells.

Slow-wave is a relatively regular periodic electrical activity, which controls the rhythm of intestinal contraction. ICCs integrate slow-wave activity with excitatory and inhibitory neurotransmission to orchestrate peristalsis. These findings demonstrated that disorders of gut motility are due to the loss of slow-wave activity as well as disturbed neurotransmission (Klein et al., 2013). PDGFRα$^+$ cells inhibit smooth muscle activity by activating purinergic receptors (Camilleri et al., 2017).

There are close apposed between axons and mast cells in the gastrointestinal mucosa (Stead et al., 1989). Mast cells can regulate peristalsis and fluid secretion *via* bidirectional brain-gut interactions between the ENS and the CNS (Schaeffer et al., 2012). Mast cells can stimulate excitatory neurons and activate the ENS network, and thereby promote powerful propulsive motility (Wang et al., 2014). Histamine and prostaglandin D2 released by mast cells have been shown to modulate the smooth muscle and fluid secretion (Wouters et al., 2016).

Regulation by ANS and CNS

The ANS, which includes the sympathetic and parasympathetic nervous systems, is a collection of afferent and efferent neurons that link the CNS with the ENS (Tait and Sayuk, 2021). Both sympathetic and parasympathetic nerves regulate gut motility by affecting the ENS circuit (Mayer et al., 2014). The postganglionic fibers of the sympathetic nerve are adrenergic nerve fibers which cause the release of norepinephrine that elicit the presynaptic inhibition of neurotransmitter release, thereby inhibiting intestinal motility. Meanwhile, the parasympathetic nerve mainly transmits excitation signals *via* the vagus nerve (Bonaz et al., 2018). Extrinsic primary afferent neurons (ExPANs) derived from spinal ganglia can regulate colon function. ExPANs regulate myenteric neuron activity and smooth muscle contraction *via* a parasympathetic spinal circuit (Smith-Edwards et al., 2019).

CNS modulates gastrointestinal motility *via* two distinct routes (Mayer and Tillisch, 2011). First, it modulates gut motility *via* the ANS. The vagus nerve is the main component of the parasympathetic nervous system, which can transmit intestinal-related signals to the CNS. Second, CNS signals induce gastrointestinal motility *via* hormonal pathways, including the hypothalamus–pituitary–adrenal axis, and hormones of the neuroendocrine stress response. For example, neurotensin released by central preganglionic neurons promotes the release of substance P (SP), which in turn stimulates peristalsis (Szurszewski et al., 2002).

Regulation by Neurotransmitters

Constipation is associated with abnormalities in intestinal neurotransmitters, which are a class of active small-molecule peptides produced in gastrointestinal endocrine cells and nerve cells. There are two type of neurotransmitters, inhibitory and excitatory ones, which play an important role in regulating the motility and secretion of the gastrointestinal tract (Schneider et al., 2019).

Inhibitory neurotransmitters include vasoactive intestinal peptide (VIP), nitric oxide (NO), adenosine triphosphate (ATP), and hydrogen sulfide (H_2S), which can induce smooth muscle relaxation and inhibit intestinal sensitivity. VIP, one of the most important neurotransmitters in the ENS, inhibits the contraction of intestinal circular muscle, maintaining the intestine in a state of relaxation, and may also participate in the secretion of intestinal fluid (Mourad and Nassar, 2000). VIP binds to its receptor ($VPAC_1$), leading to the excretion of cAMP-related HCO_3^-. This causes Na^+ and H_2O to enter the intestinal cavity, thus increasing fluid secretion (Chandrasekharan et al., 2013). Therefore, reduced VIP production may result in reduced fluid secretion, which is a possible cause of constipation or its aggravation. VIP can activate nitric oxide synthase (NOS) in the colon wall, leading to the production of the inhibitory neurotransmitter NO. NO activates cyclic guanosine monophosphate (cGMP)-dependent protein kinase by stimulating intracellular soluble guanylate cyclase, which reduces the level of intracellular Ca^{2+}, relaxes smooth muscle cells, and eventually weakens gastrointestinal motility (Beck et al., 2019). H_2S is a gasotransmitter, which plays a role in the regulation of gut motility. In addition, it can inhibit the pacemaker activity of ICCs by regulating intracellular Ca^{2+}, which in turn leads to relaxation of gastrointestinal smooth muscle (Parajuli et al., 2010). Furthermore, high concentrations of H_2S can inhibit gut motility by interacting with cholinergic and tachykinergic neural-mediated pathways (Martinez-Cutillas et al., 2015). ATP, an important neurotransmitter, inhibits peristalsis by acting on the purine receptors on intestinal nerves and muscles (Heinemann et al., 1999).

5-HT, acetylcholine (Ach), and SP are excitatory neurotransmitters, which can stimulate intestinal muscle contraction and promote intestinal peristalsis. 5-HT is a paracrine signaling molecule released from ECs, and also a neurotransmitter that produced by serotonergic neurons of the ENS (Gershon, 2012). Ach activates the gastrointestinal tract by stimulating muscle contraction and increasing the peristalsis of gastrointestinal smooth muscle. Additionally, the non-neuronal release of Ach from colonocytes, coupled with propionate stimulation, promote Cl^- secretion, *via* the paracrine action of Ach on muscarinic receptors of colonocytes (Yajima et al., 2011). SP can regulate ICCs through the tachykinin NK_1 receptor and exerts a strong contractile effect on gastrointestinal smooth muscle (Jun et al., 2004).

Regulation by Gastrointestinal Hormones

The ENS regulates gut motility through neurotransmitters and by the secretion of gastrointestinal hormones for humoral regulation. Motilin, gastrin, melatonin, calcitonin gene-related peptide (CGRP), and somatostatin have important physiological significance in the regulation of gastrointestinal motility (Penning et al., 2000). Motilin acts directly on the motilin receptor on gastrointestinal smooth muscle cells and stimulates gastrointestinal peristalsis (Xu et al., 2005). Gastrin can increase small intestinal motility (Ahmed and Ahmed, 2019). Melatonin acts on the muscularis mucosae or the myenteric plexus, is involved in regulating colonic motility, and has a bidirectional effect on gut motility (Esteban-Zubero et al., 2017). Low-dose melatonin has been shown to accelerate

intestinal transit, while high-dose melatonin decreases gut motility. CGRP has been found to exert an inhibitory effect on gut motility by inducing interneurons to trigger the peristaltic reflex (Ceccotti et al., 2018). Somatostatin inhibits intestinal secretion, peristalsis, and the release of gastrointestinal hormones by acting on the somatostatin receptors on gastric smooth muscle (John and Chokhavatia, 2017).

Regulation by Microbiota

Studies have revealed an important relationship between the intestinal microbiota and constipation (Yarullina et al., 2020). *Bacteroides* were found to be more abundant in patients with chronic constipation (Yarullina et al., 2020), as well as decreased numbers of bifidobacteria and lactobacilli, compared with healthy controls (Dimidi et al., 2017). Short-chain fatty acids (SCFAs), endotoxin, and other products are produced during intestinal microbial metabolism and may affect gut motility (Segers et al., 2019). The intestinal microbiota can regulate the release of 5-HT by ECs, which in turn, affects gut motility (Yano et al., 2015). Furthermore, the release of 5-HT from ECs in response to SCFAs stimulates 5-HT_3 receptors located on vagal sensory fibers, resulting in muscle contraction (Fukumoto et al., 2003). Serum endotoxin activity was found to be positively related to constipation in patients undergoing chronic hemodialysis (Bossola et al., 2016). Involvement of the endogenous cannabinoid system in the regulation of gastrointestinal motility has been demonstrated *in vitro* and *in vivo* (Aviello et al., 2008). Lipopolysaccharide (LPS) was shown to reduce the amplitude and frequency of myoelectric spiking activity, and this was accompanied by a slowing of gastrointestinal transit (Li et al., 2010). Both cannabinoid-1 and cannabinoid-2 receptor antagonists were able to reverse the delayed intestinal transit induced by LPS (Li et al., 2010). However, evidence remains limited; therefore, further studies are needed to elucidate the exact mechanism.

Colonic Propulsion

Several forms of contraction (mass movements, retrograde propulsion, segmentation movement) push contents through the colon. Propulsive contraction is designed to push and eventually discharge feces. The large contraction of colonic smooth muscle cells increases the intracavitary pressure, which are termed high-amplitude propagating contractions (HAPCs), the main propulsive contractile force which represent the main motor pattern related to mass movement (Vijayvargiya and Camilleri, 2019). Another motor pattern generated by enteric nerves is segmentation, which involves alternating contractions of the muscularis in a given region without forward propulsion of the luminal contents. The rhythmic slow-wave pattern naturally organizes the contractile activity of gastrointestinal muscles into phasic contractions (Sanders et al., 2014). The contents of the colon can also move in a retrograde direction, which are more pronounced after a meal, and this pattern of movement can potentially prevent rectal filling (Lin et al., 2017). The decreased frequency of HAPCs, increased low-amplitude propagated contractions, and reverse propulsive contraction frequency are related to the occurrence of constipation (Bassotti et al., 2003; Dinning and Di, 2011).

MECHANISM OF INTESTINAL ELECTROLYTE TRANSPORT

Electrolyte imbalance can lead to muscle weakness, thus accelerating the occurrence of chronic constipation. The modulation of ion channels and exchangers in epithelial cells can promote intestinal secretion, thereby enhancing gastrointestinal transit and promoting fecal excretion. This is also the mechanism through which some drugs exert their laxative effect (**Figure 3**). Under normal physiological conditions, many ion channels and exchangers exist in the intestinal epithelium, which play an important role in maintaining the balance of intestinal absorption and secretion (Kagnoff, 2014). The main mechanisms associated with ion fluxes are nutrient-coupled Na^+ absorption, electroneutral NaCl absorption, electrogenic Na^+ absorption, and Cl^- secretion (Kato and Romero, 2011).

Mechanism of Intestinal Electrolyte Absorption

Nutrient-Coupled Na^+ Absorption

Enteral nutrient-coupled Na^+ absorption involves sodium-glucose transporters and Na-amino acid cotransporters, in which Cl^- and fluid are absorbed *via* a paracellular pathway (Kato and Romero, 2011). This also occurs in the presence of an adverse osmotic gradient (Wright and Loo, 2000).

Electroneutral NaCl Absorption

The coupled NaCl absorption mechanism occurs in both the small intestine and colon and is mediated by the coupling activity of the anion exchange transporter SLC26A6 or Cl^- anion exchanger SLC26A3 and the sodium/hydrogen exchanger family (NHE2 or NHE3) on the surface of epithelial cells (Patel-Chamberlin et al., 2016; Barrett, 2017). Na^+ and Cl^- enter the cell cytosol through these transporters, and are then transported across the basolateral membrane *via* the Na^+/K^+-ATPase and K^+/Cl^- cotransporters. Studies have shown that inhibiting SLC26A3, thus, blocking intestinal fluid absorption, can be used to effectively treat the main types of constipation (Haggie et al., 2018).

Electrogenic Na^+ Absorption

Epithelial sodium channel (ENaC) is present on the superficial epithelial cells of the distal colon and rectum. Na^+ absorption occurs on the luminal epithelial membrane through the ENaC and is compensated for by the Na^+ output of basolateral Na^+/K^+-ATPase. This mechanism is important in the distal colon (Barrett, 2017). CAP1/Prss8, an *in vivo* regulator of colonic ENaC, can activate ENaC activity (Malsure et al., 2014) and is up-regulated by aldosterone.

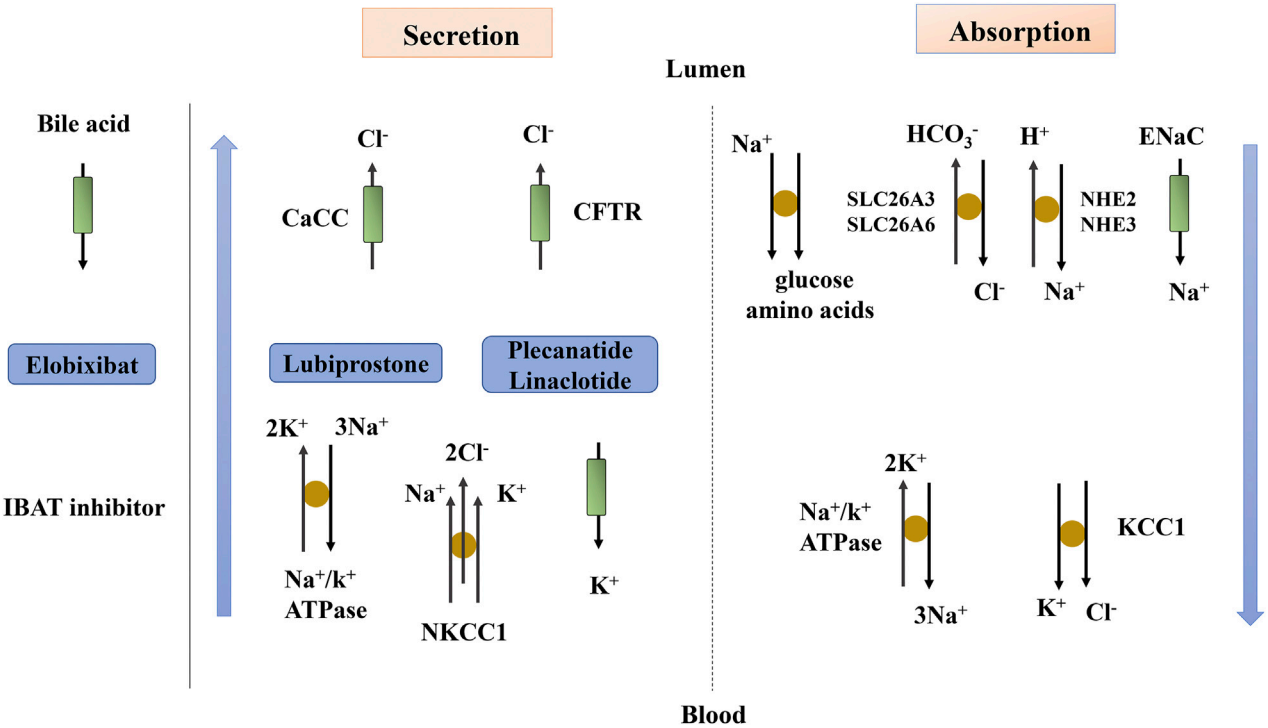

FIGURE 3 | Fluid and electrolyte transport in the intestine. The blue arrow indicates the transport of fluid beside the cells. Lubiprostone, Plecanatide and Linaclotide act through ion transport. CaCC, Ca^{2+}-activated Cl^- channel; CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial sodium channel; IBAT, ileal bile acid transporter; KCC1, K^+/Cl^- co-transporter; NHE, Na^+/H^+ exchanger; NKCC1, $Na^+/K^+/Cl^-$ co-transporter; SLC26A3, Cl^- anion exchanger; SLC26A6, anion exchange transporter.

Mechanism of Intestinal Electrolyte Secretion

Chloride ion transport in epithelial cells is the main determinant of fluid secretion, and Cl^- is mainly secreted by crypt cells in the whole small intestine and colon. The main Cl^- channels involved in intestinal fluid secretion are CFTR and Ca^{2+}-activated Cl^- channels (CaCC). CFTR is a chloride channel regulated by cAMP and cGMP, which plays a leading role in intestinal secretion (Kunzelmann et al., 2019). The increase of cAMP causes the apical membrane CFTR to open, leading to Cl^- outflow and intracellular depolarization (Dawson, 1991). Then, the increase in cAMP causes cAMP-dependent K^+ channels in the basement membrane to open, resulting in K^+ outflow and intracellular hyperpolarization to counteract the depolarization caused by the opening of apical membrane CFTR (Kunzelmann et al., 2001). The decreased intracellular Cl^- concentration induced by Cl^- outflow enhances the activity of the $Na^+/K^+/2Cl^-$ co-transporter (NKCC1) in the basement membrane, transporting Cl^- into the cell through the basement membrane. Driven by Na^+/K^+-ATPase, NKCC1, and K^+ channels, Cl^- enters the cell through the basement membrane, generating a Cl^- gradient across the epithelial cell top membrane. The activation of CaCC causes Cl^- outflow, and the secretion of Cl^- drives water transport (Jakab et al., 2013; Camilleri et al., 2017).

MECHANISM OF INTESTINAL FLUID TRANSPORT

Intestinal Fluid Transport Pathway

Colonic water absorption is the final link of intestinal water absorption in the body, and constipation is closely related to a disorder in the colonic fluid transport system. Intestinal epithelial fluid can be transported *via* paracellular and transcellular routes. Colonocytes are linked by tight junctions that impede fluid movement. This limits cell bypass absorption, and cross-cell transport becomes the main pathway of colonic fluid absorption (Sundell and Sundh, 2012). The transcellular route involves free diffusion, co-transport, and AQPs pathways (Laforenza et al., 2005). Among these, AQPs, which act as a special channel for the rapid transport of water molecules and small molecular solutes, play an important role in maintaining liquid homeostasis.

Aquaporins in the Intestinal Tract

AQPs are a family of water channel molecules (AQP0-12) that promote the movement of water from areas of low permeability to areas of high permeability under the action of osmotic gradients (Sisto et al., 2019). AQPs are located on the cell membrane where they form channels to control the inflow and outflow of water. AQPs play a pivotal role in the regulation of intestinal absorption,

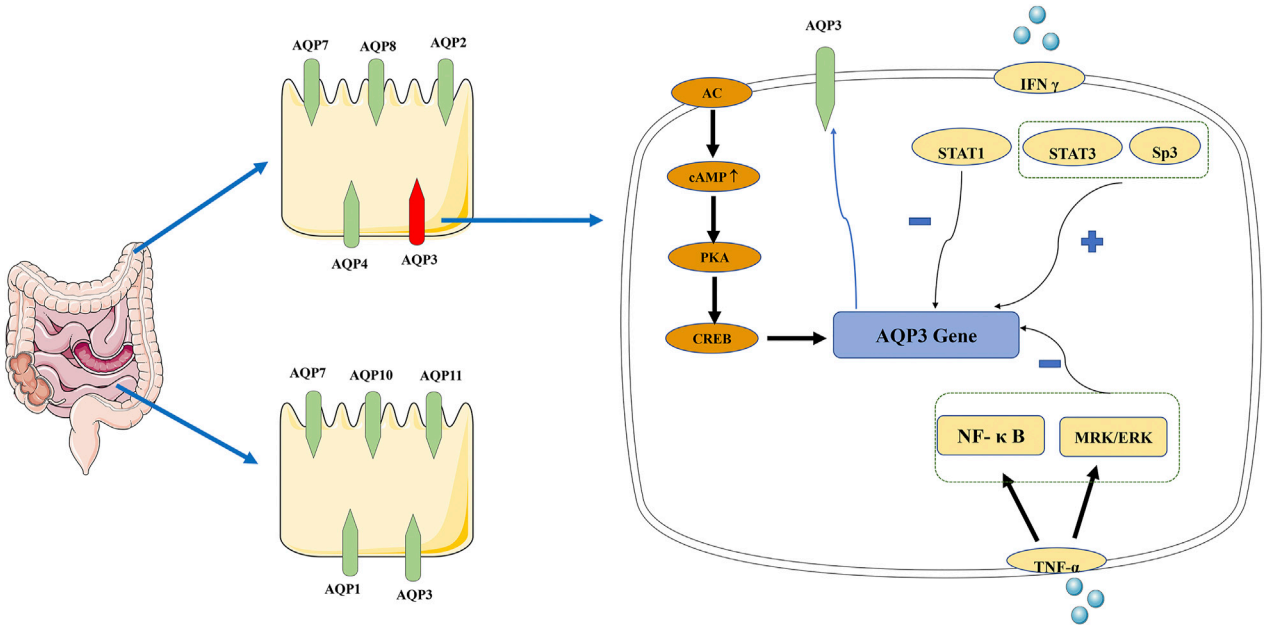

FIGURE 4 | Distribution of AQPs in intestinal tract and signal transduction mechanism of AQP3. AQP1, AQP3, AQP7, AQP10, and AQP11 are highly expressed in the small intestine, while AQP2, AQP3, AQP4, AQP7, and AQP8 are the main subtypes in the colon. "+" indicate a promoting role, "–" indicate an inhibition role. In AQP3 expression, there are two pathways: AC-mediated short-time regulation and long-term regulation at the transcriptional level. AC, adenylate cyclase; AQP, aquaporin; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; PKA, protein kinase A; TNF-α, tumor necrosis factor alpha.

secretion, and water metabolism by mediating the transmembrane transport of water molecules.

