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EDITORIAL

Toward less invasive coloproctology: The future is out there

Sameh Hany Emile, Jonathan Ragheb

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Abstract

Medical care has undergone remarkable improvements over the past few decades. One of the most important innovative breakthroughs in modern medicine is the advent of minimally and less invasive treatments. The trend towards employing less invasive treatment has been vividly shown in the field of gastroenterology, particularly coloproctology. Parallel to foregut interventions, colorectal surgery has shifted towards a minimally invasive approach. Coloproctology, including both medical and surgical management of colorectal diseases, has undergone a remarkable paradigm shift. The treatment of both benign and malignant colorectal conditions has gradually transitioned towards more conservative and less invasive approaches. An interesting paradigm shift was the trend to avoid the need for radical resection of rectal cancer altogether in patients who showed complete response to neoadjuvant treatment. The trend of adopting less invasive approaches to treat various colorectal conditions does not seem to be stopping soon as further research on novel, more effective and safer methods is ongoing.

Key Words: Toward; Less invasive; Minimally invasive; Coloproctology; Future; Colorectal surgery

Core Tip: One of the most important innovative breakthroughs in modern medicine is the advent of minimally and less invasive treatments. Coloproctology has undergone a remarkable paradigm shift as the treatment of benign and malignant colorectal conditions has gradually transitioned towards less invasive approaches. An important paradigm shift was the trend to avoid the need for radical resection of rectal cancer altogether in patients who showed complete response to neoadjuvant treatment. Another example is the trend toward non-operative management of inflammatory bowel disease and benign anorectal disorders.

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INTRODUCTION

Medical care has undergone remarkable improvements over the past few decades. One of the most important innovative breakthroughs in modern medicine is the advent of minimally and less invasive treatments. The notion that sometimes "less is more" has made clinical researchers across the world contemplate that adequate treatment of a medical or surgical condition should not necessarily be invasive. The concept of "Less is More medicine" was introduced more than a decade ago to address the unfounded presumption that providing more care is always better, as the overuse of medical care may indeed be associated with risks and harm[1].

LESS INVASIVE GASTROENTEROLOGY

The trend towards employing less invasive treatment has been vividly shown in the field of Gastroenterology. Since the first days of rigid gastrointestinal endoscopy in the 1800's until the present time, gastroenterology has evolved into a minimally invasive specialty of its own. Early gastroenterology began as a primarily diagnostic field to support surgical decision-making, which changed with the advent of Adolf Kussmaul's rigid endoscope in 1868[2]. For the first time, endoscopic tools such as biopsy forceps could be used for tissue diagnosis as well as therapeutically for relieving food impactions. Decades later, surgeries like Heller's myotomy or sleeve gastrectomy would meet their endoscopic counterparts, like per oral endoscopic myotomy and endoscopic sleeve gastroplasty.

One of the famous examples of treatment paradigm shift is the management of peptic ulcers. For several decades, selective and highly selective vagotomy was the standard of care for peptic ulcers. While effective in healing peptic ulcers, vagotomy was recognized to be a technically demanding and challenging procedure with potentially significant morbidity, particularly when it is combined with anterectomy[3]. These limitations motivated researchers to search for other equally effective yet less invasive treatments and thus proton pump inhibitors (PPIs) were developed. PPIs proved effective in the treatment of peptic ulcers with a well-tolerated safety profile[4], becoming the standard of care for peptic ulcers and replacing vagotomy which is now indicated in a select group of patients with refractory disease[3].

LESS INVASIVE COLOPROCTOLOGY

Parallel to foregut interventions, colorectal surgery has shifted towards a minimally invasive approach. Coloproctology, including both medical and surgical management of colorectal diseases, has undergone a remarkable paradigm shift. The treatment of both benign and malignant colorectal conditions has gradually transitioned towards more conservative and less invasive approaches. In particular, patients with major colorectal diseases, including colorectal cancer and inflammatory bowel disease (IBD), have benefited from the "less is more" treatment concept.

LESS INVASIVE TREATMENT OF IBD

Crohn's disease is one of the most challenging conditions to treat. Nonetheless, collective evidence has shown that early medical treatment with biological agents may reduce the need for surgery by 37%[5]. In fact, advances in medical management of Crohn's disease have led to a significant drop in the cumulative incidence of first abdominal surgery performed within five years of diagnosis from 54.8% in 1990-1995 to 17.3% in 2009-2014[6]. Similarly, it has been estimated that most patients with ulcerative colitis will be able to avoid surgery, virtue of the increasing efficacy of modern medical treatment[7]. A study spanning a period of 13 years showed a significant decrease in the incidence of colectomy performed for ulcerative colitis from 36.08/1000 patients/year before the introduction of biologic therapy to 29.99/1000 in the biologic treatment era[8]. Furthermore, minimally invasive interventions such as endoscopic stricturoplasty can be

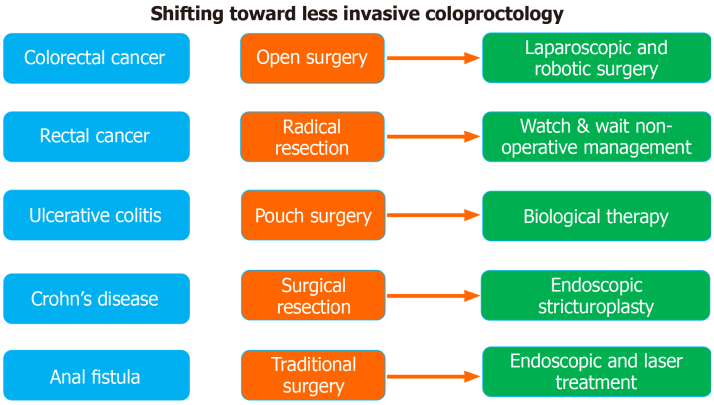

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Figure 1 Examples of the shift toward less invasive coloproctology.

considered for Crohn's disease-related strictures instead of conventional surgery[9].

LESS INVASIVE TREATMENT OF COLORECTAL CANCER

Treatment of colorectal cancer has exhibited an important shift toward less invasive management overall and specifically less invasive surgery. Surgical resection of colorectal neoplasms using a laparoscopic or robotic-assisted approach has been increasingly adopted in many hospitals in the world[10]. Minimally invasive surgery for colorectal cancer has provided tangible short-term benefits, including smaller incisions, less pain, faster recovery, and less wound-related complications, yet without compromising the oncologic outcomes[11]. Moreover, rather than doing partial colectomies for low-grade malignant polyps, endoscopic submucosal dissection or full-thickness resection can be alternatively and safely performed[12].