Many studies have shown that AQP3 is a key subtype of colonic AQPs, and its expression level affects water transport in the intestinal tract. Up-regulation of AQP3 expression in colonic epithelial cells can lead to severe constipation (Zhu et al., 2017). AQPs can be regulated (**Figure 4**) through a change in their activity or number, which is also called short-term regulation. AQPs are targets of VIP that can change the content of AQPs on the cell membrane through the cellular protein kinase A (PKA) system to regulate the membrane permeability to water. VIP up-regulates the expression of AQP3 and its mRNA by activating the cAMP-dependent PKA pathway (Itoh et al., 2003), blocking the movement of Cl^- into the intestinal cavity and that of osmotic water from the cells to the cavity (Hamabata et al., 2002). PKA also phosphorylates cAMP response element-binding protein (CREB); the phosphorylated CREB then stimulates AQPs gene transcription (Ikeda and Matsuzaki, 2015).

The other mechanism refers to the increased AQPs synthesis, mRNA, and protein expression at the transcriptional level, which is termed long-term regulation. Studies have shown that AQPs promote intestinal function through mechanisms that may involve changes in signaling. NF-κB, a key signal in the long-term regulation of AQP3, down-regulates the expression of AQP3 (Zhan et al., 2020). Tumor necrosis factor alpha (TNF-α) reduces the expression of AQP3 in HT-29 cells through MRK/ERK and NF-κB signaling (Peplowski et al., 2017). The binding of Sp3 transcription factor to the AQP3 promoter can partially prevent the down-regulation of AQP3 expression induced by TNF-α. IFN-γ, a key factor of impaired epithelial transport and barrier function, can increase epithelial permeability by inhibiting the expression of AQP3. STAT1 has been shown to partially block the down-regulation of AQP3 expression induced by IFN-γ, while STAT3 and Sp3 can increase AQP3 expression (Peplowski et al., 2018).

A growing body of studies has investigated the importance of the gastrointestinal water transport system in intestinal function and the effect of AQPs on intestinal fluid secretion and chronic constipation. However, the mechanisms of AQPs action on intestinal fluid and constipation remain unclear. Further studies on the expression and function of AQPs in the intestinal tract and the mechanism of water transport should provide information for the development of new laxatives.

ACTION MECHANISM OF DRUGS FOR TREATING CONSTIPATION

With a better understanding of the mechanisms of chronic constipation and continued advances in pharmaceutical development, an expanding array of treatment approaches have been developed. At present, drugs are the mainstay for patients with chronic constipation. Many studies have reported the efficacy and safety of laxatives in patients with constipation. Intervention with laxatives can alter the intestinal environment by affecting gut motility, ion transport, and liquid absorption/secretion, which are beneficial for patients with constipation. The main categories of approved drugs for the treatment of constipation are osmotic laxatives, stimulant laxatives, secretagogues, serotonergic agents, and ileal bile acid

TABLE 1 | Summary of drugs for the treatment of constipation.

Category	Medication	Dosage	Possible side effects	Mechanism of action	Molecular signal
Bulking agents	Insoluble fiber	25–30 g/day	Bloating	Luminal water binding increases stool volume and soften stool	Not involved
	Soluble fiber				Not involved
Osmotic laxatives	Lactulose	15–25 ml/day	Bloating, abdominal pain	Produce the osmotic gradient in the cavity, increase the moisture in the cavity and soften the stool	Not involved
	Polyethylene glycol	10–20 g/day	Abdominal distention, diarrhea		Not involved
	$MgSO_4$	5–20 g/day	Electrolyte disorder, diarrhea		Mg^{2+}↑cAMP↑AQP3↑
Stimulant laxatives	Bisacodyl	5–10 mg/day	Abdominal cramps, diarrhea. Abdominal pain	Stimulate intestinal mucosa to secrete water and electrolytes and accelerate peristalsis	TNF-α↑PGE_2↑AQP3↓
	Senna, rhubarb	No RCTs	Melanosis coli		PGE_2↑ AQP3↓ AQP8↓
Secretagogues	Lubiprostone	24–72 μg/day	Nausea, vomiting, diarrhea	Increase intestinal fluid secretion and soften stool	Cl^-↓ Na^+↑ H_2O↑
	Plecanatide	3 mg/day	Diarrhea		cGMP↑ Cl^-↓
	Linaclotide	145 μg/day			Na^+↑ H_2O↑
Serotonergic agents	Prucalopride	2 mg/day	Diarrhea, headache, nausea, abdominal pain	Selective action on 5-HT receptor	5-HT_4↑ ICCs↑
IBAT inhibitor	Elobixibat	5–15 mg/day	Abdominal pain, diarrhea	Increase the concentration of bile acid in colon and promote fluid secretion	Bile acid↑ Cl^-↓ H_2O↑
Motilin receptor agonists	Mitemcinal (GM-611)	No RCTs	Not clear	Activation of motilin receptor and promote peristalsis	motilin↑
TGP	Paeonia	No RCTs	Not clear	Improve the function of ICCs and regulate neurotransmitters to accelerate peristalsis	ICCs↑ NO↓ NOS↓ VIP↓

5-HT, 5-hydroxytryptamine; AQP, aquaporin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; IBAT, ileal bile acid transporter; ICCs, interstitial cells of Cajal; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; RCTs, randomized controlled trials; TGP, total glucosides of paeony; TNF-α, tumor necrosis factor alpha; VIP, vasoactive intestinal peptide.

transporter inhibitors (Black and Ford, 2018). Drugs used for the treatment of chronic constipation are listed in **Table 1**. The goal of such medications is to promote fecal excretion and relieve constipation-related symptoms, and to improve the patients' quality of life. However, different drugs act *via* different pathways, and a further understanding of their mechanisms, combined with that of ion transport and the expression of AQPs, may lead to the development of promising drugs.

Bulking Agents

The American Gastroenterological Association recommends that use of a fiber supplement should be the initial treatment approach for constipation (Bharucha et al., 2013). Fiber is composed of high-molecular weight food components that cannot be degraded by intestinal enzymes thus it remains in the intestinal cavity and increases fecal volume. Insoluble fibers, such as wheat bran, may alter gut motility, thereby accelerating gastrointestinal transit and increasing the frequency of stools. Soluble fiber, such as psyllium, expands after absorbing water in the intestine, thus softening and increasing the volume of feces. Although fiber supplements are effective in the treatment of constipation, adverse reactions, such as bloating, are becoming a problem with long-term therapy (Wald et al., 2008).

Osmotic Laxatives

If patients do not respond to fiber, then osmotic laxatives should be considered. Osmotic laxatives are poorly absorbed or non-absorbed substances, that produce intraluminal osmotic gradients, causing secretion of water and electrolytes into the lumen. This results in luminal water retention, an increase in stool water content and stool softening, thus facilitates stool passage (Krogh et al., 2017). These treatments are useful for patients with mild-to-moderate constipation, and the main side effects are diarrhea and abdominal distention.

Lactulose

As an osmotic laxative, lactulose has osmotic activity and can attract water to the colon cavity (Jouët et al., 2008). Since it is harmless to the human body and can effectively regulate the physiological rhythm of the colon, it is widely used to treat constipation in the elderly, pregnant women, and children. Adverse reactions are limited to the gastrointestinal system, with bloating and abdominal pain being the most common.

Polyethylene Glycol

Polyethylene glycol is a non-absorbable macromolecule belonging to the group of osmotic laxatives. Its mechanism of action is physical; it acts through local infiltration, retaining water in the colon cavity, thus softening feces, increasing fecal volume, and leading to unobstructed defecation. Polyethylene glycol can improve constipation-related symptoms (such as stool frequency and stool consistency) (Chassagne et al., 2017). Clinical studies have found that low-dose polyethylene glycol is significantly better than lactulose in improving constipation symptoms, with fewer adverse reactions (Attar et al., 1999). Polyethylene glycol can be used for the symptomatic treatment of constipation in children aged 6 months and older and in adults.

Poorly Absorbed Salts

Salt laxative $MgSO_4$ can increase the intestinal osmotic pressure, prevent the absorption of water in the colon, increase the intestinal contents, and stimulate intestinal peristalsis, resulting in rapid and severe catharsis. The increased intracellular Mg^{2+} concentration activates adenylate cyclase (AC), which leads to an increase in cAMP. The increased cAMP concentration subsequently leads to the activation of PKA, which promotes CREB phosphorylation and AQP3 gene transcription (Ikarashi et al., 2011b). Excessive use of salt laxatives may induce electrolyte disorders and are therefore, not suitable for the elderly and patients with decreased kidney-function.

Stimulant Laxatives

If the patient does not respond to osmotic laxatives, stimulant laxatives are recommended. Stimulant laxatives stimulate the intestinal mucosa and nerve plexus to secrete water and electrolytes, resulting in peristaltic contraction, thereby accelerating colonic transport. Stimulant laxatives are effective, and their side effects are known. Chronic use of stimulant laxatives does not seem to cause tolerance or rebound constipation. However, common side effects include diarrhea and abdominal pain (Wald, 2003).

Bisacodyl

Bisacodyl stimulates the secretion and motility of the small intestine and colon *via* the following mechanisms: increased secretion of TNF-α and prostaglandin E_2 (PGE_2) in the colon following the oral administration of bisacodyl. TNF-α and PGE_2, as paracrine factors, act on the colonic mucosal epithelial cells resulting in an immediate reduction in AQP3 expression, thus exerting their laxative effects (Ikarashi et al., 2011a). In a 4-weeks trial, oral bisacodyl was reported to be safe and well-tolerated (Kamm et al., 2011). However, bisacodyl is associated with abdominal cramps and diarrhea.

Secretagogues

Secretagogues are second-line drugs, the effects of which are similar to those of osmotic laxatives. Secretagogues act directly on intestinal epithelial cells, increasing fluid secretion into the intestinal cavity, thereby changing the consistency of stools and reducing the transit time in the colon (Luthra et al., 2019). However, these drugs are associated with side effects such as diarrhea when used clinically.

Lubiprostone

Lubiprostone is a prostaglandin E_1 derivative, which can activate the intestinal chloride channel type 2 on the apical surface of small intestinal enterocytes, thereby reducing epithelial permeability and promoting intestinal fluid secretion. Lubiprostone selectively activates type 2 Cl^- channels in the parietal membrane of the gastrointestinal epithelium, resulting in the excretion of Cl^- ions through the periapical membrane. Additionally, sodium and water enter the intestinal cavity passively, increasing the secretion of fluid in the intestinal cavity (Gonzalez-Martinez et al., 2014). Lubiprostone can significantly increase stool frequency, improve stool consistency, and reduce straining, which makes it effective for the treatment of constipation (Nishii et al., 2020). The results of a meta-analysis showed that adverse reactions such as nausea, vomiting, and diarrhea were common (incidence rate, 2.4–75%) (Li et al., 2016). This may be related to the rapid flow of fluid into the small intestine after taking the medicine.

Guanylate Cyclase-C (GC-C) Receptor Agonists

Plecanatide and Linaclotide are GC-C receptor agonists that target GC-C receptors on the lumen of the intestinal epithelium, resulting in increased intestinal fluid secretion (Shah et al., 2018). By activating colonic epithelial GC-C receptors, the synthesis of intracellular and extracellular cGMP is increased (Busby et al., 2010). CFTR is activated indirectly by cGMP and induces epithelial cells to secrete Cl^- and HCO_3^-, inhibits Na^+ absorption, and thus promotes intestinal water secretion (Layer and Stanghellini, 2014). Intestinal dilatation caused by increased intestinal fluid can promote intestinal movement, and therefore treat constipation. The reported efficacy of plecanatide and linaclotide is similar, and the most common side effect is diarrhea (Bassotti et al., 2018; Bassotti et al., 2019). Furthermore, increased cGMP can regulate abdominal pain (Silos-Santiago et al., 2013), thus, both drugs can relieve abdominal pain (Castro et al., 2013; Brenner et al., 2018).

Serotonergic Agents

The use of osmotic and stimulant laxatives, either alone or in combination, may be considered in first-line drug therapy. Second-line agents, such as prucalopride are indicated in patients with an inadequate response or poor tolerance to a first-line drug. Studies have reported that exploiting epithelial targets with nonabsorbable serotonergic agents could provide safe and effective therapies. Serotonin agonists stimulate intestinal secretion and motility by activating 5-HT receptors in the gastrointestinal nervous system. Unlike other older non-selective $5\text{-}HT_4$ receptor agonists (e.g., cisapride and tegaserod), prucalopride is effective for the treatment of chronic constipation and has demonstrated an excellent safety profile (Hayat et al., 2017).

Prucalopride

Prucalopride is a high-affinity $5\text{-}HT_4$ receptor agonist with colonic prokinetic activity (Vijayvargiya and Camilleri, 2019). Prucalopride functions by activating $5\text{-}HT_4$ receptors in myenteric plexus neurons and stimulates HAPCs to increase colonic motility (Miner et al., 2016). This significantly increases intestinal muscle contraction, as well as stool frequency and consistency in patients with chronic constipation. Some studies have also found that prucalopride can increase the expression of c-kit mRNA in colonic tissue of rats with constipation, and then improve the function of ICCs, so as to promote colonic motility. It is effective at improving stool frequency, stool consistency and straining. The most common side effects include diarrhea, headache, nausea, and abdominal pain (Daniali et al., 2019). Multicenter, double-blind, randomized, placebo-controlled trials have demonstrated that

prucalopride is superior to placebo in the short to medium term and can improve constipation in both men and women across a broad spectrum of ages and ethnicities (Camilleri et al., 2016). Nevertheless, this drug is considerably more expensive than conventional therapy. With knowledge that 5-HT_3 receptors can participate in the activation of propulsive motility and secretory responses in the gut, 5-HT_3 agonists have been developed and tested for the treatment of constipation (Mawe and Hoffman, 2013).

Ileal Bile Acid Transporter Inhibitor

Bile acid can activate the secretory activity of colonic epithelial cells (Mekjian et al., 1971). Therefore, up-regulation of the colonic bile acid concentration can be used to treat patients with constipation. Bile acid, a natural laxative in the human body, has garnered attention for the treatment of chronic constipation because of its ability to promote colonic epithelial secretion (Keely et al., 2007). However, its efficacy and safety need to be further confirmed in large scale studies.

Elobixibat

Bile acid acts as a physiological laxative by activating AC, increasing mucosal permeability, and inhibiting apical Cl^-/OH^- exchange to alter the transport of electrolytes and water in the lumen. Elobixibat can block the enterohepatic circulation of bile acid, up-regulate the synthesis of bile acid reaching the colon, and stimulate the secretion of fluid and electrolytes, thereby increasing fecal water content and gut motility (Mekjian et al., 1971). Increased gut motility facilitates stool passage. Few adverse reactions have been associated with this drug and they include abdominal pain and diarrhea. Abdominal pain with elobixibat may be related to its ability to induce dilatation and contraction (Taniguchi et al., 2018).

Motilin Receptor Agonists

As a motilin receptor agonist, mitemcinal (GM-611) can stimulate and promote peristalsis of the gastrointestinal tract by acting on the motilin receptor (Sudo et al., 2007). This effect has been observed in animal models; however, due to a lack of clinical outcome data, the clinical significance of these studies has not been clearly demonstrated. Therefore, further clinical trials are required to confirm the efficacy and safety of mitemcinal in this population (Mozaffari et al., 2014).

Probiotics

Probiotic consumption can regulate the intestinal microbiota of patients with constipation, which in turn, can improve gut motility. Some studies have shown that probiotics can be helpful for treating patients with constipation (improved stool frequency and stool consistency) with very few side effects (Ohkusa et al., 2019; Zhang et al., 2020). These studies have mainly involved the *bacteroides*, bifidobacterial, and lactobacilli. Recently, the positive impacts of SCFAs on gut motility and constipation were established (Chu et al., 2019). Nevertheless, since the effects of probiotics may be strain-specific and the exact mechanism of action remains unknown, more studies and randomized controlled trials are needed to confirm the effects of probiotics in patients with constipation (Dimidi et al., 2020).

Traditional Chinese Medicine

Traditional Chinese Medicine (TCM) has a role in promoting gastrointestinal motility and has been used to treat constipation for more than 1,000 years in China. In recent years, there have been many reports about TCM in the treatment of constipation and the improvement of gastrointestinal function (Cirillo and Capasso, 2015). Therefore, TCM has garnered increasing attention as a promising alternative treatment for constipation. However, few studies have investigated its therapeutic mechanisms. Thus, the long-term efficacy and side-effect profiles of these medicines in modern medicine need to be determined. Further studies are needed to determine the exact mechanism for the observed recovery of intestinal function. Importantly, the composition of TCM is complex.

Senna and rhubarb are anthraquinone laxatives used widely in the treatment of intestinal constipation. Sennoside A exerts a laxative effect through its main bioactive component. Rheinanthrone, the active metabolite of sennoside A, can increase the production of PGE_2 (Kon et al., 2014), thus participating in the regulation of intestinal peristaltic reflex. Emodin in rhubarb may also regulate water transport and absorption *via* the cAMP-dependent PKA/p-CREB signal pathway to change AQP3 (Zheng et al., 2014). Owing to its toxicity to the kidney and liver, we suggest that special attention should be paid to patients with kidney and liver diseases when using Senna drugs for a long period (Cao et al., 2018).

Total glucosides of paeony (TGP) are extracted from the root of Paeonia Lactiflora Pall. Studies have shown that TGP can improve the function of ICCs, block inhibitory neurotransmitters such as NO, NOS, and VIP, and increase the fecal volume and water content, as well as intestinal transit rate (Zhu et al., 2016).

CONCLUSION AND PROSPECT

As a common disease that seriously impacts the quality of life and mental health of patients, chronic constipation has attracted widespread attention. In general, impaired gut motility, fluid secretion/absorption, and electrolyte transport can result in decreased intestinal transit, reduced fluid secretion, and increased fluid reabsorption, which will eventually lead to chronic constipation. Further studies on the abnormal changes of the ENS, ANS, CNS, endocrine signaling, and microbiota would aid our understanding of constipation from the perspective of gut motility. Intestinal fluid and electrolyte transport are also strongly correlated with chronic constipation. As a subject for future research, ion channels and AQPs play critical roles in the transport of fluid. At present, studies on ion transport and AQPs in constipation are limited, and many complex mechanisms have not been clarified. Further experiments are warranted to demonstrate this mechanism.

With extended symptom duration, severity, and frustration, the occurrence of additional symptoms will also increase.

Therefore, patients with chronic constipation usually require active treatment. The choice of therapeutic drugs should focus on the effectiveness of relieving the symptoms of constipation, the improvement of the intestinal environment, and the effectiveness and safety of long-term use. Preferentially, fiber/osmotic/stimulant laxatives should be considered. If these measures fail, prescription laxatives with different mechanisms of action may be used. Modifying the gut luminal environment through gut motility, fluid, and electrolytes will affect transit and secretion in the gut, thereby benefiting patients with chronic constipation. Intestinal fluid transport mediated by ion channels and AQPs is the key mechanism through which many laxatives exert their effects. An in-depth understanding of ion channels and water channels in constipation will provide a scientific basis for the development of synergistic and/or antagonistic drugs targeting specific channels. Nevertheless, further studies are still required to resolve the problem. Recently, the action of probiotics on gut motility was shown to be beneficial for constipation. However, the positive effects of probiotics depend on the specific probiotics used and the level applied. Therefore, the use of probiotics in the treatment of chronic constipation is promising and further studies are required. We hope that a better understanding of the pathogenesis of constipation and the mechanism of drug action may create new targets for the treatment of diseases that remain a major scourge worldwide.

AUTHOR CONTRIBUTIONS

Y-YC and Y-PT conceived and designed the review. QZ searched the literature and drafted the manuscript. D-QX and S-JY additions and revisions in manuscript. JY and L-MX examined the literature and made the figures. R-JF edited the manuscript. Y-YC and Y-PT made a critical revision of the review. All authors approved the final version of the manuscript.

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GLOSSARY

5-HT 5-hydroxytryptamine
AC adenylate cyclase
Ach acetylcholine
ANS autonomic nervous system
AQPs aquaporins
ATP adenosine triphosphate
CaCC Ca^{2+} activated Cl^- channel
cAMP cyclic adenosine monophosphate
CFTR cystic fibrosis transmembrane conductance regulator
cGMP cyclic guanosine monophosphate
CGRP calcitonin gene-related peptide
CNS central nervous system
CREB cAMP response element-binding protein
ECs enterochromaffin cells
EGCs enteric glial cells
ENaC epithelial sodium channel
ENS enteric nervous system
ExPANs extrinsic primary afferent neurons
GC-C guanylate cyclase-C
H_2S hydrogen sulfide
HAPCs high-amplitude propagating contractions
ICCs interstitial cells of Cajal
LPS lipopolysaccharide
NKCC1 $Na^+/K^+/2Cl^-$ co-transporter
NO nitric oxide
NOS nitric oxide synthase
PDGFRα$^+$ platelet-derived growth factor receptor α-positive
PGE_2 prostaglandin E_2
PKA protein kinase A
SCFAs short-chain fatty acids
SP substance P
TCM Traditional Chinese medicine
TGP Total glucosides of paeony
TNF-α tumor necrosis factor alpha
VIP vasoactive intestinal peptide

frontiers in Pharmacology

REVIEW
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New Developments in Prokinetic Therapy for Gastric Motility Disorders

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Prokinetic agents amplify and coordinate the gastrointestinal muscular contractions to facilitate the transit of intra-luminal content. Following the institution of dietary recommendations, prokinetics are the first medications whose goal is to improve gastric emptying and relieve symptoms of gastroparesis. The recommended use of metoclopramide, the only currently approved medication for gastroparesis in the United States, is for a duration of less than 3 months, due to the risk of reversible or irreversible extrapyramidal tremors. Domperidone, a dopamine D2 receptor antagonist, is available for prescription through the FDA's program for Expanded Access to Investigational Drugs. Macrolides are used off label and are associated with tachyphylaxis and variable duration of efficacy. Aprepitant relieves some symptoms of gastroparesis. There are newer agents in the pipeline targeting diverse gastric (fundic, antral and pyloric) motor functions, including novel serotonergic 5-HT_4 agonists, dopaminergic $D_{2/3}$ antagonists, neurokinin NK_1 antagonists, and ghrelin agonist. Novel targets with potential to improve gastric motor functions include the pylorus, macrophage/inflammatory function, oxidative stress, and neurogenesis. In the current review, we discuss the use of pharmacological approaches with potential to enhance motor functions in the management of gastroparesis.

Keywords: aprepitant, domperidone, erythromycin, functional dyspepsia, gastroparesis, ghrelin, prucalopride, relamorelin

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INTRODUCTION: DEFINITIONS AND CURRENTLY AVAILABLE PROKINETIC TREATMENTS

Gastroparesis is characterized by upper gastrointestinal symptoms including nausea, vomiting, early satiety, postprandial fullness, bloating, and upper abdominal pain, as well as slow gastric emptying of solids in the absence of gastric outlet or intestinal obstruction. In clinical practice, the most common etiologies of gastroparesis are diabetes mellitus, idiopathic, iatrogenic (post-surgical or medication), diseases affecting the neural control arising in the brain and spinal cord (such as Parkinson disease, which may also be associated with effects of dopaminergic agents), and diseases that damage intrinsic nerves or smooth muscle, often as a result of tissue infiltration (such as scleroderma) or muscle degeneration (as in amyloidosis).

Prokinetic agents are medications that amplify and coordinate gastrointestinal muscular contractions (Acosta and Camilleri, 2015), including coordination between different segments of the gut, thereby enhancing propulsion of intra-luminal contents. Some prokinetics are active in selective areas of the gastrointestinal tract, whereas the activity of other prokinetics is more generalized and reflects the location of receptor targets of the pharmacological agents. In

the current review, we discuss the use of prokinetics in the management of gastric motility disorders.

In most countries, only two medications are approved or available for the treatment of gastroparesis: metoclopramide and domperidone. Both agents are antagonists at dopamine-2 (D_2) receptors. The effect of the endogenous transmitter, dopamine, is to inhibit the release of acetylcholine (ACh), and this results in decrease in motility of the stomach and proximal small bowel (Tonini et al., 2004). These inhibitory effects of endogenous dopamine are reversed by D2 receptor antagonists. In general, metoclopramide and domperidone were equally effective for relief of symptoms, although central nervous system adverse effects were more common with metoclopramide (Patterson et al., 1999; Camilleri et al., 2013). Current guidelines recommend liquid formulation metoclopramide, 5 to 10 mg orally, 30 min before meals and at bedtime in patients with gastroparesis. Higher doses are not recommended in order to avoid side effects. A new intra-nasal formulation of metoclopramide has also been approved by the Food and Drug Administration (FDA) of the United States.

Domperidone is available for physician prescription through FDA's program for Expanded Access to Investigational Drugs (https://www.fda.gov/drugs/investigational-new-drug-ind-application/how-request-domperidone-expanded-access-use). The recommended starting dose of domperidone is 10 mg, t.i.d., which could be increased (if necessary) to 20 mg, t.i.d., and at bedtime. Domperidone has been associated with cardiac dysrhythmias which led to its removal in Europe from the over-the-counter space to availability only by prescription. It is generally recommended that domperidone be avoided if the corrected QTc interval on the patient's electrocardiogram is > 470 ms in males and >450 ms in females.

An off-label prokinetic approach for gastroparesis involves the use of inhibitors of acetyl cholinesterase. Neostigmine is a short-acting agent that was shown to accelerate gastric emptying of liquids in patients who are critically ill and have delayed gastric emptying; this was associated with induction of an irregular increase in gastric and duodenal contractility (Bortolotti et al., 1995; Lucey et al., 2003). Neostigmine has a short duration of action, is administered parenterally by slow intravenous or intramuscular injection. Therefore, its use is limited to the hospital setting with electrocardiogram monitoring because of the potential to induce vagotonia and bradycardia.

Pyridostigmine has a longer duration of action; it is not approved for treatment of gastroparesis; it is available in liquid or tablet formulation and, based on clinical experience, it is used at doses up to 60 mg, t.i.d. There are, as yet, no clinical trials documenting its clinical efficacy in gastroparesis. In an open-label case series, pyridostigmine was beneficial in relief of symptoms in children with such upper gastrointestinal motility problems as chronic intestinal pseudo-obstruction, delayed small bowel transit accompanying gastroparesis, and chronic constipation associated with failure to thrive. Effective dosing ranged between 0.25 and 2.0 mg/kg/day (Manini et al., 2017).

The serotonergic 5-HT_4 receptor agonist, cisapride, was associated with symptomatic benefit in patients with gastroparesis based on short-or medium-term placebo-controlled trials (Corinaldesi et al., 1987; Camilleri et al., 1989; Richards et al., 1993a), and on long-term, open-label studies (Abell et al., 1991). Although cisapride accelerated gastric emptying, it did not necessarily enhance glycemic control, over the long-term (Braden et al., 2002). Cisapride is no longer available in most countries due to withdrawal as a result of cardiovascular concerns (cardiac arrhythmias due to inhibition of the human ether-à-go-go-related gene [hERG] potassium channel).

Other medications available in a few countries, clebopride, cinitapride, and mosapride, are not reviewed in detail here in view of the relatively weak evidence of efficacy in gastroparesis. There are no controlled trials of clebopride (D2 antagonist) in gastroparesis other than in dyspepsia with radiologically delayed gastric emptying, a criterion not currently accepted for gastroparesis (Bavestrello et al., 1985). Cinitapride (a 5-HT_1 and 5-HT_4 agonist and 5-HT_2 antagonist) was superior to placebo in a parallel design study of 19 patients with dyspepsia associated with postprandial distress and mildly to moderately delayed gastric emptying (Portincasa et al., 2009). Mosapride (a 5-HT_4 agonist) enhanced gastric emptying in gastroparesis associated with treatment with interferon, but had no significant effects on symptoms (Kawamura et al., 2012).

There is, therefore, significant and unmet clinical need to develop new prokinetic agents for gastric motility disorders. Over the past few years, it has been appreciated that the upper gastrointestinal symptoms that are consistent with gastroparesis may arise from diverse gastric motor dysfunctions that constitute potential targets for pharmacological agents, thus, expanding the spectrum of therapeutic approaches.

Gastric Motor Dysfunctions

There are three dominant motor dysfunctions that can result in diverse manifestations or symptoms: gastric emptying, gastric accommodation, and pyloric dysfunction. It is relevant to note that, in patients with upper gastrointestinal symptoms, there are about 25% of patients with delayed gastric emptying, about 25% with impaired gastric accommodation, and about 25% with the combination of both gastric motor dysfunctions (Park et al., 2017; Chedid et al., 2019a). In addition, among patients with gastroparesis, a subset of those with antral hypomotility also has evidence of pylorospasm (Mearin et al., 1986).

In this article, new developments in prokinetic therapy for these motility disorders are reviewed. Prior to exploring the pharmacological approaches using prokinetics, it is useful to review the overall principles regarding the methods used to measure those gastric motor functions as they are used in pharmacodynamic assessment of the therapeutic approaches.

Identification of disorders of gastric emptying requires an accurate gastric emptying test. The optimal gastric emptying diagnostic tests typically involve measurements at standard times (e.g., 0, 0.5, 1, 2, 3, and 4 h) over 4-h by scintigraphy or by stable isotope breath tests. The symptoms associated with retardation of gastric emptying are nausea, vomiting, and upper abdominal bloating, but pain is not a quintessential symptom of delayed gastric emptying. A significant relationship has been demonstrated between the acceleration of gastric emptying and the improvement of symptoms. Thus, using a meta-regression analysis, it was demonstrated that acceleration in

gastric emptying $T_{1/2}$ of 20.4 min was associated with a1-unit reduction in the severity of symptoms. This analysis was based on standardized mean difference in order to account for differences in the measurements of symptoms between studies (Vijayvargiya et al., 2019). Normal values and performance characteristics of the scintigraphic test have been published (Szarka et al., 2008; Camilleri et al., 2012). Disorders of gastric emptying can be ameliorated by targeting specific receptors, including serotonergic 5-HT_4, dopamine $D_{2/3}$, and neurokinin NK_1 receptors.

Disorders of gastric accommodation are typically associated with postprandial distress syndrome, a component of functional dyspepsia. In fact, approaches to enhance postprandial accommodation have been associated with reduced symptoms of functional dyspepsia, for example, by using the serotonergic 5-HT_{1A} agonist, buspirone, or by using acotiamide, an antagonist of acetylcholinesterase and antagonist of presynaptic M_1 and M_2 muscarinic receptors. These muscarinic receptors are involved in inhibition of acetylcholine release. Therefore, by antagonizing those receptors and by inhibiting acetylcholinesterase, acotiamide leads to an increased local level of acetylcholine, which is an excitatory transmitter in the enteric nervous system and parasympathetic nerve pathways.

There are currently three direct and one indirect measurements of gastric accommodation. The three direct methods are: first, single-photon emission computerized tomography (SPECT) imaging (Bouras et al., 2002), second, measurement of proximal gastric volume by barostat, whereby the pressure of air within an infinitely compliant polyethylene balloon is clamped (maintained constant by an electronic pump aspirating or infusing air), and the continuous monitoring of the intra-balloon volume provides a measurement of gastric tone. A third method is intraluminal high resolution manometry in the proximal stomach (Tack et al., 1998; Carbone et al., 2017). In addition, an indirect measurement of gastric accommodation can be obtained through ingestion of a nutrient drink at constant rate until the maximum tolerated volume (MTV) is reached]; this measurement assesses gastric sensation (Tack et al., 2003). However, in addition, it provides an indirect measure of accommodation if the MTV is less than ~750 kilocalories, since there is a linear correlation between the MTV and gastric accommodation volume measured by a barostat when the MTV is below 750 kcal (Tack et al., 2003). There have been attempts to use two-dimensional imaging of the area of the proximal stomach immediately after food ingestion to estimate gastric accommodation; however, these measurements were subsequently found to be inaccurate relative to three-dimensional imaging and the 2-D imaging method therefore requires further validation (Orthey et al., 2018a; Orthey et al., 2018b; Chedid et al., 2019b).

Disorders of pyloric function are difficult to assess noninvasively and two approaches are available, requiring intraluminal measurements. These are antropyloroduodenal manometry and the Endoflip (endoscopic functional lumen imaging probe) device. The former uses closely-spaced manometric sensors to measure the pressure profile and identifies pyloric activity by the combination of phasic and tonic contractions, as well as the combination of antral and duodenal phasic pressure activity in the manometric tracing (Nelson et al., 2016). The Endoflip device is a longer (8 or 16 cm) probe consisting of 16 paired impedance planimetry electrodes mounted on a catheter and located within a balloon that is filled with a conductive fluid (typically distended with 40–50 ml fluid); excitation electrodes at either end of the balloon generate a low electric current. The impedance electrodes measure voltage and, using the voltages, the device calculates the cross-sectional areas (CSA) using Ohm's Law (resistance = voltage/current) at each electrode interval. A solid-state pressure transducer is located at the distal end of the balloon. Thus, by measuring the pressure simultaneously with the CSA, it is possible to calculate a distensibility index (Vosoughi et al., 2020).

Novel Pharmacotherapies: Current State of Evidence

Symptoms may result from diverse pathophysiological disorders including accelerated or slow emptying, reduced gastric accommodation, gastric dysrhythmias, or duodenal mechanisms. It has therefore been proposed that future treatment of gastric motor dysfunctions and related symptoms should be based on identified pathophysiology or "actionable biomarkers" that is putative mechanisms that are associated with induction of the symptoms suggesting gastroparesis and that can be normalized with specific treatments, such as treatments targeting5-HT_4, dopamine D_2 and D_3, and NK_1 receptors. The available and investigational prokinetic agents discussed below for gastric motility disorders are summarized in **Table 1**.

Novel 5-HT_4 Receptor Agonists Targeting Gastric Emptying

Several "new generation" 5-HT_4 receptor agonists are selective for 5-HT_4 receptors withouthERG effects (De Maeyer et al., 2006; Tack et al., 2012); these include prucalopride, velusetrag, naronapride, and felcisetrag.

Prucalopride is approved by the European Medicines Agency (EMA) and the FDA for the treatment of chronic constipation. In a randomized, placebo-controlled, cross-over study involving 34 patients (28 idiopathic, six diabetic) with gastroparesis, patients received prucalopride, 2 mg once daily, or placebo for 4 weeks, with a 2-weeks washout between treatments. Prucalopride was efficacious in relieving symptoms based on total Gastroparesis Cardinal Symptom Index, subscales of nausea/vomiting, fullness/satiety, and bloating/distention, as well as improvement in the overall Patient Assessment of Upper Gastrointestinal Disorders-Quality of Life score (Carbone et al., 2019). Similarly, velusetrag, was reported to be efficacious in the treatment of diabetic and idiopathic gastroparesis (Kuo et al., 2021a).

Intravenously administered felcisetrag significantly accelerated gastric emptying, small bowel transit and colonic transit compared to placebo in patients with gastroparesis with previously confirmed delayed gastric emptying. Felcisetrag was well tolerated (Chedid et al., 2021). In a previous double-dummy, parallel-group, randomized trial, felcisetrag (TAK-954), administered to mechanically ventilated patients with enteral feeding intolerance defined as gastric residual volume ≥200 ml, led to a greater proportion of patients with normal gastric retention compared to four doses of 10 mg metoclopramide (Chapman et al., 2021).

TABLE 1 | Current and investigational prokinetic drugs for gastric motility disorders.