An even more interesting paradigm shift was the trend to avoid the need for radical resection of rectal cancer altogether in patients who showed complete response to neoadjuvant treatment. Habr-Gama *et al*[13] introduced the concept of watch-and-wait non-operative management for rectal cancer in 2015. With the recent advances in neoadjuvant treatments, organ-sparing treatment of rectal cancer has become an option for several patients who otherwise were deemed indicated for radical proctectomy. The introduction of total neoadjuvant therapy (TNT) has further expanded the scope of non-operative treatment of rectal cancer as TNT was associated with more than twice the odds of achieving a complete response as compared to standard treatment. With the use of TNT, approximately 30% of patients with rectal cancer may have the chance to avoid radical surgery and be treated non-operatively[14].

LESS INVASIVE TREATMENT OF ANORECTAL CONDITIONS

Akin to colorectal cancer and IBD, coloproctologists have started to adopt less invasive approaches for benign and frequently diagnosed colorectal conditions such as hemorrhoids, anal fistulas, and pilonidal sinus disease. Although excisional hemorrhoidectomy is considered the standard of care for grade III-IV hemorrhoidal disease owing to its effectiveness, adverse effects namely severe postoperative pain are challenging and sometimes dissuade patients from receiving treatment[15]. These limitations have led to the development of less invasive techniques such as Doppler-guided hemorrhoidal artery ligation[16] and hemorrhoid laser dearterialization[17] which conferred satisfactory results with acceptably low recurrence rates and less postoperative pain compared to excisional hemorrhoidectomy. Similarly, minimally invasive options were devised for complex anal fistulas in an attempt to achieve healing and preserve the anal sphincter muscles. These techniques included video-assisted anal fistula treatment, fistula laser therapy, and stem cell treatment[18-20]. Figure 1 illustrates different examples of the shift toward less invasive coloproctology.

IMPACT OF MINIMALLY INVASIVE TREATMENTS ON QUALITY OF LIFE

The impact of less invasive treatment approaches on the quality of life of the patients with colorectal diseases has been explored in the literature. Compared to the more invasive open surgery, minimally invasive colorectal resections are associated with better cosmetic outcomes and greater patient satisfaction[21]. Laparoscopic resection of colorectal cancer is also associated with shorter hospital stays and higher quality of life scores than open resection on the short term[22]. Similarly, the adoption of a non-operative management approach may confer better quality of life. In a matched-controlled study, Hupkens *et al*[23] found that watch-and-wait strategy for rectal cancer conferred better quality of life than did radical resection in terms of physical and cognitive function, physical and emotional roles, and global health

status. Non-operative treatment was also associated with fewer defecation, sexual, and urinary functional adverse events. Also, minimally invasive treatment of benign anal conditions may confer better quality of life than conventional treatments. A randomized controlled trial showed that laser hemorrhoidoplasty was followed by higher scores of 36-item Short Form Health Survey questionnaire at six months than Milligan and Morgan hemorrhoidectomy[24].

CONCLUSION

The trend of adopting less invasive approaches to treat various colorectal conditions does not seem to be stopping soon as further research on novel, more effective, and safer methods is ongoing. Perhaps this could be considered an overly optimistic prediction, but the authors of this article anticipate that in the near future, most cases of colorectal cancer and IBD will be subject to non-operative, minimally invasive treatment. However, only time will tell if this prediction proves valid.

FOOTNOTES

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OPINION REVIEW

Garg incontinence scores: New scoring system on the horizon to evaluate fecal incontinence. Will it make a difference?

Petr Tsarkov, Inna Tulina, Parvez Sheikh, Darya D Shlyk, Pankaj Garg

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Abstract

The main aim of this opinion review is to comment on the recent article published by Garg *et al* in the *World Journal of Gastroenterology* 2023; 29: 4593–4603. The authors in the published article developed a new scoring system, Garg incontinence scores (GIS), for fecal incontinence (FI). FI is a chronic debilitating disease that has a severe negative impact on the quality of life of the patients. Rome IV criteria define FI as multiple episodes of solid or liquid stool passed into the clothes at least twice a month. The associated social stigmatization often leads to significant under-reporting of the condition, which further impairs management. An important point is that the complexity and vagueness of the disease make it difficult for the patients to properly define and report the magnitude of the problem to their physicians. Due to this, the management becomes even more difficult. This issue is resolved up to a considerable extent by a scoring questionnaire. There were several scoring systems in use for the last three decades. The prominent of them were the Cleveland Clinic scoring system or the Wexner scoring system, St. Marks Hospital or Vaizey's scores, and the FI severity index. However, there were several shortcomings in these scoring systems. In the opinion review, we tried to analyze the strength of GIS and compare it to the existing scoring systems. The main pitfalls in the existing scoring systems were that most of them gave equal weightage to different types of FI (solid, liquid, flatus, *etc.*), were not comprehensive, and took only the surgeon's perception of FI into view. In GIS, almost all shortcomings of previous scoring systems had been addressed: different weights were assigned to different types of FI by a robust

statistical methodology; the scoring system was made comprehensive by including all types of FI that were previously omitted (urge, stress and mucus FI) and gave priority to patients' rather than the physicians' perceptions while developing the scoring system. Due to this, GIS indeed looked like a paradigm shift in the evaluation of FI. However, it is too early to conclude this, as GIS needs to be validated for accuracy and simplicity in future studies.

Key Words: Fecal incontinence; Scoring system; Urge; Stress; Flatus

Core tip: Several scoring systems were used to assess fecal incontinence (FI), among which the most commonly used were Wexner's, Vaizey's, and FI Severity Index scoring systems. However, there are major lacunae and shortcomings in these scoring systems. A new scoring system, Garg incontinence scores (GIS), attempted to sort out the lacunae in the existing scoring systems. In the commentary, we analyzed the GIS while comparing it to the existing scoring systems. GIS seemed to be a major improvement over the existing scoring systems as almost all shortcomings of previous scoring systems have been addressed. However, this needs to be validated in further studies.

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INTRODUCTION

A common definition of fecal incontinence (FI) is "involuntary expulsion of rectal contents (liquid or solid feces or flatus) through the anus and the inability to defer a bowel movement for at least 15 minutes". The symptoms should have been present for a duration of ≥ 1 mo, and the patient's age should be ≥ 4 years, with previously achieved control[1]. In 2006, the Rome Foundation laid down diagnostic criteria for FI, and these were called Rome III criteria. These were subsequently revised in 2016 and were then known as Rome IV criteria[2]. In both these criteria, FI is defined as the accidental passage of liquid or stool into the clothes on several occasions. Incidentally, the involuntary or loss of control over flatus was not made a part of these criteria[2]. For Rome III criteria, at least one FI event per month is required for definition of FI, but for Rome IV, it was modified to at least two episodes of FI per month.

FI is a common problem, and it is estimated that this problem afflicts 1%–15% of the population worldwide[2-8]. The common causes are tears/trauma to the perineal region after difficult and problematic deliveries, after colorectal surgery, especially anal fistula surgery, after radiotherapy to the lower abdomen, *etc.*

The prevalence of FI in inflammatory bowel disease (IBD) is high, as recent studies have shown that FI can occur in up to 21% (as per Rome IV criteria) of patients with ulcerative colitis[2]. The incontinence rates remained high even when the patients were in remission, and understandably, this has led to a lot of anxiety, psychological disturbances, increases in symptoms, and poorer quality of life[2]. The incontinence rates in IBD are about 12 times higher than the prevalence rates in the wider population[9]. The risk of FI increases significantly in parous women with IBD[10].

FI due to gynecological trauma (traumatic vaginal birth) can occur in up to 8% of women[8]. The perineal tears involving the external anal sphincter (EAS) (3rd degree) and the tears extending through the EAS (4th degree) are one of the commonest risk factors for FI[11]. The risk of incontinence is also high (1.5 times higher) for instrument-assisted deliveries[12]. Incidentally, the symptoms often do not manifest until several years after the injury, and various factors such as hormonal changes during menopause, accelerated aging of traumatically damaged sphincter muscles, or decompensation of compensatory mechanisms probably contribute to this delay[1]. In primiparous women, it is possible to prove occult or at least minimal sphincter injuries in ~35% of cases[8,13]. The delivery with utilization of forceps, the occipital–posterior position of the child, and prolonged delivery represent independent risk factors for subsequent FI[8,13]. It is estimated that ~13% of women experience varying degrees of incontinence or stool urgency after first delivery [14]. As these are mostly young women, the impact of incontinence on their quality of life is substantial[15].

Loose stools are commonly ignored but pose a major risk of FI[16]. In this type of FI, the stoppage of drugs such as laxatives can be curative. In some patients, special diets such as low fructose or lactose can also decrease the frequency of loose stools and help to maintain normal stool form. Psyllium husk has been shown to improve FI in clinical trials; a feat that no other fiber supplements like carboxymethylcellulose or gum arabic can achieve[17]. Medications can also cause or aggravate FI. These drugs are laxatives, such as lactulose, docusate, or bisacodyl; cancer medications, such as cyclophosphamide, 5-fluorouracil, or paclitaxel; antibiotics, such as cephalosporins, penicillins, macrolides or Amphotericin B-liposomal; antacids that contain magnesium, arsenic trioxide, orlistat, quetiapine, rivastigmine, donepezil, sweeteners and caffeine[18].

Understandably, FI leads to profound physical, emotional and mental issues and even social isolation in many patients. Incidentally, the clinical objective evaluation and satisfactory management of FI have not been developed so far, and a significant amount of work still needs to be done in this difficult field.

The individual indices should be compared with functional tests to demonstrate FI. There are various tests that can help in the evaluation of FI.

Anorectal manometry helps in anorectal physiology testing, which can give insight and objectively document pelvic floor function[19]. The manometry can be inconsistent with physical examination and is incapable of predicting the response to the therapy, but the manometric evaluation can be of significant help in guiding the therapy[19]. It is not necessary that the anal tone is low in incontinence patients. In some patients with FI, the anal tone may be high or normal; for example, when an incomplete evacuation or anismus is present[19]. In FI patients with constipation, the rectum may be hyposensitive[20], whereas, in FI patients with IBS, post-radiation, diarrhea, and urgency, the rectum may be hypersensitive. In diseases such as IBD, autoimmune disorders like scleroderma, or post-radiation, rectal compliance may be decreased[20].

Endoanal ultrasound (EAUS) is helpful for assessing the integrity of both the sphincters objectively and can help detect their injuries[21]. The EAUS is economical, available easily to surgeons, and demonstrates the sphincters well, especially the internal sphincter. Magnetic resonance imaging (MRI) is a good alternative method for imaging the deeper parts of the sphincter complex and assessing associated rectal and pelvic prolapse[21].

The testing of the neurophysiology of the anorectum can be done with electromyography (EMG) and pudendal nerve terminal motor latency (PNTML) testing[21]. EMG can help to identify the defects in the anal sphincters and associated nerve injury[21]. The evaluation and assessment of neuromuscular integrity between the anal sphincter and the pudendal nerve can be done through PNTML. However, as both these techniques are invasive, they are not commonly used[21].

Defecography, with or without fluoroscopy or MRI, can help in the assessment of defecation in a dynamic motion. In incontinence patients, this test helps in confirming the inability to hold stool, which can give insight into the severity of the problem, and further recognize malfunctioning evacuation and/or associated prolapse of pelvic organs contributing to FI[21].

Last but not least, the lower gastrointestinal endoscopy (colonoscopy or sigmoidoscopy) may be indicated in patients with FI who have suggestive symptoms. Endoscopy can help to rule out diseases like IBD and malignancy in FI patients [21].

In spite of all the diagnostic tests available, the clinical assessment of FI is the initial step in the management. As FI can be of several types like solid stool FI, flatus (gas), liquid, urge, *etc*., it is pertinent to clinically evaluate the disease with maximum objectivity. To achieve this goal, many scoring systems have been published in the last 35 years[22-24]. The first scoring system that was published and subsequently became popular was the Cleveland Clinic or Wexner scoring system[22]. It was published in 1993 by Jorge and Wexner[22] (Table 1). Subsequently, the next one was published in 1999 by Vaizey *et al*[23], and it was widely cited as St. Marks Hospital or Vaizey's[23] scores (Table 2). After this, a few more scoring methods were published, but none of them became popular. The only one among them that was more relevant was the FI Severity Index (FISI) published by Rockwood *et al*[24] in 1999 (Table 3)[24]. After a gap of two decades, a new scoring system to assess FI has been recently published by Garg *et al*[25] and Armstrong *et al*[26] (Table 4).