Drug name	Disease	Effect on gastric motor function	GP symptoms	Ref. #
5-HT4 receptor agonist				
Prucalopride	IG and DG	↑ GE	Improved	Carbone et al. (2019)
Velusetrag	IG and DG	↑ GE	Improved	Kuo et al. (2021a)
Felcisetrag	IG and DG	↑ GE	Not studied	(Chapman et al., 2021; Chedid et al., 2021)
Tegaserod	FD	↑ GA	Mixed effects	(Vakil et al., 2008; Tack et al., 2010)
D2/3 receptor antagonist				
Trazpiroben	IG and DG	↑ volume to fullness, No Δ in GE	Improved	Kuo et al. (2021b)
Ghrelin receptor agonist				
Relamorelin	DG	↑ GE, ↑ antral contractions	Improved	(Shin et al., 2013a; Shin et al., 2013b; Lembo et al., 2016; Nelson et al., 2016; Camilleri et al., 2017; Camilleri et al., 2020)
Muscarinic M1/2 receptor antagonist				
Acotiamide	FD	↑ GE and GA	Improved	Kusunoki et al. (2012)
Motilin receptor agonist				
Erythromycin	IG and DG	↑ GE, ↑ fundic and antral contractions, ↓ pyloric contractions	Improved	(Janssens et al., 1990; Catnach and Fairclough, 1992; Richards et al., 1993b; Parkman et al., 1995)
Azithromycin	Gastroparesis	↑ GE	Not studied	Larson et al. (2010)
Clarithromycin	FD	↑ GE	Not studied	Bortolotti et al. (1999)
NK1 receptor agonist				
Aprepitant	IG and DG	↑ GA, No Δ in GE	Improved	(Jacob et al., 2017; Pasricha et al., 2018)
Tradipitant	IG and DG	Not studied	Improved	Carlin et al. (2021)
Opioid antagonists [non-selective (NS) or peripherally active (PAMORA)]				
Naloxone [NS]	FD and IG	No Δ in GE	Not studied	Narducci et al. (1986)
MNTX [PAMORA]	opioid-induced gastric delay	No Δ in GE	Not studied	Wong et al. (2010)
Naloxegol [PAMORA]	opioid-induced gastric delay	No Δ in GE	Not studied	Halawi et al. (2018)
Phosphodiesterase-5 Inhibitor				
Sildenafil	Gastroparesis with uremia	No Δ in GE	Not studied	Dishy et al. (2004)

Abbreviations: DG, diabetic gastroparesis; FD, functional dyspepsia; GA, gastric accommodation; GE, gastric emptying; GP, gastroparesis; IG, idiopathic gastroparesis; MNTX, methylnaltrexone; PAMORA, peripherally active μ-opioid receptor antagonist.

Velusetrag and felcisetrag (TAK-954) had no significant effects on coronary tone (demonstrated in dog, pig or human coronary arteries) or cardiac rhythm (hERG channel potassium conductance) or platelet function), or other off-target actions (Beattie et al., 2013). Felcisetrag has high affinity (pK(i) = 9.4) for human recombinant 5-HT_{4c} receptors and >2,000-fold selectivity for those receptors compared to 78 other receptors (including all other 5-HT receptors, several non-5HT receptors), transporters or ion channels tested (Beattie et al., 2011).

Another potential mechanism to enhance neuromuscular function in the stomach is an anti-inflammatory effect that may facilitate vagal stimulation. This has been demonstrated with the 5-HT_4 agonist, prucalopride, which modified responses of T2helper cells and shortened post-operative ileus (Matteoli et al., 2013; Bosmans et al., 2017; Stakenborg et al., 2019). There is evidence that macrophages that are not derived from circulating monocytes, are resident in the gut, are distinct from CD206 (also called M2) macrophages, and can impact enteric nervous system function (De Schepper et al., 2018). This anti-inflammatory mechanism may be relevant since some animal models have inflammation and oxidative stress-induced damage to the enteric nervous system and pacemaker cells (discussed below).

Targeting Sensations With D_2/D_3 and NK_1 Antagonists

For upper gastrointestinal disorders associated with increased gastric sensation, such as functional dyspepsia, the dopaminergic $D_2/_3$

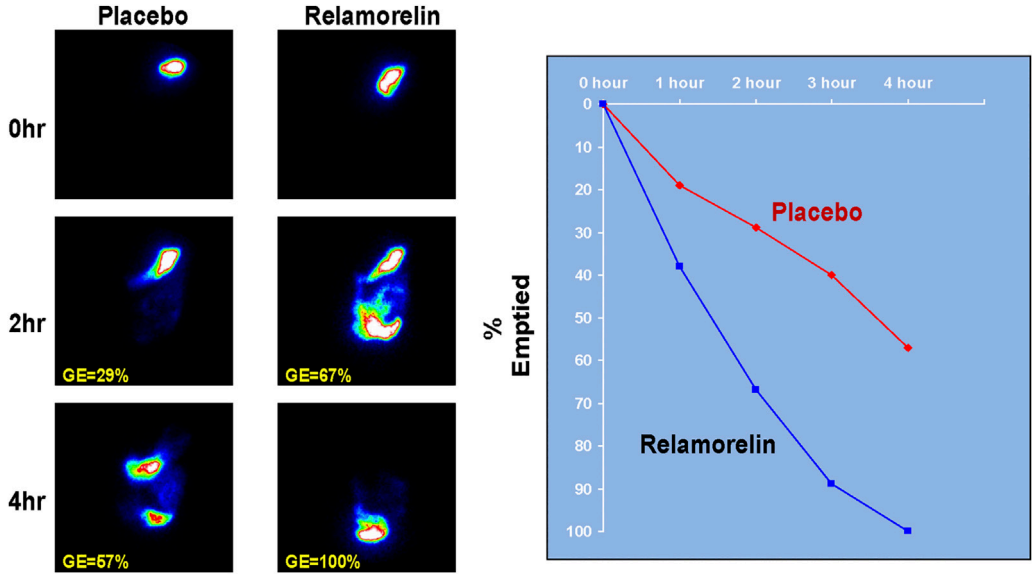

FIGURE 1 | Effect of relamorelin on gastric emptying in a patient with type 1 diabetes with gastroparesis. Aadapted from ref. 51, Shin A, et al. Clin Gastroenterol Hepatol 2013; 11:1,453–1,459.

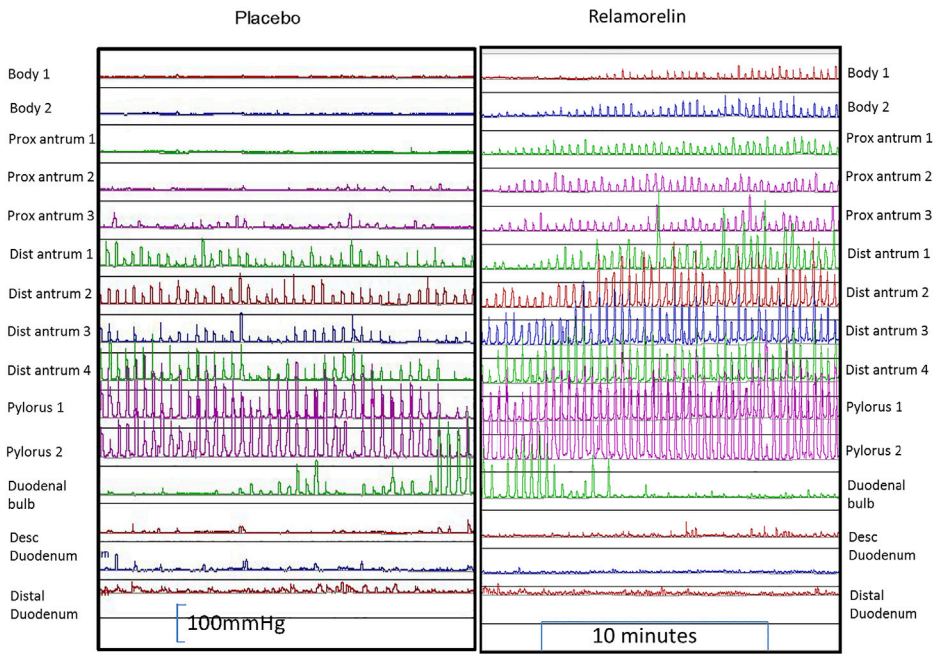

FIGURE 2 | Effect of relamorelin on antral motility in a healthy subject. Reproduced with permission from ref. 29, Nelson A, Camilleri M, et al. Neurogastroenterol Motil 2016; 28:1,705–1,713.

antagonist, trazpiroben (TAK-506) administered for 1 week significantly increased the volume to fullness during a nutrient drink test, compared to baseline (Kuo et al., 2021b). Moreover, in a placebo-controlled trial, the NK_1 receptor antagonist, aprepitant, improved multiple symptoms of gastroparesis including nausea (Pasricha et al., 2018). These beneficial effects may reflect the known effects of NK1 receptor antagonists on the vomiting center in the brainstem, akin to the action associated with reduced chemotherapy-induced emesis. Another potential mechanism for the symptomatic benefit may be related to increased fasting and accommodation volumes of the stomach without deleterious effect on gastric emptying, which has been demonstrated in healthy controls (Jacob et al., 2017). The novel NK_1 receptor antagonist, tradipitant, improved several symptoms of gastroparesis in a 4-weeks, randomized, controlled trial (Carlin et al., 2021). The symptomatic benefit

was most marked in patients with vomiting among the baseline symptoms; it was interesting to note that improvement of nausea was associated with improvement of all the other symptoms evaluated.

Ghrelin Receptor Agonist

Ghrelin is predominantly located in the stomach. It is an appetite-stimulating 28 amino acid hormone. Administration of a pharmacological dose of recombinant human ghrelin increased proximal gastric tone through central and peripheral effects (Peeters, 2003; Tack et al., 2006), and in some studies, it also accelerated stomach emptying in patients with gastroparesis [reviewed in ref. (Camilleri et al., 2009).].

A synthetic pentapeptide ghrelin receptor agonist, relamorelin, is 15–130 times more potent than natural ghrelin (Van der Ploeg et al., 2014). At a dose of 100 mg subcutaneously (s.c.), relamorelin accelerated gastric emptying of solids **Figure 1** in patients with either type 1 or type 2 diabetes mellitus with prior documentation of delayed gastric emptying (Shin et al., 2013a; Shin et al., 2013b). Relamorelin also increased distal antral contraction frequency without impeding gastric accommodation or altering postprandial satiation in healthy volunteers **Figure 2** (Nelson et al., 2016), which differentiates its effects from those of the macrolide antibiotic, erythromycin. In phase 2A and 2B, randomized, controlled trials in patients with diabetic gastroparesis, relamorelin improved clinical symptoms and appears to be safe, other than the induction of (typically postprandial) hyperglycemia which is attributable to the acceleration of gastric emptying (Lembo et al., 2016; Camilleri et al., 2017; Camilleri et al., 2020). It has therefore been recommended that proactive steps should be taken to control postprandial glycemia in diabetics receiving relamorelin (Camilleri et al., 2020).

Motilin Receptor Agonists

The most common motilin receptor agonists are the macrolide antibiotics that stimulate gastrointestinal motilin receptors, especially gastric receptors. Erythromycin improves gastric emptying and transiently improves symptoms before there is downregulation of the motilin receptor, typically in about 4 weeks after onset of therapy (Richards et al., 1993b; Thielemans et al., 2005), manifesting as tachyphylaxis or reduced treatment efficacy. One of the attractions of erythromycin is that it stimulates fundic and antral contractions, while inhibiting pyloric contractility (Janssens et al., 1990; Catnach and Fairclough, 1992; Parkman et al., 1995). The current recommended dose for hospitalized patients with gastroparesis is 1.5–3 mg/kg (by i.v. infusion over 45 min) every 6–8 h, and 125 mg b.i.d., orally for outpatient gastroparesis management for a few weeks. The liquid formulation is often preferred in order to enhance pharmacokinetics in patients with marked delay in gastric emptying. Side effects experienced with erythromycin treatment include abdominal pain, nausea, and diarrhea. Caution should be taken when erythromycin is co-administered with agents that alter or are metabolized by cytochrome P450 (CYP) 3A4 (e.g., diltiazem or verapamil or domperidone) because the drug interactions may be associated with risk for sudden cardiac death (Ray et al., 2004).

Azithromycin and clarithromycin are other macrolides that accelerate gastric emptying (Bortolotti et al., 1999; Larson et al., 2010). There are no randomized, placebo-controlled trials to assess efficacy. Use of these agents should be balanced with the potential of tachyphylaxis due to downregulation of the target receptors, cardiac risk as mentioned above, or development of bacterial resistance to these useful antimicrobial agents.

Targeting the Gastric Fundus

Mechanistic studies showed that acotiamide enhanced gastric accommodation and gastric emptying of a liquid meal (Kusunoki et al., 2012) and improved symptoms in patients with functional dyspepsia (Matsueda et al., 2012). Some 5-HT_4 receptor agonists also enhance gastric accommodation, such as tegaserod in dyspeptics with normal gastric emptying (Tack et al., 2010). This provides a rationale for their use in functional dyspepsia. However, two randomized controlled trials that studied tegaserod in patients with functional dyspepsia demonstrated mixed effects on dyspepsia symptoms (Vakil et al., 2008).

Recent data using simultaneous measurement of gastric accommodation and emptying of a solid egg meal suggests that there is a direct relationship between the degree of gastric accommodation and the gastric emptying lag duration and $T_{1/2}$, suggesting that, in some patients, impaired emptying may result from excessive gastric accommodation with delayed movement of solid food from the fundus to the antrum of the stomach (Wang et al., 2021). This observation suggests that stimulation of the proximal stomach with reduced gastric accommodation may actually enhance gastric emptying in patients with gastroparesis.

It is not surprising, therefore, that erythromycin is associated with marked acceleration or dumping of food from the stomach since, as a motilin receptor agonist and stimulant of cholinergic mechanisms, it enhances both fundic contraction as well as antral motor function, thereby having a dual effect on mechanisms associated with increased longitudinal axial forces in the antrum in healthy participants and patients with dysmotilities and acceleration of gastric emptying (Surrenti et al., 1996; Coulie et al., 1998; Liau et al., 2001). Further studies on the potential of fundic and antral stimulants to improve gastric symptoms would be of considerable interest.

Targeting the Pylorus

It is increasingly recognized that patients on long-term opioid medications may present with gastroparesis (Hasler et al., 2019). The mechanisms associated with the effects of opiates or opioid medications are reviewed elsewhere (Camilleri and Sanders, 2020). Opioids can induce pyloric dysfunction in addition to inhibition of antral motor function, both of which contribute to delayed gastric emptying. It is, therefore, relevant to assess whether targeting the pylorus or inhibiting the effects of opioids might be a therapeutic approach for gastric emptying delay attributed in part to pyloric dysfunction.

Although the classical pharmacological approach to treating the pylorus in gastroparesis involves botulinum toxin injection and there is open-label experience to suggest efficacy especially with higher dose injections (Coleski et al., 2009), two placebo-controlled trials did not demonstrate efficacy (Arts et al., 2007; Friedenberg et al., 2008).

Two pharmacological approaches have been pursued to reverse pyloric dysfunction in gastroparesis. One approach is

the use of sildenafil (Watkins et al., 2000), a phosphodiesterase-5 inhibitor which mimics the effect of nitric oxide by increasing intracellular cGMP. A reduced expression of neuronal nitric oxide synthase in the pylorus of diabetic mice was reversed by treatment with insulin and by sildenafil. However, in gastroparesis associated with uremia, there was no significant effect of sildenafil on gastric emptying (Dishy et al., 2004).

A second approach to reverse pyloric contractility involves use of opioid antagonists. In an older study, naloxone did not stimulate gastric emptying in healthy subjects or in patients with gastric hypomotility associated with functional dyspepsia or idiopathic gastroparesis (Narducci et al., 1986). Two studies have tested the potential of peripherally active μ-opioid receptor antagonists (PAMORA) in the setting of codeine-induced delay in gastric emptying in opioid-naïve healthy participants. At the doses approved for treatment of chronic opioid-induced constipation (OIC), short-term administration of methylnaltrexone (s.c. 0.30 mg/kg) or naloxegol (25 mg) daily did not inhibit the retardation of gastric emptying induced by codeine in healthy, opioid-naïve volunteers (Wong et al., 2010; Halawi et al., 2018).

As stated above, there is evidence of pyloric relaxation by erythromycin by stimulating the inhibitory nerves of the pylorus; however, the long-term effects of erythromycin on the pylorus are unclear and they have not been studied extensively.

Targeting M_2 Macrophages and Oxidative Stress

Gastroparesis may also result from abnormal function of enteric mechanisms which may be targeted by macrophage-based immune dysregulation, as demonstrated in diabetic mice with delayed gastric emptying (Cipriani et al., 2018). Reduced pacemaker cells [interstitial cells of Cajal (ICCs), and platelet-derived growth factor receptor alpha (PDGFRα) fibroblast-like cells] and numbers of nitrergic neurons and CD206 positive macrophages have been reported in some studies in patients with idiopathic or diabetic gastroparesis (Grover et al., 2011; Grover et al., 2012; Grover et al., 2017), though the ICC results were not confirmed in patients with idiopathic gastroparesis (Bernard et al., 2014; Herring et al., 2018). Damage to the pacemaker cells may occur because of depletion of anti-inflammatory resident M_2 macrophages expressing heme oxygenase-1 (HO_1), allowing oxidative stress to damage the pacemaker cells or enteric nerves, as evidenced from animal models of gastroparesis (Choi et al., 2008; Cipriani et al., 2018). However, hemin, an heme oxygenase (HO_1) inhibitor, failed to reverse delayed gastric emptying in a randomized, controlled trial in humans. Unfortunately, the pharmacokinetics of the hemin in that trial were insufficient to conclusively test whether countering oxidative stress can restore normal ENS function and gastric emptying (Bharucha et al., 2016).

Potential Pharmacological Promotion of Neuronal Cell Differentiation

As in other tissues, there is a dynamic balance in the enteric nervous system between cell loss by apoptosis, phagocytosis of dead cells by resident macrophages, and restoration of new cells from stem cells. Similarly, in the enteric nervous system, the loss of neurons is replenished by neurogenesis precursor cells that behave like stem cells and are prominent in the submucosal zone and in the muscular layers (Kulkarni et al., 2017). It has been shown, *in vitro,* that a selective estrogen receptor β (ERβ) agonist, LY3201, stimulated glial-to-neuron cell differentiation. It also increased recovery of neurons in the damaged myenteric plexus in two *in vivo* models of enteric neuronal damage in mice, specifically the damage resulting from administration of a high-fat diet, or the serosal application of the cationic detergent, benzalkonium chloride (D'Errico et al., 2018).

The potential for neurogenesis and nerve growth factors to restore normal propulsion has been demonstrated in both animal and human studies. In rats, exogenous brain-derived neurotrophic factor (BDNF) increased myoelectric activity and peristalsis in the gastrointestinal tract and colon (Chai et al., 2003; Grider et al., 2006). In addition, exogenous recombinant human BDNF and neurotrophin-3 were shown to accelerate gastrointestinal and colonic transit in healthy human volunteers and in patients with constipation respectively (Coulie et al., 2000). Thus, neurogenesis has the potential to improve enteric nervous system function in patients with gastroparesis.

CONCLUSION

There has been extensive research into the effects of diverse classes of medications targeting different pathophysiological mechanisms including defective contraction or coordination of the stomach manifesting with symptoms and objective retardation of gastric emptying. The findings reported augur well for the development of effective treatments for gastroparesis. These include immune (such as macrophage) modulation, reversal of oxidative stress, direct pharmacological therapies targeting pivotal receptors without inducing adverse effects, and endoscopic pyloromyotomy. Further validation studies and approval by regulatory agencies should lead to opportunities to resolve the significant unmet clinical need in patients with gastroparesis.