The Wexner scores were developed and published in 1993 and became popular. Even after the development of simple, easy-to-use scores, why was a need felt for the development of other scoring systems, such as Vaizey and FISI?[22] Moreover, the Vaizey scores also rose in popularity to almost the same magnitude as the Wexner scores. The reasons could be that there were shortcomings in the Wexner scoring system that the Vaizey scores attempted to improve upon. So that brings us to the question: when Wexner and Vaizey scores were popular, was a new score, GIS, really needed now? If yes, has GIS added substantially to the clinical evaluation of FI?

Wexner's scores included three types of FI, solid, liquid and flatus[22], and Vaizey's scores added another type of incontinence which was urge FI (inability to defer bowel motion/defecation for at least 15 min). This addition was a valuable enhancement as urge FI is a different type of FI and is distinct from solid, liquid or flatus incontinence, and is known to occur in isolation in several patients. Along with this, Vaizey scores also included a column of "need to take constipating medicines"; it was not present in the Wexner scoring system[23]. Apart from this, Vaizey's scores were similar to Wexner's. The Vaizey and Wexner scoring systems have been widely cited and have become popular in recent decades[27]. The strong points of both scoring systems have been the ease of use and understanding[27]. However, there were a few lacunae in both these scores, which have been pointed out and highlighted by Garg *et al*[25] and perhaps corrected too. Both these scores give equal weighting (hence scores) to different types of FI (solid, liquid, flatus and urge) [22,23]. Expectedly, this was done for the sake of simplicity, but from the statistical point of view, this was a gross error. The different types of FI are a full spectrum, and it would be unscientific to give equal weighting to all types. We are in agreement with Garg *et al*[25] that ease-of-use is an important ingredient of a scoring system, but it should not be at the cost of scientific accuracy. An optimum balance has to be maintained between the two. The systems should be easy to use and convenient, but the scientific quotient and statistical accuracy cannot be sacrificed. FISI score perhaps failed to become popular as it was on the opposite extreme[24]. It became too complicated to be utilized by practicing physicians as it tried to assign different weights to different types of incontinence[24]. Moreover, this scoring system had short-comings in its research methodology. The sample size was too small, the questionnaire was not filled by the respondents physically but was sent to them by email, filling the same scores in different cells was not permitted while assigning weights, *etc*.[24].

Therefore, to summarize, on one extreme are scoring systems (like Wexner and Vaizey) that are easy to use but not scientifically sound, and on the other extreme is a scoring system (FISI) that lost its simplicity while upgrading scientific and statistical soundness[24]. For this matter, GIS manages to strike the balance of scientific accuracy and simplicity[25]. Garg *et al*[25] utilized robust statistical techniques, such as the interviewee and interviewer were both blinded, the sample

Table 1 Wexner scoring[25]

	Never	Rarely	Sometimes	Usually	Always
Solid	0	1	2	3	4
Liquid	0	1	2	3	4
Gas	0	1	2	3	4
Wears a pad	0	1	2	3	4
Lifestyle alteration	0	1	2	3	4

Rarely: < 1/mo; sometimes: < 1/wk to ≥ 1/mo; usually: < 1/d to ≥ 1/wk; always: ≥ 1/d. Citation: Garg P, Sudol-Szopinska I, Kolodziejczak M, Bhattacharya K, Kaur G. New objective scoring system to clinically assess fecal incontinence. *World J Gastroenterol* 2023; 29: 4593-4603.

Table 2 Vaizey's scoring[25]

	Never	Rarely	Sometimes	Weekly	Daily
Solid stool incontinence	0	1	2	3	4
Liquid stool incontinence	0	1	2	3	4
Gas incontinence	0	1	2	3	4
Alteration in lifestyle	0	1	2	3	4
	No	Yes			
Need to wear a pad or plug	0	2			
Constipating medication	0	2			
Lack of ability to defer defecation for 15 min	0	4			

Never: No episodes in last 4 wk; rarely: 1 episode in last 4 wk; sometimes: ≥ 1 in last 4 wk but < 1/wk; weekly: ≥ 1/wk to < 1/d; always: ≥ 1/d. Citation: Garg P, Sudol-Szopinska I, Kolodziejczak M, Bhattacharya K, Kaur G. New objective scoring system to clinically assess fecal incontinence. *World J Gastroenterol* 2023; 29: 4593-4603.

Table 3 Fecal incontinence severity index scoring[25]

	≥ 2 times/d (patient/surgeon scores)	Once/d (patient/surgeon scores)	≥ 2 times/wk (patient/surgeon scores)	Once/wk (patient/surgeon)	1-3 times/mo (patient/surgeon scores)
Gas	12/9	11/8	8/6	6/4	4/2
Mucous	12/11	10/9	7/7	5/7	3/5
Liquid	19/18	17/16	13/14	10/13	8/10
Solid	18/19	16/17	13/16	10/14	8/11

Citation: Garg P, Sudol-Szopinska I, Kolodziejczak M, Bhattacharya K, Kaur G. New objective scoring system to clinically assess fecal incontinence. *World J Gastroenterol* 2023; 29: 4593-4603.

size was bigger, an upgraded EuroQol (EQ-5D+) descriptive system-4D3L was utilized, all the proforma were filled by the same interviewer physically (not through email or telephone), *etc*[25]. The weight calculation was also done by an appropriate statistical method[25].

The GIS has another improvement over the earlier scoring systems like Wexner's and Vaizey's. Unlike them, the GIS gave importance to the patients' and laypersons' perceptions rather than the surgeons' perceptions[25]. It is a significant improvement because the scoring system has to be from the patients' point of view when it is being developed for them. It is possible that the earlier scoring systems (Vaizey's and Wexner's) presumed that the patients' and surgeons' perceptions would be similar. However, Garg *et al*[25] and other studies[24] clearly demonstrated that there could be significant differences between the patients' and surgeons' perceptions regarding the different types of incontinence.