AUTHOR CONTRIBUTIONS

MC–conceptual study design, literature review, drafting, revising and finalizing the article JA–literature review, revising and finalizing the article.

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Therapeutic Effects of Berberine Hydrochloride on Stress-Induced Diarrhea-Predominant Irritable Bowel Syndrome Rats by Inhibiting Neurotransmission in Colonic Smooth Muscle

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The etiology of diarrhea-predominant irritable bowel syndrome (IBS-D) is complicated and closely related to neurotransmission in the gastrointestinal (GI) tract. Developing new strategies for treating this disease is a major challenge for IBS-D research. Berberine hydrochloride (BBH), the derivative of berberine, is a herbal constituent used to treat IBS. Previous studies have shown that BBH has potential anti-inflammatory, antibacterial, analgesic, and antidiarrheal effects and a wide range of biological activities, especially in regulating the release of some neurotransmitters. A modified IBS-D rat model induced by chronic restraint stress was used in all experiments to study the effects of BBH on the GI tract. This study measured the abdominal withdrawal reflex (AWR) response to graded colorectal distention (CRD; 20, 40, 60, and 80 mmHg) and observed the fecal areas of stress-induced IBS-D model. Experiments were conducted using organ bath techniques, which were performed *in vitro* using strips of colonic longitudinal smooth muscle. Inhibitory and excitatory neurotransmitter agents were added to each organ bath to observe contractile responses on the strips and the treatment effect exerted by BBH. The IBS-D rat model was successfully induced by chronic restraint stress, which resulted in an increased defecation frequency and visceral hypersensitivity similar to that of humans. BBH could reduce 4-h fecal areas and AWR response to CRD in IBS-D. The stress-induced IBS-D model showed upregulated colonic mRNA expression levels of 5-hydroxytryptamine-3A receptor and downregulated expression levels of neuronal nitric oxide synthase. Meanwhile, BBH could reverse this outcome. The responses of

Abbreviations: BBH, berberine hydrochloride; IBS-D, diarrhea-predominant irritable bowel syndrome; GI, gastrointestinal; CRD, colorectal dilation; AWR, abdominal withdrawal reflex; qRT-PCR, quantitative real-time polymerase chain reaction; EFS, electrical -field stimulation; L-NAME, Nω-nitro-L-arginine methyl ester hydrochloride; MRS2500, (1R*,2S*)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy) bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt; α,β-MeATP, α, β-methyleneadenosine 5′-triphosphate trisodium salt; CCh, carbachol

substances that regulate the contraction induced by related neurotransmission in the longitudinal smooth muscle of IBS-D colon (including the agonist of acetylcholine, carbachol; NOS inhibitor, L-NAME; and $P2Y_1$ receptor antagonist, MRS2500) can be inhibited by BBH. In summary, BBH promotes defecation frequency and visceral hypersensitivity in IBS-D and exerts inhibitory effects on contractile responses in colonic longitudinal smooth muscle. Thus, BBH may represent a new therapeutic approach for treating IBS-D.

Keywords: berberine hydrochloride, diarrhea-predominant irritable bowel syndrome, colonic longitudinal smooth muscles, inhibitory effects, neurotransmission

INTRODUCTION

Irritable bowel syndrome (IBS) is a prevalent gastrointestinal (GI) disease characterized by abdominal pain and change in bowel habits. IBS has affected 11.2% of the general population worldwide (Ford, 2020). Many factors, such as heredity, diet, mentality, social culture, and inflammatory substances, influence its onset. IBS incurs a heavy economic and social burden on an individual, as well as the society. The Rome IV standard is the latest diagnostic criteria used to categorize patients with IBS into four types. Diarrhea-predominant IBS(IBS-D) is a subtype associated with rectal urgency, increased defecation frequency, abdominal bloating, and loose to watery stools and accounts for 23.4% of patients with IBS (Clarke et al., 2009). Currently, drugs targeting neurotransmitter receptors, such as loperamide, iludoline, alosetron and some antidepressants, are used to treat patients with IBS-D (Lacy, 2016). However, a standard treatment algorithm has not been established yet for IBS-D (Corsetti and Whorwell, 2017).

Current research has shown that GI motility and mucus secretion, as well as the sensitivity of the GI tract to mechanical or chemical sensory stimuli, can be altered by abnormalities in the enteric nervous system (ENS) (Gwynne and Bornstein, 2007; Peiris et al., 2017). The intestinal plexus is involved in regulating intestinal peristalsis. The study on the rat model of IBS induced by neonate maternal separation shows that the increase of colonic motility is related to the up-regulation of L-type calcium (Zhang et al., 2010). Other results suggest that the transient receptor potential vanilloid type-1 (TRPV1 or VR1) immunoreactive nerve fibers in colonic biopsies from patients with IBS can be activated by intestinal chemical mediators and release neurotransmitters such as substance P and calcitonin gene-related peptide. Moreover, the increase of TRPV1 nerve fibers may be related to visceral hypersensitivity and pain in IBS (Akbar et al., 2008). In short, the changes in GI motility involved in IBS-D are closely related to the mechanical relaxation and contraction of GI smooth muscle, which are jointly regulated by transmitters released in ENS, especially in the intermuscular plexus.

As an important non-adrenergic and non-cholinergic neurotransmitter regulating smooth muscle contraction, nitric oxide (NO) is widely distributed in the GI tract and has important biological functions, especially in the regulation of GI motility and visceral sensation (Konturek and Konturek, 1995). In addition, the effects of NO-mediated antinociception and anti-diarrhea in the IBS-D rat model have been confirmed (Paragomi et al., 2014). Intestinal peristalsis depends on the contraction of smooth muscle and is regulated by interstitial cells of Cajal (ICC) in the GI tract (Lammers et al., 2011). NO is produced from arginine in a reaction catalyzed in the intestine by NO synthases (NOS), in which neuronal NOS (nNOS) plays an important role in regulating GI peristalsis, and the distribution of nNOS immunoreactive positive products in GI mucous epithelial cells, myenteric plexus and submucosal ganglion cells has been confirmed (Ravella et al., 2013; Tomuschat et al., 2017). NO is an unstable lipid-soluble small molecular compound, which is synthesized and diffused to the target ICC by diffusion, activating calcium pumps dependent on cyclic guanosine monophosphate (cGMP) protein kinase, preventing calcium ion influx, or promoting calcium ion transmembrane transport out of cells. It also participates in cell-to-cell information transmission (Toda and Herman, 2005; Cain and Snutch, 2011). Therefore, as a messenger between nNOS positive neurons and GI smooth muscle cells, the increase of NO reduces the influx of calcium ions and directly promotes the relaxation of smooth muscle.

The neurohormone 5-hydroxytryptamine (5-HT or serotonin) is considered to be an important intestinal neurotransmitter, about 95% of which exist in the intestinal tract in the human body. It plays an important role in regulating GI motility and secretion, coordinating reflexes, and regulating sensory information in and out of the intestine. Within the bowel, serotonin is synthesized by the enterochromaffin (EC) subtype of enteroendocrine cells and by serotonergic neurons in the myenteric plexus (Gershon and Tack, 2007). $5\text{-}HT_3$ receptors are widely distributed in peripheral and central nervous tissues, through which 5-HT acts to excite enteric neurons (Derkach et al., 1989). Current studies have confirmed that $5\text{-}HT_3$ receptor antagonists can effectively inhibit intestinal urgency, prolong intestinal and colorectal conduction and relieve the symptoms of IBS-D (Oh et al., 2001).

The concept of purinergic signaling stems from studies that were designed to identify the non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter in the gut (Burnstock et al., 1972). Purine receptors are broadly classified as P1 for nucleosides (adenosine) and P2 for nucleotides (ATP). They can be subdivided into adenosine (A_1, A_{2A}, A_{2B}, A_3), $P2X_{1\text{-}7}$, and $P2Y_{1,2,4,6,11-14}$. Most of these receptors are expressed in the GI tract where they are known to be involved in the physiological

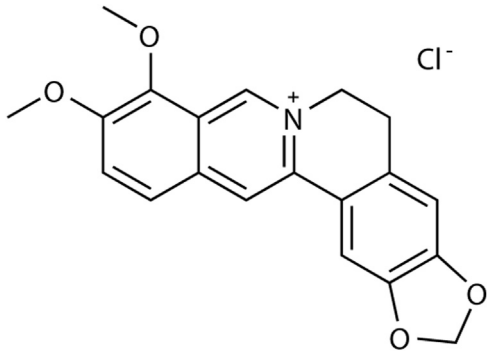

FIGURE 1 | Structure of BBH.

regulation of gut reflexes in animal models. Endogenous purines are critical regulators of neurotransmission in the human ENS acting at $P2X_1$, $P2X_2$, and $P2X_3$ channels, as well as inhibitory P2Y or A_3 receptors. These purinergic receptors and signaling pathways are essential as they are potential therapeutic targets for inflammatory bowel diseases (IBDs), IBS and diarrheal disorders (Liñán-Rico et al., 2015).

To sum up, neurotransmission may contribute to the homeostatic maintenance of the ENS and play an important role in the pathogenesis of IBS-D.

Rhizoma coptidis is a traditional Chinese herb widely used as a remedy for GI diseases, particularly against diarrhea (Chen et al., 2015). Berberine hydrochloride (BBH; its structure is shown in **Figure 1**), a berberine derivative, is a benzylisoquinoline alkaloid found in *Rhizoma coptidis* and has potential therapeutic effects as an antiemetic, antimicrobial, anti-inflammatory, and antinociceptive substance. BBH may play a role in the treatment of diarrhea (Cheng et al., 2009). A systematic review and meta-analysis about children and adults demonstrated that berberine was generally effective in improving clinical cure rates and shortening the duration of diarrhea (Yu et al., 2020). Studies on colitis models induced by intracolonic instillation have shown that BBH can significantly reduce visceral sensitivity and the frequency of defecation in rats, which may be achieved through NO-mediation (Tang et al., 2013). Furthermore, BBH inhibits GI motility in rodents and is closely related to the endogenous opioid system (Chen et al., 2015). These results implied that BBH might exert a therapeutic effect on IBS-D. However, its mechanism has not been fully clarified to date, and no further studies have reported the role of BBH in regulating the pharmacology of digestive system-related neurotransmitters. The present study mainly explores how the neurotransmission of the GI tract affects IBS-D and whether BBH can treat IBS-D in rats through GI neurotransmitters.

MATERIALS AND METHODS

Chemicals and Reagents

The Krebs solution used in this study was composed of 120 mmol/L NaCl, 5.9 mmol/L KCl, 25 mmol/L $NaHCO_3$, 1.2 mmol/L $Na_2HPO_4{\cdot}12H_2O$, 1.2 mmol/L $MgCl_2{\cdot}6H_2O$, 2.5 mmol/L $CaCl_2$, and 11.5 mmol/L dextrose. The following materials were used in the study: BBH (Aladdin, China), pinaverium bromide (30 mg/kg, Abbott Products SAS, France), TRIzol reagent (Thermo Fisher, United States), RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher, United States), SuperReal PreMix Plus [SYBR Green; a special reagent for quantitative real-time polymerase chain reaction (qRT-PCR) by chimeric fluorescence method; Tiangen, China], dimethyl sulfoxide (DMSO; <0.2%; Thermo, United States; used as a medium to dissolve BBH in all experiments), tetrodotoxin (TTX; 1 μmol/L, Thermo Fisher, United States), carbachol (CCh; 1×10^{-9}–1×10^{-5} mol/L; Sigma-Aldrich, United States), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME; 10 μmol/L; Sigma-Aldrich, United States), α,β-methyleneadenosine 5′-triphosphate trisodium salt (α,β-MeATP; 100 μmol/L; Tocris Bioscience, United Kingdom), and (1R*,2S*)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo [3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500; 1 μmol/L; Tocris Bioscience, United Kingdom). All reagents were dissolved in distilled water except for KCl, which was distilled in Krebs solution.

Animal Preparation

Male Sprague–Dawley (SD) rats (aged 4 weeks and weighing 100 ± 10 g) were purchased and fed in specific pathogen-free animal house in strict accordance with the Guide to Animal Use and Care published by the Research Center for Laboratory Animals (Guangzhou University of Chinese Medicine, China). The rats were maintained in adaptable circumstance with 12 h/12 h light/dark cycle, 20–25°C environmental temperature, and 50–70% humidity. The study was reviewed and approved by the Institutional Review Board of Guangzhou University of Chinese Medicine. All procedures used in this study that involved animals were reviewed and approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine (IACUC protocol number: S2018020).

Stress-Induced IBS-D Rat Model and Administration

The IBS-D rat models used in all experiments were induced by "chronic restraint stress" as described in our previous study (Zhou R. T. et al., 2018) to investigate the effects of BBH on the GI tract. The pathogenesis of IBS remains speculative and may be multifactorial and thus may cause trouble to the research on the modeling mechanism and corresponding drugs under multiple factors. We were concerned that the model induced by single-factor stimulation can stably simulate the symptoms of diarrhea or increased defecation in humans with high visceral sensitivity while excluding the organic lesions of IBS-D. In our previous studies (Zhou TR. et al., 2018), we showed that the abnormalities of neurotransmission in this model occurred in the colon but not in the jejunum.

All the male rats were randomly divided into two groups (50 rats in the model group and 10 rats in the control group) after

FIGURE 2 | Graph of the excrement before and after ImageJ analysis.

7 days of adaptation. The rats in the model group were subjected to restraint stress using an elastic bandage to restrict the movement of the upper body and forelimbs and then anesthetized with ether. Their foreshoulders, upper forelimbs, and thoracic trunk were wrapped in elastic bandage for 2 h each day for 14 days to produce a steady and consistent amount of stimulation to restrict but not prevent movement. The control animals were anesthetized with ether but not restrained. The rats subjected to chronic restraint stress were randomly divided into five groups, namely, the model group (without any drug treatment), the positive drug group (30 mg/kg pinaverium bromide), and three BBH groups with different BBH doses (25, 50, and 100 mg/kg). Then, they were subjected to pharmacological treatment for 14 days. The drugs were given by intragastric administration according to body mass volume and did not exceed 10 ml/kg once a day. The control and model groups were treated with the same volume of normal saline. Specific BBH doses were based on previous studies (Tang et al., 2013; Gong et al., 2014).

Measurements of Fecal Area and Visceral Hypersensitivity

We evaluated the rat model by measuring fecal areas using ImageJ software (Schneider et al., 2012) and observing visceral hypersensitivity responses to colorectal distention (CRD) (Al-Chaer et al., 2000). Rat fecal areas in 4 h were recorded by capturing vertical photos after fixing the height and shooting mode of the digital camera (Bai et al., 2009). The graph of the excrement before and after ImageJ analysis is shown in **Figure 2**. Behavioral responses to CRD were then assessed in all groups by measuring the abdominal withdrawal reflex (AWR) using a semiquantitative score. A catheter (8Fr) was inserted into the descending colon of the rat anesthetized with ether and secured by taping the attached tubing to the rat's tail with the outer end of the balloon at about 1 cm from the anus. Then, the catheter was connected with a sphygmomanometer through a three-way valve. AWR measurement consisted of a visual observation of animal response to graded CRD (20, 40, 60, and 80 mmHg) by blinded observers and the assignment of an AWR score: 0, no behavioral response to CRD; 1, brief head movement followed by immobility; 2, contraction of abdominal muscles; 3, lifting of abdomen; 4, body arching and lifting of pelvic structures. The rats were given CRD for 20 s every 4 min, and the expansion was repeated five times for each intensity in the threshold intensity of CRD and AWR measurement.

Total RNA Extraction and qRT-PCR

The expression of 5-hydroxytryptamine-3A receptor (5-HT_{3A}R), nNOS, dynein light chain 8 (DLC8 or LC8), and solute carrier family 17 member 9 (SLC17A9) at the mRNA level were evaluated by qRT-PCR. Briefly, colonic tissues (50–100 mg) were eviscerated from SD rats euthanized by CO_2 asphyxiation to prepare homogenates after the end of the last administration. Total RNA was isolated from the colonic tissues using TRIzol solution in accordance with the manufacturer's instructions and treated with RNase-free DNase. Reverse transcription was performed in a reaction system with RevertAid First-Strand cDNA Synthesis Kit. qRT-PCR reactions (20 μl) were set up by the addition of 8 μl of primer mix (containing 10 μM reverse and forward primers), 2 μl of diluted cDNA template, and 10 μl of TaqMan Gene Expression Master Mix (2× SuperReal PreMix Plus). qRT-PCR was performed with a Bio-Rad qRT-PCR System. The relative abundance of glyceraldehyde-3-phosphate dehydrogenase mRNA was used to normalize the levels of mRNAs of interest. All primers used for qPCR are listed in **Table 1**.

Preparation of the Colonic Longitudinal Smooth Muscle Strips

Strips of colonic longitudinal smooth muscle were used in *in vitro* experiments through standard organ bath techniques, and the mechanical activity of the muscle strips was recorded as changes

TABLE 1 | Primers used for qRT-PCR in this study.

Proteins	Upstream primer sequence (5′–3′)	Downstream primer sequence (3′–5′)	Quantity of product (bp)
GAPDH	TGGCCTCCAAGGAGTAAGAAAC	TGGAATTGTGAGGGAGATGCTC	109
5-HT_{3A}R	AGAACTGCTCTCTGACCTTCAC	TTGTCCGACCTCACTTCTTCTG	94
nNOS	TGGCAAATCGCACAAAGCTC	TACGGGTTGTTAAGGACCACAG	102
LC8	TAGCATGGACTGTGCCAAAC	TGGTGCCCTGTACAAAACAC	140
SLC17A9	TTCCTGCCAGTTTGTTCAGC	AATGCTTGACAGACCAAGGC	113

in isometric force. The strips of colonic longitudinal smooth muscles (10 mm long), eviscerated from euthanized SD rats, were cut and suspended in a culture dish. The sample strips were equilibrated in organ baths (the volume of each bath is 5 ml; LE13206, Harvard Apparatus Corporation, United States), which were continuously perfused with Krebs solution that was bubbled with a mixture of 5% CO_2 and 95% O_2 (pH 7.4) and maintained at 37°C. The strips were suspended in the direction of the longitudinal muscle. One end of each tissue was connected to a fixed hook, and the other end was connected to a flexible hook. The surgical suture was connected to a force transducer (BR4740-ISO510A, Harvard Apparatus Corporation, United States), which was connected to a computer through an amplifier to record mechanical activity. Changes in tension due to the relaxation or contraction of muscles were recorded through an analog-to-digital board connected to a computer (PL33508, ADInstruments, Powerlab Corporation, Australia). Data were digitized using LabChart Reader (Harvard Apparatus Corporation, United States; LabChart, New Zealand) by a computer. Electrical-field stimulation (EFS; 40 V, 2–30 Hz, 0.5 ms pulse duration, 10 s) was applied *via* two platinum ring electrodes, which were connected to a stimulator (LE12406, Harvard Apparatus Corporation, United States) and attached to each strip. The tissues were given basal tension at 1.4 g after 0.5 h of non-tension adaptive treatment and equilibrated for approximately 1 h prior to the commencement of the experiments. Each colonic tissue was weighed and recorded after blotting on filter paper at the end of the experiments.