Table 4 Garg incontinence scores[25]

Incontinence type	Weight	Frequency			Maximum score
		Never (points)	Occasional (points) (≤ 1 episode/wk)	Common (points) (> 1 episode/wk)	
Solid	8	0	1	2	16
Liquid	8	0	1	2	16
Urge	7	0	1	2	14
Flatus	6	0	1	2	12
Mucus	6	0	1	2	12
Stress	5	0	1	2	10
Total					80

Score in a cell = Weight for that incontinence type × frequency points. For example, a person with occasional liquid incontinence would have an 8 × 1 = 8 score. Maximum possible score = 80 (total incontinence), minimum score possible = 0 (no incontinence). Citation: Garg P, Sudol-Szopinska I, Kolodziejczak M, Bhattacharya K, Kaur G. New objective scoring system to clinically assess fecal incontinence. *World J Gastroenterol* 2023; 29: 4593-4603.

Table 5 Comparison of existing scoring systems with new scoring system[25]

	Wexner	Vaizey	FISI	GIS
Comprehensive	No	No	No	Yes
FI type included: Urge FI	No	Yes	No	Yes
FI type included: Mucous FI	No	No	Yes	Yes
Presence of confounding parameters like "Need to wear a pad", "Need to take constipating medicine", and "Alteration of lifestyle"	Yes	Yes	No	No
Assigning weights to each FI by an objective method	No	No	No	Yes
Inclusion of patient perceptions (*n*)	0	0	34	50
Inclusion of laypersons' perceptions (*n*)	0	0	0	50
Simple and easy to use	+++++	+++++	+	+++++
Detailed structured definitions	No	No	No	Yes
In-depth disability scores based on an objective description system	No	No	No	4D3L [modified EQ-5D+ (EuroQol)] used

FI: Fecal incontinence; FISI: Fecal Incontinence Severity Index; NSS: New scoring system. Citation: Garg P, Sudol-Szopinska I, Kolodziejczak M, Bhattacharya K, Kaur G. New objective scoring system to clinically assess fecal incontinence. *World J Gastroenterol* 2023; 29: 4593-4603.

Therefore, basing the scoring system on laypersons' and patients' perceptions added to the scientific quotient of GIS.

Last but not least, the GIS is the most comprehensive as it includes the incontinence types such as mucus, urge, and stress FI, which were omitted by all previous scoring systems. The authors of the published study compared different scoring systems (Wexner's, Vaizey's and Garg's) in a table that is being reproduced here (Table 5).

CONCLUSION

So, it seems that the GIS is a major improvement over the existing scoring systems, as almost all shortcomings of previous scores have been addressed. Due to this, GIS indeed looks like a paradigm shift. However, it is too early to conclude this. GIS has not been validated in a published study[25], which the authors stated that they would do in the next phase[25]. Only when this new scoring system is utilized, validated, and its efficacy corroborated by clinicians across the world will it be considered a benchmark in objective clinical assessment of FI.

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FOOTNOTES

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REVIEW

Why is early detection of colon cancer still not possible in 2023?

Valeria Tonini, Manuel Zanni

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Abstract

Colorectal cancer (CRC) screening is a fundamental tool in the prevention and early detection of one of the most prevalent and lethal cancers. Over the years, screening, particularly in those settings where it is well organized, has succeeded in reducing the incidence of colon and rectal cancer and improving the prognosis related to them. Despite considerable advancements in screening technologies and strategies, the effectiveness of CRC screening programs remains less than optimal. This paper examined the multifaceted reasons behind the persistent lack of effectiveness in CRC screening initiatives. Through a critical analysis of current methodologies, technological limitations, patient-related factors, and systemic challenges, we elucidated the complex interplay that hampers the successful reduction of CRC morbidity and mortality rates. While acknowledging the advancements that have improved aspects of screening, we emphasized the necessity of addressing the identified barriers comprehensively. This study aimed to raise awareness of how important CRC screening is in reducing costs for this disease. Screening and early diagnosis are not only important in improving the prognosis of patients with CRC but can lead to an important reduction in the cost of treating a disease that is often diagnosed at an advanced stage. Spending more sooner can mean saving money later.

Key Words: Colorectal cancer; Colorectal cancer screening; Colorectal screening test; Colon and rectal cancer

Core Tip: Colorectal cancer (CRC) screening is a fundamental tool in the prevention and early detection of a prevalent and lethal cancers. Despite advancements in screening, the effectiveness of CRC screening programs remains less than optimal. This paper examined the multifaceted reasons behind the persistent lack of effectiveness in CRC screening initiatives. This study aimed to raise awareness of how CRC screening can reduce costs. Screening and early detection improve the prognosis of patients with CRC and result in an important reduction in the cost of treating advanced disease. Spending more sooner can mean saving money later.

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INTRODUCTION

From the latest reports of the National Cancer Institute, the number of "cancer survivors" is soaring, and projections are alarming. This phenomenon is due to the natural increase in population numbers, amplified by the lengthening of the average life span and to improved treatments that allow increasing survival for cancer patients.

As of January 2022, it was estimated that there were 18.1 million cancer survivors in the United States. This represents approximately 5.4% of the population. The number of cancer survivors is projected to increase by 24.4%, to 22.5 million, by 2032 and to 26.0 million by 2040. Over the next decade, the number of people who have lived 5 or more years after their cancer diagnosis is projected to increase approximately 30% to 16.3 million. Most (67%) survivors are currently age 65 or older. It is estimated that by 2040 74% of cancer survivors in the United States will be 65 or older.

In the light of these data, our first goal has been to treat patients with cancer and then to seek more effective and often more expensive treatments to achieve a patient's cure or otherwise increase survival. However, if cancer survivors are increasing day by day, how are we going to take care of this growing volume of patients in need of treatment in the future? What strategies should we adopt to deal with this problem? Obviously, the first measure is undoubtedly to implement information campaigns on anti-cancer lifestyles and to put in place screening programs for early detection of the disease. It is intuitive that cancer costs less when diagnosed at an early stage, thus limiting expenses to surgery, length of stay, and follow-up. If it is diagnosed at a more advanced stage, costs will increase in an attempt to keep the disease under control for as long as possible.