Measurement of the Contraction of Colonic Longitudinal Smooth Muscles

After washing in organ bath and equilibration, BBH was added to each bath in a sequence of concentrations (1.5625, 3.125, 6.25, 12.5, 25, 50, 100 and 200 μmol/L) to observe its effect on the spontaneous contraction of colonic longitudinal smooth muscle. Then, the 50% effective concentration (EC_{50}) of BBH was calculated to determine its most effective concentration. In addition, the contractile responses of the BBH- and DMSO-treated groups were compared.

CCh (1×10^{-9}–1×10^{-5} mol/L) was added to the organ bath, and the contractile responses of the longitudinal muscular strips of the control and model groups were compared. After the contraction induced by CCh reached a plateau, the muscle strips were washed with Krebs solution three times and equilibrated for 1 h before the next experiment.

The tissues were stimulated by EFS to induce neuron-mediated contraction, and the frequency–response curve was obtained at an interval of 3 min. Substances able to modulate neurotransmission pathways, including TTX (1 μmol/L), L-NAME (10 μmol/L), α,β-MeATP (100 μmol/L), and MRS2500 (1 μmol/L), were added to each organ bath before EFS to observe the contractile responses in the strips.

The strips were preincubated in a single concentration of BBH (40 μmol/L) to observe its effects on neurotransmitters such as L-NAME (10 μmol/L), α,β-MeATP (100 μmol/L), MRS2500 (1 μmol/L), and TTX (1 μmol/L) during spontaneous or EFS-induced colonic contraction, respectively. In this *in vitro* study, each strip was subjected to a 1-h equilibration before the next experiment.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). $p < 0.05$ was considered statistically significant, and n indicates the number of samples. Statistical analysis and curve fit were performed using GraphPad Prism (GraphPad Software, San Diego, CA, United States). Calculations were performed using SPSS 20.0 on the basis of the number of individual tissue segments. Non-pairwise comparisons were performed using Student's *t*-test. ANOVA was used in testing three or more variables for statistical significance. Nonlinear and linear regression analyses were also utilized as needed.

RESULTS

Establishment of the IBS-D Rat Model

We counted and measured the fecal areas of the control and IBS-D rats on days 0, 7, 14, 21, and 28. The fecal areas of the IBS-D group on days 14, 21, and 28 significantly increased ($p < 0.05$) compared with those of the control group (**Figure 3A**). Nevertheless, the fecal areas of both groups had no discrepancy on the 0th and seventh days. The change in 4-h fecal areas showed that the defecation frequency of the IBS-D group increased with the passage of time, which is consistent with the change in the bowel habit of clinical patients with IBS-D. This result indicated that an IBS-D rat model was successfully established by chronic restraint stress.

All rats were subjected to graded CRD (20, 40, 60, and 80 mmHg) and given AWR scores. The IBS-D group showed a remarkable increase in AWR compared with the control group (**Figure 3B**). These changes were substantial for all the intensities

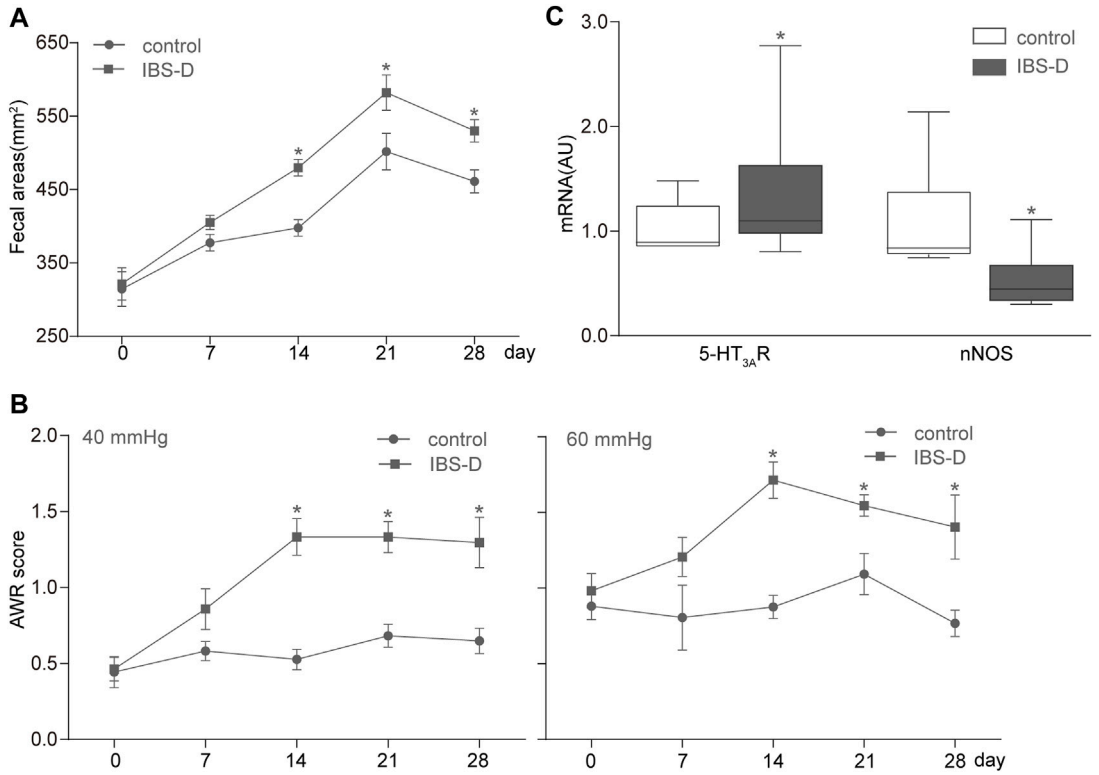

FIGURE 3 | Establishment of the IBS-D rat model. **(A,B)** Measurement results of fecal areas and AWR scores at 40 and 60 mmHg on days 0, 7, 14, 21, and 28. **(C)** Box and whisker plots of the distribution and mean expression levels of 5-HT_{3A}R and nNOS in the colonic tissues of the control and IBS-D groups. Data are represented as mean ± SEM. n = 6–12 rats per group. *denotes $p < 0.05$ compared with the control group.

of CRD; hence, the applied chronic restraint stress successfully established the IBS-D rat model and resulted in a visceral hypersensitivity similar to that of humans.

Furthermore, qRT-PCR analysis of mRNA levels showed that the expression of 5-HT_{3A}R increased, whereas that of nNOS decreased in the IBS-D group compared with the control group (**Figure 3C**).

BBH Ameliorated Symptoms in IBS-D Rats

The 4-h fecal areas and AWR scores (under 40 and 60 mmHg CRD) of the IBS-D group significantly increased ($p < 0.05$ or $p < 0.01$) on days 21 and 28 compared with the control group. This finding indicated the presence of increased defecation frequency and visceral hypersensitivity, which were consistent with the characteristics of hyperalgesia in clinical patients with IBS-D. Furthermore, the fecal areas and AWR scores of the positive drug group (30 mg/kg pinaverium bromide) and BBH groups (50 and 100 mg/kg) decreased obviously ($p < 0.05$ or $p < 0.01$) compared with the model group (no medication). In brief, 50 and 100 mg kg^{-1} BBH considerably reduced the 4-h fecal areas and improved the high sensitivity of visceral neurons. Although the AWR scores of the (25 mg/kg) BBH group decreased significantly compared with the model group ($p < 0.05$), no obvious change in fecal areas was observed ($p > 0.05$). **Figures 4A,B** only show the data of fecal areas and AWR scores at 40 and 60 mmHg CRD on days 21 and 28.

The results of qRT-PCR analysis showed that chronic restraint stress changed the colonic mRNA expression levels of 5-HT_{3A}R and nNOS in the model group compared with the control group. However, the LC8 and SLC17A9 mRNA expression in the model and control groups had no statistical difference. Pinaverium bromide (30 mg/kg) treatment inhibited the mRNA expression of 5-HT_{3A}R and nNOS in the model group. qRT-PCR analysis of mRNA levels in BBH-treated rats showed similar effects as pinaverium bromide. BBH treatment led to the downregulation of 5-HT_{3A}R mRNA level and the upregulation of nNOS level in the IBS-D group (**Figure 4C**). BBH (25, 50, and 100 mg/kg) had no effect on LC8 and SLC17A9 mRNA expression in the GI tract of rats. These changes show the treatment effects of BBH. BBH may improve the symptoms of visceral hypersensitivity and abnormal defecation frequency in the IBS-D group by regulating the mRNA expression levels of 5-HT_{3A}R and nNOS to achieve a treatment effect.

Effect of BBH on the Colonic Contractile Responses of Isolated Longitudinal Smooth Muscles

3.1.1 BBH Inhibited the Spontaneous Contraction of Isolated Colonic Longitudinal Smooth Muscles

Colonic longitudinal smooth muscle strips were prepared in an organ bath, and the basic tension of spontaneous contraction

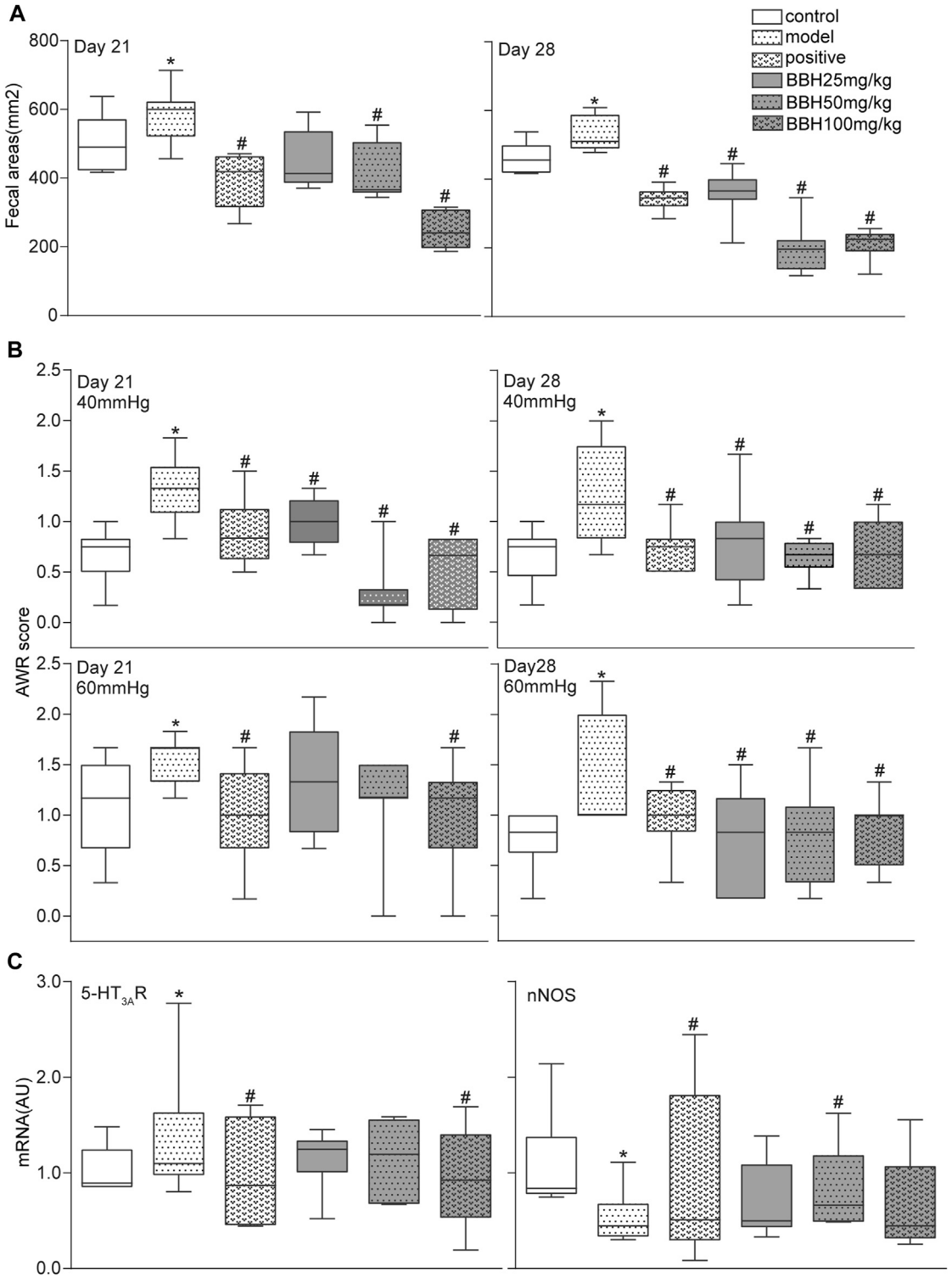

FIGURE 4 | BBH ameliorated symptoms in IBS-D rats. Box and whisker plots of the distribution and mean of fecal areas, AWR scores, and the expression levels of 5-HT_{3A}R and nNOS of the control group, model groups, and four treatment groups on days 21 and 28. The AWR scores were measured in response to graded CRD (40 and 60 mmHg). n = 6–12 rats per group. *denotes $p < 0.05$ compared with the control group. #denotes $p < 0.05$ compared with the model group.

occurred within a few minutes. The addition of seven cumulative concentrations of BBH (1.5625, 3.125, 6.25, 12.5, 25, 50, 100, and 200 μmol/L) successively led to a decrease in the tension of colonic longitudinal muscles in a concentration-dependent manner ($p < 0.01$). Meanwhile, DMSO did not affect the strips. The EC_{50} of BBH was 25.17 μmol/L. Thus, the BBH concentration of 40 μmol/L was used in further experiments (**Figures 5A,B**).

3.1.2 BBH Inhibited the EFS-Induced Contraction of the TTX-Treated Isolated Colonic Longitudinal Smooth Muscles

The intestinal nerve activated by EFS can simulate the contraction produced by various nerve cells *in vivo*; thus, it was used to observe neurogenic contraction response. We explored the effect of BBH on the contraction of isolated longitudinal colonic smooth muscle under TTX. The tissue was preincubated with BBH (40 μmol/L) for 3 min. TTX (1 μmol/L) was added to the organ bath to evaluate the contractile response. EFS was induced using 2–30 Hz for 10 s. The TTX-treated contractions and EFS-induced tissues in the control and IBS-D groups had a significant difference ($p < 0.05$) in contractile responses; thus, the contractile responses to EFS were mediated *via* neural stimulation (**Figures 5C–F,I,J**). The results of BBH + TTX-treated tissues in the IBS-D group showed no significant differences compared with those of TTX-treated tissues ($p > 0.05$). Nevertheless, the effect of the diastole produced by TTX preincubated with BBH was decreased compared with that produced by TTX only in the control group ($p < 0.05$; **Figures 5E–J**).

3.1.3 BBH Inhibited the Cholinergic Contractile Responses of Isolated Colonic Longitudinal Smooth Muscles

In previous studies, we showed that the contractile responses of longitudinal smooth muscle by cholinergic neurotransmission are inhibited in the jejunum but excited in the colon of IBS-D rat model induced by chronic restrain stress. The IBS-D group had higher ($p < 0.05$) contractile tension to the movement of isolated colon induced by CCh (1×10^{-7}–1×10^{-5} mol/L) compared with the control group (**Figures 6A,B,E**). The EC_{50} obtained in the IBS-D group (0.4906 μmol/L) in the presence of CCh was relatively lower than that in the control group (2.527 μmol/L) as shown in **Figure 6E**. Moreover, pretreatment with 40 μmol/L BBH for 10 min could eliminate the colonic contraction responses caused by CCh in the control and IBS-D groups. The contractile tensions of BBH + CCh-treated colonic tissues in the control group were significantly lower ($p < 0.05$) than those of CCh-treated tissues (1×10^{-7}–1×10^{-5} mol/L) (**Figures 6A,C,F**). Preincubation with 40 μmol/L BBH could significantly reduce the contractile tension of CCh-treated (1×10^{-9}–1×10^{-5} mol/L) colonic tissue compared with the IBS-D group treated with CCh alone ($p < 0.05$ or $p < 0.01$; **Figures 6B,D,G**). Furthermore, the comparison of the colonic contractile responses of the control and IBS-D groups treated with BBH and CCh showed that the tension of the IBS-D group was much lower than that of the control group. In brief, the antagonistic effect of BBH on colonic smooth muscle was more obvious in the IBS-D group than the control group (**Figures 6C,D,F,G**).

3.1.4 BBH Inhibited the EFS-Induced Contractions Elicited in the Presence of NO Blockade of Isolated Colonic Longitudinal Smooth Muscles

The contractile responses of EFS-induced colon tissues of the IBS-D group increased significantly compared with those of the control group ($p < 0.05$; **Figures 7A,B,G**). The contraction caused by EFS after preincubation with L-NAME (10 μmol/L) was higher ($p < 0.05$) than that by EFS induction only in the control and IBS-D groups (**Figures 7A–D,H**). Colonic tissues in the control group pretreated with L-NAME showed a remarkably lower EFS-induced contraction than those in the IBS-D group at 5–30 Hz (**Figures 7C,D,H**). Preincubation with BBH (40 μmol/L for 10 min) significantly reduced the combined contraction stimulated by L-NAME and EFS (5–30 Hz, 10 s) in the control and IBS-D groups ($p < 0.05$) and had a remarkable impact on the IBS-D group (**Figures 7C–F,I,J**).

3.1.5 BBH Inhibited the EFS-Induced Contractile Responses of Isolated Colonic Longitudinal Smooth Muscles Treated With $P2Y_1$ Receptor Antagonist

α,β-MeATP (100 μmol/L) was added to the organ bath, and MRS2500 (1 μmol/L) was added 2 min later. Then, changes in the tension of colonic tissues without EFS were observed. α,β-MeATP could inhibit the spontaneous contraction of colonic smooth muscle in the control and IBS-D groups. Meanwhile, this relaxing effect on the colon could be antagonized by BBH. The results showed significant differences between the BBH- and α,β-MeATP-treated control group, and similar results were observed in the BBH- and α,β-MeATP-treated IBS-D group ($p < 0.05$; **Figures 8A–C**). Indeed, the inhibitory effect of α,β-MeATP on colonic spontaneous contraction could be completely antagonized by the addition of MRS2500. Significant differences were observed between the BBH- and MRS2500-treated control groups ($p < 0.05$), whereas no significant difference ($p > 0.05$) was observed between the BBH- and MRS2500-treated IBS-D groups (**Figures 8A,B,D**). In addition, EFS-induced (2–30 Hz, 10 s) contractions were significantly increased in the MRS2500-treated IBS-D group compared with the untreated IBS-D group ($p < 0.05$; **Figures 8F,H,L**). The contractions in the MRS2500-treated IBS-D group were significantly decreased by the co-application of BBH (40 μmol/L) at the presented EFS compared with that in the IBS-D group treated with MRS2500 only ($p < 0.05$; **Figures 8H,J,L**). By contrast, the comparison between MRS2500-treated contractions and EFS-induced contractions had no substantial differences in the control group, and BBH + MRS2500-treated contractions and MRS2500-induced contractions also had no remarkable differences (**Figures 8E,G,I,K**).