The cancers we will have to address first will obviously be the most frequent ones, and among them is colorectal cancer (CRC). CRC is the third most common cancer in males and the second most common in females worldwide[1] and accounts for 10% of the total cancer burden[2]. Globally, nearly 2 million new cases of CRC (including anus) and more than 900000 deaths occur each year[3]. Incidence rates are approximately 4-fold higher in transitioned countries compared with transitioning countries, but there is less variation in the mortality rates because of higher fatality in transitioning countries[3]. The highest incidence rates of CRC are observed in European regions, Australia/New Zealand, and North America[3,4]. Lifetime risk of CRC is similar between females and males, 4.1% and 4.4%, respectively[5]. The dominant risk factor for CRC is age. Age-specific incidence and mortality increase dramatically over a lifetime, from 6 and 1 per 100000 people aged 30-34 years to 228 and 105 per 100000 people aged 80-84 years, respectively[5,6]. In 2021, Fang *et al*[7] performed an analysis of the clinical characteristics of CRC in the Chinese population (cohort of 13328 patients) and found that 58.1% of CRC cases were observed in individuals over 60-years-old. According to an even more recent study of the Chinese population, age > 65 years is a significant risk factor for developing CRC with an odds ratio of 1.4[8]. The 5-year survival rate for stage I colon cancer is 91% but drops to 72% for locally advanced disease and 14% for stage IV[4].

CRC occurs sporadically in 65%-70% of CRC cases, while the remaining 30%-35% of cases are genetic or familial forms, which should be recognized as early as possible and included in a close follow-up program. Polyps are considered precancerous lesions. About two-thirds of CRC cases develop through the adenomacarcinoma sequence, while the remaining one-third of CRC cases originate from the serrated pathway[9]. The neoplastic degeneration of a colorectal polyp to CRC occurs over a very long period, and we would therefore have a lot of time to recognize this polyp early and remove it before it becomes cancer. Resection of the polyp in CRC screening reduces the incidence and mortality of cancer [9,10]. In any case, we could still remove the cancer at a very early stage of the disease. Therefore, we are facing a disease that could be prevented by a simple endoscopy and instead bring an exaggerated number of new cases and deaths every year. Unfortunately, even in the most advanced countries and in those where screening programs are active, diagnosis is often late when the cancer is already in an advanced stage. As reported in the latest guidelines of the American Society of Colorectal Surgery[11], the diagnosis of CRC is made in 70% of cases when the patient is already symptomatic, often with symptoms such as hemorrhage or occlusion that require emergency surgery.

CRC incidence and mortality have declined over time (Figure 1) due to improvements in exposure to risk factors, treatment of diagnosed CRC, and widespread uptake of screening[5]. The observed trend correlates with an increase in the proportion of eligible individuals upgrading with screening[12]. From 2000 to 2018, CRC incidence and mortality decreased from 56 and 20 to 37 and 13 per 100000, respectively[13] while the proportion of individuals aged 50 years to 75 years who are up-to-date with screening increased from 34.6%[14] to 67.0%[15]. The benefit of colorectal screening in

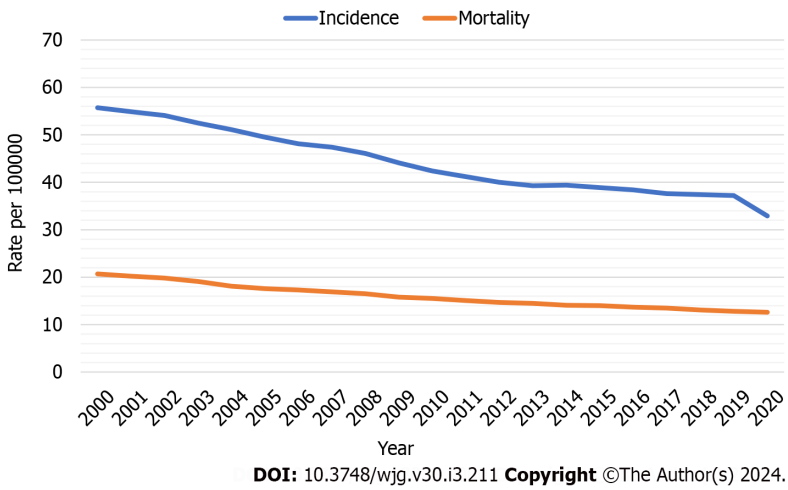

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Figure 1 Colorectal cancer incidence and mortality rates, 2000 to 2020, United States Surveillance Epidemiology and End Results Program. Incidence and mortality are reported per 100000 people.

preventing specific deaths is between 25% and 50%[4,16].

The use of screening and the resulting early detection of more cases, in addition to having benefits in terms of survival and quality of life, could also have economic benefits[17]. In fact, despite a higher initial cost because the exams are performed on a large number of individuals considered healthy, early detection of CRC results in a global cost reduction. The positive cost impact in the lower cancer stages (stages I-II) can be explained by less invasive surgery, shorter hospital stays, fewer emergency admissions and outpatient visits within 12 mo of diagnosis, and less use of chemotherapy and biologic drugs. The potential cost savings associated with an early diagnosis are greater in patients age 18-64[18].

Gheysariyeha *et al*[19] conducted a systematic review with cost-effectiveness results showing that annual fecal immunochemical test (FIT), colonoscopy every 10 years, sigmoidoscopy every 5 years, and biennial high-sensitivity guaiac-based fecal occult blood test (HSgFOBT) and Stool DNA Test every 3 years were cost-saving strategies compared to not screening. In most of the studies, FIT in comparison with other strategies was cost-saving (less costly and more effective).

SCREENING TESTS

CRC is an ideal target for screening because it arises from precursors that take a long time (up to 10 years) to evolve into a malignancy, offering a window of opportunity for polypectomy and cancer prevention[20]. Current CRC screening methods are divided into invasive and noninvasive tests[21]. Noninvasive tests include stool-based tests, blood tests, and radiological examinations. Stool-based tests available are HSgFOBT, FIT, and fecal DNA test (Multitarget stool DNA, MT-sDNA, Cologuard®). Blood-based tests include Epi proColon, which detects circulating methylated SEPT9, and tests that detect microRNA and plasma protein biomarkers. Radiological examinations include computed tomographic colonoscopy (CTC) and capsule endoscopy (double-contrast barium enema is practically no longer applied in clinical practice)[21]. Invasive tests include flexible sigmoidoscopy and colonoscopy, which offer direct visualization and detection of a colon polyp or neoplasm with the advantage of obtaining a pathological specimen[21]. These are complemented by novel emerging screening modalities such as stool-based microbiome testing, urine-based screening tests using liquid chromatography-mass spectrometry or nuclear magnetic resonance spectroscopy, and magnetic resonance colonography. However, the novel tests are not optimal in terms of accuracy and depend on colonoscopy in case of abnormal results[22-24]. Currently, there are no data on whether the new screening strategies have an impact on CRC incidence and mortality, and they cannot, therefore, be recommended for CRC screening[22-24].

HSgFOBT

HSgFOBT detects colorectal polyps and cancers through an oxidation reaction of guaiaconic acid by hydrogen peroxide when the heme group is present in the stool sample. Sensitivity and specificity for CRC are 0.50-0.75 and 0.96-0.98[23], while for advanced adenomas are 0.06-0.17 and 0.96-0.99, respectively[23]. A 2019 meta-analysis showed that HSgFOBT screening led to a reduction in CRC-related mortality but did not reduce the incidence of CRC[25]. HSgFOBT has been largely replaced by FIT because it requires more samples, avoidance of red meat and drugs that can cause false positives, and because a positive test could be due to bleeding from anywhere in the gastrointestinal tract[5].

FIT

FIT is a screening test that detects the presence of the intact globin portion of human hemoglobin in stool using antibodies [26]. Considering the cutoff of 20 μgHb/g stool, the sensitivity and specificity for CRC are 0.74 and 0.94, respectively[23, 24], while sensitivity and specificity for advanced adenoma are 0.23 and 0.96[21]. A 2015 study demonstrated a reduction

in CRC mortality with biennial FIT but no change in CRC incidence[23].

Unlike HSgFOBT, FIT requires only a stool sample, is not influenced by the individual's diet or medications, and does not present abnormal results in the presence of upper gastrointestinal bleeding because hemoglobin is partially digested before reaching the colon[26]. FIT is the most common noninvasive CRC screening modality among average-risk individuals. In a 2020 analysis, CRC detection rates were similar when four rounds of FIT in alternate years were compared with a single flexible sigmoidoscopy and a single colonoscopy[27]. An Italian intention-to-screen study evaluated the effectiveness of a 2-year screening program with FIT and found a stable 28% decrease in annual CRC incidence after 8 years[28].

Multi-target stool DNA testing

The multi-target stool DNA testing (mt-sDNA screening test, also called Cologuard) is an Food and Drug Administration-approved noninvasive CRC screening tool. Cologuard uses a biomarker panel that analyzes a person's stool sample for DNA markers as well as blood in the stool. The sensitivity and specificity for CRC are 0.93 and 0.85, respectively[23]. For advanced adenoma, the sensitivity is 0.43 and the specificity is 0.89[23,24]. With perfect adherence, mt-sDNA reduces the incidence of CRC by 66%[29]. Challenges of screening with mt-sDNA include cost and a high false-positive rate compared with FIT[29-31]. Overall, mt-sDNA is better than FIT in differentiating advanced precancerous lesions from non-neoplastic or negative findings[32]. However, its specificity is lower, which may result in more colonoscopies[31,22].

CTC

First described in the literature in 1994, CTC (also called CT colonoscopy, virtual colonography, and virtual colonoscopy) uses traditional computed tomography with image reconstruction techniques (3D rendering) to visualize the inner wall of the colon without the use of an endoscopic probe[33]. Sensitivity for adenomas 10 mm or greater is 0.89, and specificity is 0.94[23,24]. For adenomas 6 mm or larger, sensitivity is 0.86, and specificity is 0.88[23,24]. The advantages of CTC are less invasiveness, no need for procedural sedation, and low complication rate. Disadvantages are the need to prepare the bowel, exposure to radiation, the need to undergo colonoscopy in cases of positive results, and extracolonic findings involving further examination and potential overtreatment. The use of CTC is limited due to the lack of trained radiologists and imaging centers offering the test[24].

Colon capsule

Colon capsule (CCE) is a noninvasive colon imaging technique involving the ingestion of a wireless pill-sized camera that takes images as it travels through the gastrointestinal tract. The first generation of CCE (PillCam-Colon) showed a sensitivity of 69% and specificity of 86% for detecting a polyp ≥ 6 mm in size[34]. The second generation of CCE (PillCam-Colon 2), which offers an adaptive frame rate and wider viewing angle, showed better accuracy in detecting polyps ≥ 6 mm in size, with a sensitivity of 84% and specificity of 88%[35]. It does not require air inflation, sedation, or the use of radiation and thus allows minimally invasive and painless colon evaluation. However, the rate of complete CCE examinations is only 67%[36], and 32% of CCEs result in referral to colonoscopy (polyps ≥ 10 mm)[37]. Interpretation of CCE also requires a physician skilled in reading capsule endoscopy and often takes longer than performing a colonoscopy[36]. The European Society for Gastrointestinal Endoscopy has proposed CCE as a screening tool in patients at average risk, in patients with incomplete colonoscopy, in patients who refuse conventional colonoscopy, and in patients with contraindications to conventional colonoscopy[38,39].

Blood-based tests

The detection of circulating and cell-free tumor DNA in blood has opened up the potential for blood-based tests for CRC and advanced malignancies, such as the search for SEPT9 DNA, C9orf50, KCNQ6, CLIP4, miRNA, interleukin-6, lectin serine protease 1 mannan binding, and integrin alpha 11[40-42]. Currently, only Epi proColon has been approved by the Food and Drug Administration as a blood-based screening test. Epi proColon detects circulating methylated SEPT9 DNA and has a sensitivity and specificity of 0.68 and 0.79 for CRC and 0.22 and 0.79 for advanced adenomas, respectively[43]. In general, a blood-based test is attractive because of its minimal invasiveness and the possibility of being combined with other routine tests. Adler *et al*[44] reported that 97% of people who refuse screening with colonoscopy accept a non-invasive test, and 83% choose a blood test. It can be offered to medium-risk individuals who have refused other screening tests, with annual testing and a recommendation to have a colonoscopy if the result is abnormal. The United States Preventive Services Task Force has not approved serum methylated septin-9 for medium-risk screening because of low accuracy[5,21,24]. As of 2021, a blood test must have a specificity of 90% and a sensitivity of 74% for CRC compared to an accepted standard (such as colonoscopy) to meet approval thresholds[33]. Unless high sensitivity is achieved, blood-based CRC screening can cause false positive results, unnecessary colonoscopies, and consequently adverse events. It will be essential to determine and improve test accuracy, cost, and the appropriate clinical work-up after abnormal results[45].

Colonoscopy

Colonoscopy is the most common screening modality in the United States and allows visual examination of the entire colon and rectum for polyps and CRC. Sensitivity is 0.89-0.95 and specificity is 0.89 for adenomas 10 mm or larger[23]. For CRC, the sensitivity is 0.18-1.00[23,24].