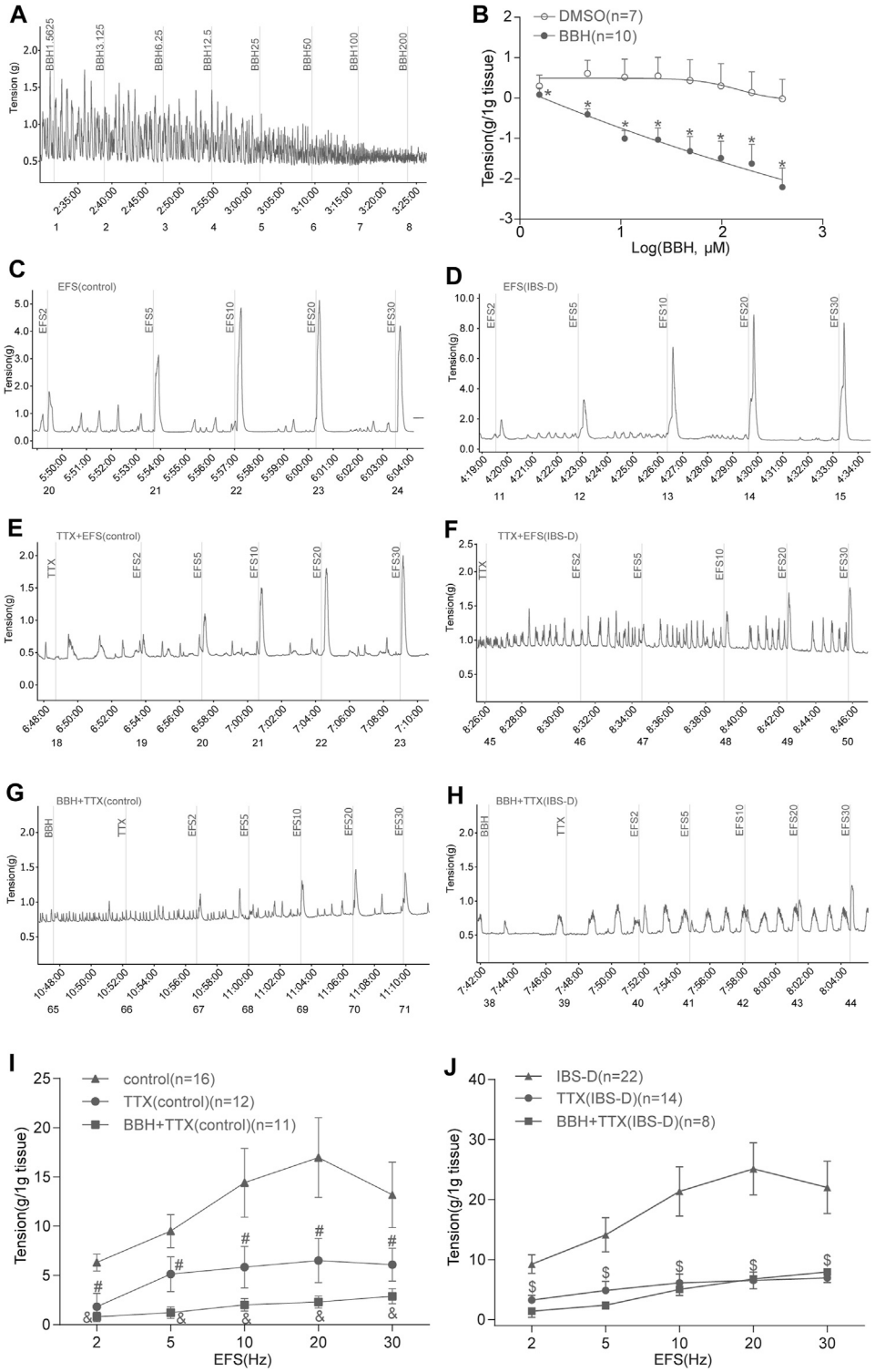

FIGURE 5 | BBH inhibited spontaneous and EFS-induced contractions of TTX-treated isolated colonic longitudinal smooth muscle. **(A,B)** Mechanical recording and linear regression curve of the cumulative log concentration–response of the effect of BBH (1.5625–200 μmol L^{-1}) on the spontaneous contractions of the isolated colonic longitudinal smooth muscle. The EC_{50} of BBH was 25.17 μmol/L. DMSO had no effect. *denotes $p < 0.01$ compared with the vehicle DMSO group. BBH: $n = 10$, DMSO: $n = 7$. **(C–J)** Mechanical recording **(C–H)** and histogram **(I–J)** of the effect of TTX and BBH + TTX treatments on the EFS-induced contraction response of colonic longitudinal smooth muscle in the control and IBS-D groups. **(C)**: EFS-induced control group ($n = 16$). **(D)**: EFS-induced IBS-D group ($n = 22$). **(E)**: TTX-treated control group ($n = 12$). **(F)**: TTX-treated model group ($n = 14$). **(G)**: BBH + TTX-treated control group ($n = 11$). **(H)**: BBH + TTX-treated IBS-D group ($n = 8$). **(I)**: BBH inhibited EFS-induced contractions of TTX-treated colonic tissues in the control group. # denotes $p < 0.05$ compared with the EFS-induced control group & denotes $p < 0.05$ compared with the TTX-treated control group. **(J)**: BBH inhibited the EFS-induced contractions of TTX-treated colonic tissues in the IBS-D group. $ denotes $p > 0.05$ compared with the EFS-induced IBS-D group. Error bars represent mean ± SEM.

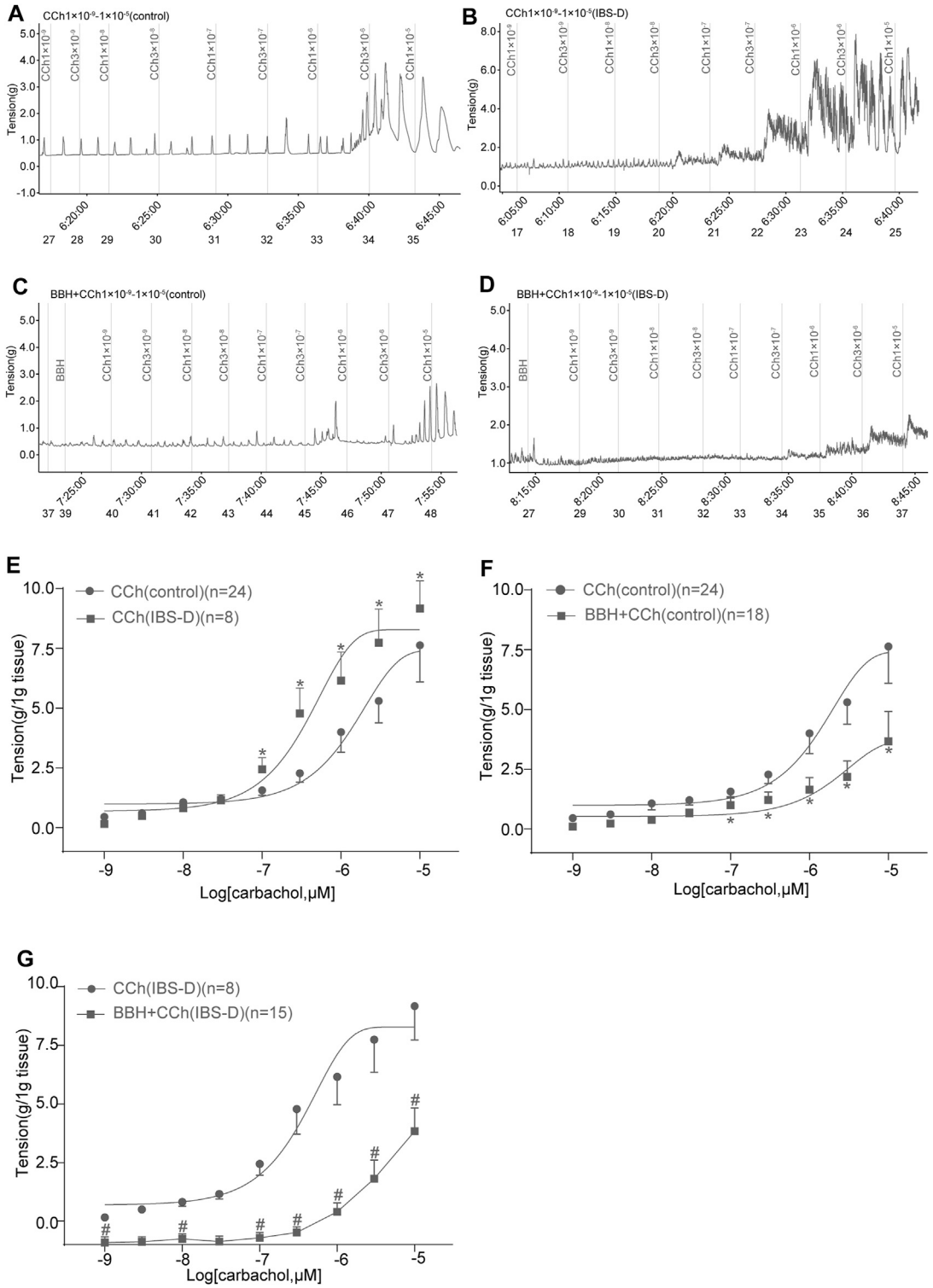

FIGURE 6 | BBH inhibited the cholinergic contractile responses of isolated colonic longitudinal smooth muscles. Mechanical recording **(A–D)** and cumulative log concentration–response curve **(E–G)** of the effect of CCh and BBH + CCh treatments on the contractions of isolated colonic longitudinal smooth muscles in the control and IBS-D groups. **(A)**: CCh-induced control group (n = 24). **(B)**: BBH + CCh-induced control group (n = 18). **(C)**: CCh-induced IBS-D group (n = 8). **(D)**: BBH + CCh-induced IBS-D group (n = 15). **(E)**: Cholinergic contractile responses in the control and IBS-D groups. The EC_{50} obtained in the IBS-D group was 0.4906 μmol/L, whereas that in the control group was 2.527 μmol/L * denotes $p < 0.05$ compared with the CCh-induced control group. **(F)**: BBH inhibited the cholinergic contractile responses in the control group. * denotes $p < 0.05$ compared with the CCh-induced control group. **(G)**: BBH inhibited the cholinergic contractile responses in the IBS-D group. # denotes $p < 0.05$ compared with the CCh-induced IBS-D group. Error bars represent mean ± SEM.

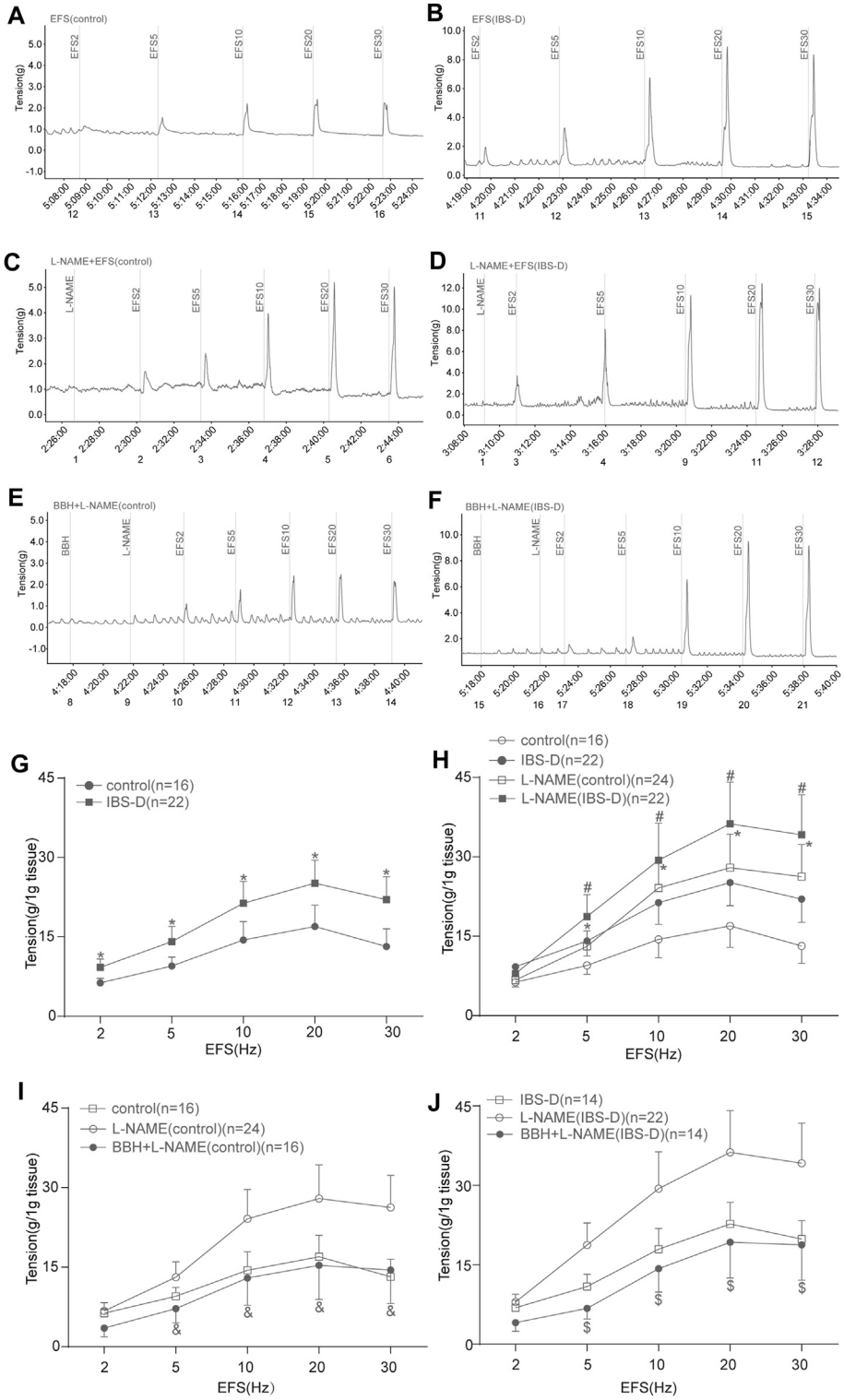

FIGURE 7 | BBH inhibited the EFS-induced nitrergic contractile responses of isolated colonic longitudinal smooth muscles. Mechanical recording **(A–F)** and line chart **(G–J)** of the effect of L-NAME and BBH + L-NAME treatments on the EFS-induced contraction response of colonic longitudinal smooth muscles in the control and IBS-D groups. **(A)**: EFS-induced control group ($n = 16$) **(B)**: EFS-induced IBS-D group ($n = 22$) **(C)**: L-NAME-induced control group ($n = 24$). **(D)**: L-NAME-induced IBS-D group ($n = 22$). **(E)**: BBH + L-NAME-induced control group ($n = 16$). **(F)**: BBH + L-NAME-induced IBS-D group ($n = 14$). **(G)**: EFS-induced contractile responses in the control and IBS-D groups. * denotes $p < 0.05$ compared with the EFS-induced control group. **(H)**: EFS-induced nitrergic contractile responses in the control and IBS-D groups. * denotes $p < 0.05$ compared with the EFS-induced control group; # denotes $p < 0.05$ compared with the EFS-induced IBS-D group. **(I)**: BBH inhibited the EFS-induced nitrergic contractile responses in the control groups & denotes $p < 0.05$ compared with the L-NAME-induced control group. **(J)**: BBH inhibited the EFS-induced nitrergic contractile responses in the IBS-D groups. $ denotes $p < 0.05$ compared with the L-NAME-induced IBS-D group. Error bars represent mean ± SEM.

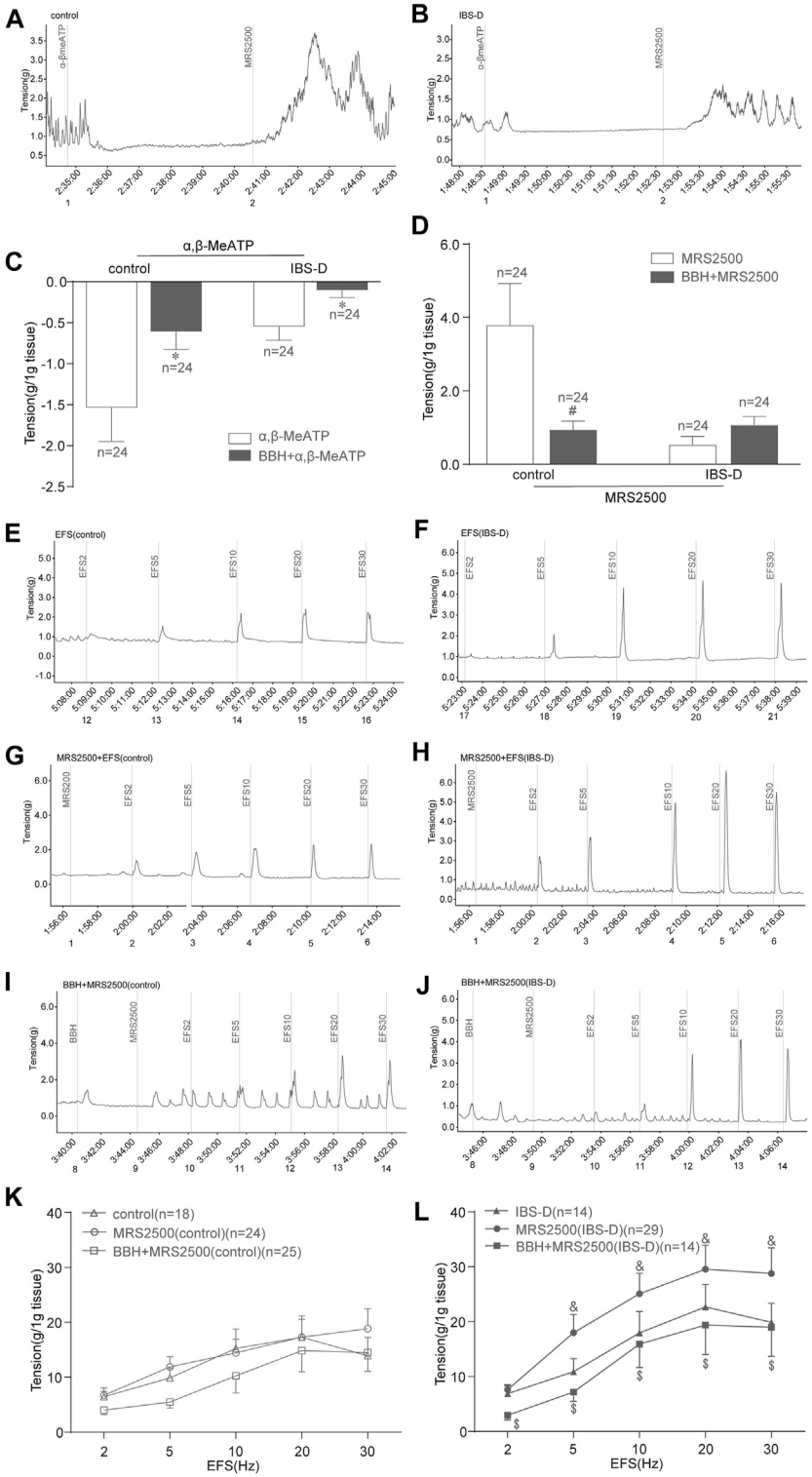

FIGURE 8 | BBH inhibited the EFS-induced contractile responses of isolated colonic longitudinal smooth muscles treated with $P2Y_1$ receptor antagonist. Mechanical recording **(A, B, and E–J)**, histogram **(C,D)**, and line chart **(K,L)** of the inhibitory effect of BBH on the tension of the α,β-MeATP-treated and MRS2500-treated isolated colonic longitudinal smooth muscles in the control and IBS-D groups. **(A)**: α,β-MeATP-treated ($n = 24$) and MRS2500-treated ($n = 24$) control groups. **(B)**: α,β-MeATP-treated ($n = 24$) and MRS2500-treated ($n = 24$) IBS-D groups. **(C)**: *denotes $p < 0.05$ compared with the α,β-MeATP-treated group. **(D)**: # denotes $p < 0.05$ compared with the MRS2500-treated control group. **(E)** EFS-induced control group ($n = 18$). **(F)**: IBS-D group ($n = 14$). **(G)**: EFS + MRS2500-treated control group ($n = 24$). **(H)**: EFS + MRS2500-treated IBS-D group ($n = 29$). **(I)**: BBH + MRS2500-treated and EFS-induced control group ($n = 25$). **(J)**: BBH + MRS2500-treated and EFS-induced IBS-D group ($n = 14$). **(K)**: Inhibitory effect of BBH on the MRS2500-treated control group. **(L)**: Inhibitory effect of BBH on the MRS2500-treated IBS-D group & denotes $p < 0.05$ compared with the EFS-induced IBS-D group. $ denotes $p < 0.05$ compared with the EFS + MRS2500-induced IBS-D group. Error bars represent mean ± SEM.

DISCUSSION

The etiology of IBS-D is complicated, and its physiological and pathological mechanisms have not been elucidated yet . Current drug treatment alleviates symptoms but has limited effect. Therefore, a safe, effective, and targeted drug for IBS-D is urgently needed.

BBH is a derivative of berberine, which is the major constituent of *Rhizoma coptidis*, a herbal constituent used to treat IBS. Previous studies have shown that BBH has potential anti-inflammatory, antibacterial, analgesic, and antidiarrheal effects and a wide range of biological activities, especially in regulating the release of some neurotransmitters, which are involved in the pathological process of various neurological diseases. A recent study (Yu et al., 2019) has shown that intestinal immune disorders are closely related to the pathogenesis of IBS. Cytokines are a part of intestinal immune regulation. Intestinal inflammation leads to abdominal pain, which is closely related to the nuclear factor kappa-B signaling pathway in the immune response. BBH can inhibit intestinal mucosal inflammation in IBS rats by affecting the signal pathway, thus reducing intestinal motility and visceral hypersensitivity. Berberine can significantly reduce the levels of pro-inflammatory cytokines (TNF, IFN-γ, KC, IL-7) in colon tissue and improve the symptoms of intestinal injury and colitis model induced by dextran sulfate sodium in mice (Yan et al., 2012). Berberine can inhibit the immunoreaction caused by T helper (Th)1, Th2, and Th17 to improve the inflammatory symptoms of ulcerative colitis. On the other hand, it can reduce the expression of zonula occludens-1 protein related to tight junction (TJ) protein in the colon by inhibiting Th17 reaction (Li et al., 2016). TJ protein is a component of the epithelial barrier that prevents GI flora from affecting the environment of luminal epithelial tissue. These findings suggest that the therapeutic potential of berberine against IBD is partially realized by improving the intestinal microenvironment.

However, IBS is a functional bowel disease characterized by abdominal pain with changes in defecation habits or abnormal fecal characteristics. The degree of pain or discomfort in patients with IBS is related to the high sensitivity of the colon and rectum. Research on pain (Gold and Gebhart, 2010) suggests that continuous peripheral afferent stimulation is a necessary and sufficient condition for chronic pain, and cutting off afferent stimulation can effectively relieve pain and discomfort. One of the best examples is that local rectal anesthesia can quickly relieve abdominal pain, discomfort, and tenderness in IBS (Feng et al., 2012). Another experiment conducted on a human colon biopsy showed that intestinal mediators can induce increased visceral sensitivity (Balestra et al., 2012). We paid attention to this point in a previous study (Zhou R. T. et al., 2018). Tissue samples were taken from the antrum, duodenum, middle ileum, and colon of the modified stress-induced model rats, and then sectioned and stained chemically. The results showed that compared with the control group, no obvious pathological changes were observed in the tissue samples of the model, and organic lesions could be basically excluded. In the preparation of visceral hypersensitivity animal model, colonic injection of sodium butyrate solution (110 mg/ml) twice a day for 3 days resulted in non-inflammatory visceral hyperalgesia in rats (Lian et al., 2010). In addition, almost no GI inflammatory response were observed in IBS mice induced by *Trichinella spiralis* infection, but the intestinal hypersensitivity persisted (Wang et al., 2014). Therefore, the primary focus of our exploration in IBS-D rat model and the pharmacological mechanism of BBH is not inflammation, but the regulation of intestinal nervous system homeostasis.

GI dysfunction in IBS-D is closely associated with the colon (Kanazawa et al., 2008; Manabe et al., 2010). Our previous studies confirmed that the abnormal neurotransmission function in the IBS-D rat model occurs in the colon rather than in the jejunum. Increased defecation frequency is one of the diagnostic criteria that can distinguish IBS-D from the other IBS subtypes, and the abnormal GI motility function of IBS-D is often associated with high visceral sensitivity. The purpose of this work was to provide insights into the treatment effects and mechanisms of BBH in an IBS-D rat model. The major finding of this study is that chronic restraint stress results in a remarkable alteration in defecation frequency, visceral hypersensitivity, and intestinal neurotransmission in IBS-D rats. These stress-induced changes in intestinal function in rats, which indicate a homeostasis imbalance in the ENS, are similar to stress-induced changes in intestinal motility in humans. We found that BBH exerted a therapeutic effect on stress-induced IBS-D rats by inhibiting neurotransmission in colonic smooth muscle. The 4-h fecal area of the IBS-D group was remarkably different compared with that of the control group starting on the 14th day as shown in the Image-J analysis. The rats were then tested for behavioral responses to CRD. The behavioral effect of colon distention was recorded using a semiquantitative scale to measure AWR, which is a kind of unconscious motor reflex similar to visceral motor reflex (Al-Chaer et al., 2000), in response to a range of CRD intensities (20, 40, 60, and 80 mmHg). The results showed that the IBS-D group had substantially higher AWR scores compared with the control group. The changes were relevant for our modified rat model and indicated the successful establishment of the IBS-D rat model, which had increased defecation frequency and visceral hypersensitivity similar to those of humans, by chronic restraint stress. These changes in the model were also verified by the results of qRT-PCR analysis. The mRNA expression levels of neurotransmission-related transmitters or receptors, such as 5-HT_{3A}R, were increased, whereas that of nNOS was decreased in the IBS-D group compared with the control group. Meanwhile, BBH could reverse this outcome and showed an effect similar to that of positive drugs. Thus, the increased defecation frequency and visceral hypersensitivity in the IBS-D model induced by restraint stress may be related to changes in GI tract neurotransmission, which lead to the imbalance of ENS homeostasis. This mechanism is perhaps important in the physiology and pathology of IBS-D. Moreover, BBH could reduce abnormal defecation frequency and improve visceral hypersensitivity in IBS-D rats probably by changing the colonic neurotransmission in the IBS-D model.

NO produced by nNOS is an inhibitory small molecular neurotransmitter. The absence or severe deficiency of nNOS can lead to the failure or weakening of nitrergic neurotransmission. LC8 is not only a part of the dynamic protease complex, but also has the activity of nNOS inhibitor. When it binds to nNOS, it can make the structure of nNOS dimer unstable and inactive, thus affecting the transmission of nitrergic nerves (Jaffrey and Snyder, 1996; Chaudhury et al., 2008). The neurotransmission of purine is related to ATP, while the transport of nucleotides in cells cannot cross the cell membrane freely, and its storage and transshipment must be transmembrane by means of vesicle (Burnstock, 2014). The protein encoded by human or mouse SLC17A9 gene is a vesicular nucleotide transporter capable of carrying nucleotides such as ATP, ADP, and GTP, so the novel technique of SLC17A9 staining can specifically identify purinergic vesicles of intestinal neuronal varicosity (Larsson et al., 2012; Hiasa et al., 2014). Once released, the stored ATP and nucleotide molecules in the vesicle bind to specific P2X ion channel receptors or P2Y metabolic receptors on the surface of vesicular secretory cells or adjacent cells and produce biological effects (Burnstock and Novak, 2013).

The results of our study showed that LC8 and SLC17A9 mRNA expression in the model and control groups had no statistical difference, and BBH (25, 50, and 100 mg/kg) had no effect on LC8 and SLC17A9 mRNA expression in the GI tract of rats. We speculate that this may be related to other targets of BBH acting on IBS-D. LC8 can not only connect with nNOS, but also interact with Myosin Va. LC8 is a transporter that connects nNOS and Myosin Va (Chaudhury et al., 2011). Myosin is one of the three superfamily molecular motors in eukaryotes, which participates in a wide range of cellular physiological processes and functions, such as intracellular substance transport, transcriptional regulation, signal transduction, cell movement, and smooth muscle contraction. Myosin Va is the most widely distributed in the nervous system of humans and mice (Hammer and Wagner, 2013). In DBA mice with Myosin Va gene deficiency, the expression of Myosin Va in nerve endings was significantly decreased or deleted, resulting in decreased purinergic inhibitory junction potential and nitrergic inhibitory junction potential (Chaudhury et al., 2011). The *in situ* proximity ligation assay (PLA) used to study protein–protein interaction showed that SLC17A9 and Myosin Va were simultaneously expressed in the neuronal varicosity. In DBA mice, the PLA signals of SLC17A9 and Myosin Va were missing, and the exocytosis-entry rate of the varicosity membrane was low, suggesting that Myosin Va plays an important role in the transport of purinergic vesicles to the varicosity membrane and exocytosis (Chaudhury et al., 2012). Our team has carried out a series of studies on the role of Myosin Va in GI neurotransmission and the mechanism of IBS-D. Combined with the above analysis, we believe that the regulatory mechanism of BBH on neurotransmitter release-related proteins such as nNOS, LC8, and SLC17A9 may depend on the regulation of Myosin Va.

Our further research proved that the isolated colonic neurotransmission of IBS-D model induced by chronic restraint stress changed, and BBH exerted a treatment effect on the IBS-D model by blocking the neurotransmission of acetylcholine (ACh) and nitrergic agents and by inhibiting $P2Y_1$ receptor antagonist-induced contractile responses in the colonic smooth muscles of IBS-D rat.

The addition of TTX blocked the neurotransmission of ENS and remarkably decreased the contractile responses of colonic smooth muscles in the control and IBS-D groups compared with the non-TTX-treated group. TTX preincubated with BBH continued to inhibit these responses in normal rats but had no effect on the IBS-D group. It is possible that the responses of BBH found in the present study are TTX sensitive, showing that they are originated by neural-mediated activity. Therefore, what neurons and neurotransmitters are involved in this neural-mediated activity? We then made the following exploration.

Cholinergic neurons are not only the earliest species and ontogeny neurons, but also have the largest number in the ENS. As the main excitatory motor neurotransmitter, ACh has an excitatory effect on the GI tract. ACh receptors (AChRs) in the postsynaptic membrane convert the ACh signals released by motor neurons into endplate potentials to stimulate the action potential and contraction of muscle fibers. The main neurotransmitter in this reflex arch is ACh. The agonist of ACh, CCh (1×10^{-9} 1×10^{-5} mol/L), could considerably promote the contractile responses in colons of stress-induced IBS-D rats, which is consistent with published data (Guarino et al., 2017). Pretreatment with a single BBH concentration strongly inhibited the CCh-induced contractions in the IBS-D rat colon *in vitro*. Our study is the first to demonstrate that BBH exerts an inhibitory effect on CCh-induced colonic contraction. However, further research is necessary to determine whether this inhibitory effect against ACh operates on muscarinic receptors, nicotinic AChRs, or both.

L-NAME, a NOS inhibitor, can suppress the production of NO and inhibit the relaxation of smooth muscles and NANC relaxant responses to induce contraction. The present study (Suthamnatpong et al., 1993) reported that NO plays an important role in the relaxation of longitudinal muscle preparations from rat proximal colon. NO could activate calcium-activated and ATP-sensitive K^+ channels in human colon strips (Sahin et al., 2001). K^+ channels play an important role in the regulation of membrane excitability and tonus in various smooth muscle cells (Li et al., 1999). The activation of these channels causes smooth muscle membrane hyperpolarization, which leads to relaxation. By contrast, the inhibition of these channels produces membrane depolarization and smooth muscle contraction (Gil et al., 2010). The qRT-PCR analysis of mRNA levels showed that the expression of nNOS was decreased in stress-induced IBS-D rat colon compared with the control group, and BBH may upregulate the expression of nNOS in IBS-D. Stimulation with EFS *in vitro* showed that the colonic longitudinal muscles of IBS-D rats had higher contractile tension when incubated by L-NAME compared with the control group, and BBH could inhibit this contractile response. Overall, the obtained data indicated that the etiology of IBS-D may be

caused by reduced nNOS expression and NO release. Therefore, the inhibitory effect of BBH on contraction may be caused by the promotion of nitrergic neurotransmission in the colonic longitudinal muscle of IBS-D rats. These results suggest that BBH may increase the synthesis of NO in the colon of rats. *In vitro* experiments showed that BBH could exert a diastolic effect through the nitrergic neurotransmission of IBS-D, indicating that BBH may be an NO donor.

In addition, our research showed that BBH exerts significant effects on the treatment of diarrhea. Abnormal ion transport in the intestinal tract is closely related to the occurrence of diarrhea (Coté and Buchman, 2006). Secretory diarrhea refers to diarrhea caused by excessive secretion of water and electrolytes in intestinal mucosa crypts beyond the absorption capacity of intestinal villous epithelial cells (Schiller, 1999). Different types of ion channels are present on the intestinal cell membrane, such as sodium–potassium pump, calcium channel, chloride channel, and sodium–hydrogen exchanger. Changes in cellular second messengers such as cAMP, cGMP, and calcium-related transport channels; hormone secretion from endocrine tumors, bile salts, and long-chain fatty acids; and inflammatory mediators may stimulate mucosal cells and lead to secretory diarrhea (Field, 2003). cAMP can increase the probability of opening low conductance chloride channels and promote more chloride ion transport to the extracellular space; at the same time, cAMP can also enhance the activity of sodium and potassium dichloride cotransporter in the basement membrane and promote more sodium and potassium pump and potassium channel plasma transporters to enter the top membrane of intestinal epithelial cells, resulting in diarrhea caused by the destruction of electrolyte absorption balance of intestinal epithelial cells (Tabcharani et al., 1991). The opening of cAMP-activated potassium channels on the basement membrane can promote the repolarization of cells, counteract the depolarization effect caused by the opening of chloride channels in the basement membrane, and make chloride ions secrete into the intestinal cavity. The increase of chloride ion content promotes the secretion of intestinal electrolytes, leading to diarrhea (Kunzelmann et al., 2001). Studies on the antisecretory effects of berberine in rat ileum have been reported, and the alkaloid berberine may inhibit intestinal ion secretion and mucosal adenylate cyclase and Na-K-ATPase activities *in vitro* (Tai et al., 1981). Evidence provided in a previous study (Taylor et al., 1999) shows that secretory diarrhea is also modulated by the ENS, and berberine inhibits ion transport in human colonic epithelia. Data suggest the inhibition of basolateral K^+ conductance on epithelial cells as the mechanism of action of this antidiarrheal drug. However, further studies are needed to confirm whether its antidiarrheal mechanism is related to its inhibition of intestinal secretion in IBS-D rats.

Purinergic neurotransmitters are responsible for NANC inhibitory responses in the GI tract. MRS2500 is considered the most potent $P2Y_1$ antagonist and has been proven to be inactive on other purine receptors such as P2X, $P2Y_{12}$, and $P2Y_{13}$. α,β-MeATP is an unselective P2X receptor agonist (Alexander et al., 1999). A study indicated that α,β-MeATP, a stable analog of ATP, mimics endogenous purinergic mediator and causes the inhibition of spontaneous contractions in human and rat colons (Martínez-Cutillas et al., 2014). Our study showed that α,β-MeATP could inhibit the spontaneous contraction of colonic smooth muscle in the control and IBS-D groups in the absence of EFS induction. This relaxing effect on the colon could be antagonized by BBH. In addition, the inhibitory effect of α,β-MeATP on colonic spontaneous contraction could be completely antagonized by the addition of MRS2500. This is consistent with the results of previous studies (Martínez-Cutillas et al., 2014). The spontaneous contractile responses of colonic smooth muscle were restored in the control and IBS-D groups by BBH, and the effect of MRS2500 in the control group could be inhibited by BBH (had no effect on the IBS-D group). Interestingly, MRS2500 produced a greater contractile effect than EFS induction, and BBH inhibited these responses but only in the IBS-D group. These results indicated that $P2Y_1$ receptors participate in the regulation of ENS and the coordination of intestinal movement in rats and play an important role in colonic motility disorders in IBS-D rats. Therefore, BBH acts on the $P2Y_1$ receptor to treat IBS-D. Thus, the mechanism of BBH action was mediated by purinergic neurotransmission.

In general, the differences in the colon of IBS-D rat model from that of normal animal were as follows: 1) The mRNA expression of 5-HT receptors was activated, whereas that of nNOS was decreased. 2) Cholinergic receptors were activated, and colon contraction was promoted. 3) NOS was inhibited, NO release was decreased, and diastolic effect was inhibited. 4) $P2Y_1$ receptor was activated and impeded the diastolic effect. The GI tract contractile responses of IBS-D rats can be improved by BBH by inhibiting the effect exerted by cholinergic, nitrergic, and purinergic neurotransmitters. In this work, neuropharmacological studies using GI tissue samples may help develop novel agents that target receptors that control smooth muscle contractility. However, additional studies are still needed to confirm the potential pharmacological target protein for BBH in the treatment of IBS-D.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine. Guangzhou University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

YL drafted the manuscript and contributed to data analysis and figure preparation. JH participated in the design of the study, performed the experiments and statistical analysis. HC and BT participated in the design of the research and reviewed the manuscript. ZH assisted in the performance and help in reviewing the manuscript. YZ assisted in the performance and the recording of experiments. WY, TZ, ZW, and LL assisted in the performance. All the authors have read and approved the submission of the manuscript.

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