Cancer mortality is 29%-68% lower among people who undergo screening colonoscopy than those who do not[16,46-48]. The effectiveness of screening colonoscopy for CRC prevention was further quantified by a recent large randomized trial[49]. The 10-year risk of CRC was 0.98% among participants invited to undergo screening colonoscopy compared with 1.20% among those assigned to receive usual care. Screening colonoscopy was performed in only 42% of participants

invited for screening. In analyses adjusted to estimate the effect of screening if all participants randomly assigned to screening actually underwent screening, the risk of CRC decreased from 1.22% to 0.84% (31% reduction) and the risk of death from CRC decreased from 0.30% to 0.15% (50% reduction)[49].

The disadvantages of colonoscopy are its invasiveness, risk of complications, need for bowel preparation, resource burden, and associated costs. Because of the financial and psychosocial barriers to adherence, colonoscopy is best reserved as the second stage of a two-stage screening cascade[50].

Flexible sigmoidoscopy

Flexible sigmoidoscopy is another option for direct visualization of the distal colon. Studies in the United Kingdom, Italy, and United States have reported a reduction in CRC incidence of 23% and 18%-23% and CRC mortality of 22%-31%[51-53]. However, due to the inability to evaluate the entire colon, the overall reduction in CRC incidence and CRC-related mortality is greater for colonoscopy than for flexible sigmoidoscopy[54]. The resources required for flexible sigmoidoscopy are similar to a colonoscopy, but colonoscopy is needed for follow-up of a positive FIT and for those with polyps on flexible sigmoidoscopy. Consequently, rates of screening flexible sigmoidoscopy have declined in the United States[22].

WHY ARE THESE SCREENING MODALITIES NOT ENOUGH?

Although screening has had a positive effect on incidence and mortality, as previously reported, a significant percentage of CRC patients arrive at the hospital with urgent symptoms and advanced neoplasia[55]. About one-third of patients with CRC present as a surgical emergency[55].

Large bowel obstruction accounts for nearly 80% (15%-30% of CRCs) of CRC-related emergencies, while perforation accounts for the remaining 20% (1%-10% of CRCs)[56-59]. The most common site of CRC obstruction is the sigmoid colon, with 75% of tumors located distal to the splenic flexure[60]. Perforation occurs at the tumor site in almost 70% of cases and proximal to the tumor site in about 30% of cases[56,61]. Emergency surgery for CRC is associated with a worse prognosis than elective surgery, with lower overall and recurrence-free survival rates[59,62,63].

Such a high rate of urgent presentations of CRC should give pause to the still unsatisfactory results of screening. The ineffectiveness of early detection is due to the suboptimal accuracy of screening tools (particularly for polyps/adenomas), the poor adherence, the absence of screening programs in some areas of the world, the coronavirus disease 19 (COVID-19) pandemic, and the early onset of CRC.

SCREENING ADHERENCE AND SCREENING PROGRAM

Despite the various modalities offered for CRC screening, it is still underutilized. In the United States, screening rates remain around 60%[21,64]. Adherence to CRC screening is particularly poor among underserved populations, including low-income and African American and Hispanic populations. Over the past four decades, CRC incidence rates have decreased by 33.9% in United States Whites but only 6.6% in African Americans[2]. In 2015, 62.4% of males and females reported using a screening test for CRC[65]. Reported screening was lower among those aged 50-64 years (57.9%) than those aged 65-75 years (71.8%)[65]. The lowest use of screening for CRC was reported by people without a usual source of health care (26.3%) and uninsured people (25.1%)[65]. Adherence rates are no better in Asia-Pacific countries, ranging from 21.0% in South Korea to 62.9% in Thailand[66,67]. Participation rates ranging from 26% to 73% have been reported in Europe[68]. The European Union guidelines have proposed acceptable and desirable CRC screening adherence rates above 45% and 65%, respectively, and colonoscopy adherence among those with a positive primary screening test result above 90%[69,70]. The National Roundtable on CRC proposed an 80% adherence goal for primary screening, and the United States Multi-society Task Force on CRC set an 80% goal for colonoscopy adherence in patients with a positive FIT result[69,71,72]. Several factors play a role in influencing patient participation and sustained adherence. Barriers to screening include high costs, lack of adequate education about CRC, poor consideration of the benefits of screening, a sense of fatalism, or simply fear of screening tests[68,73].

The screening modality has an impact on the adherence rate. In general, the rule applies that more invasive tests have lower adherence rates[74]. In the COLONPREV randomized trial[75], patients underwent either colonoscopy or FIT, and the authors found participation rates of 25.0% and 34.2%, respectively. Similarly, in a meta-analysis comparing colonoscopy with CTC, the participation rates were 20.0% and 29.0%, respectively[68,76].

To achieve the highest level of adherence, it might be better to offer participants a choice because the "best" strategy is the one they will consistently adhere to[76]. Each step in effective CRC screening is associated with specific barriers. Each of these steps can occur in the opportunistic health care setting, such as independent private practices or individual hospitals. However, there are data demonstrating that implementation of programmatic or organized screening can result in improved adherence with CRC screening and benefits for outcomes[77]. An organized screening program is defined by the following characteristics: (1) An explicit policy with specified screening methods and intervals; (2) A defined target population; (3) A management team responsible for implementation; (4) A healthcare team for decision-making and assistance; (5) A quality assurance structure; and (6) A method for identifying cancer occurrence in the population[77,78]. Organized screening programs use a variety of evidence-based approaches to improve CRC screening uptake by members of the target population. These include sending patients invitations from their primary care provider, sending reminder letters, phone calls, sending fecal occult blood test/FIT kits to patients' homes, and population-based public

awareness campaigns[79-83]. Combinations of interventions have been associated with greater increases than single components[31]

In a randomized trial, Libby *et al*[84] compared the rate of HSgFOBT adherence in 3 groups: invitation letter alone; invitation letter plus a prewarning letter; and the former two plus a CRC and screening information booklet. HSgFOBT uptake was highest in the group that received all three mailings. At the provider level, a recommendation to be screened from a primary care provider/general practitioner (GP) is clearly effective in raising participation[31]. Providing GPs with a list of their patients who were noncompliant with CRC screening resulted in a small increase in FIT screening at 1 year[85]. Boguradzka *et al*[86] found a higher participation rate for patients who received GP counseling on CRC screening than for those who received an information pamphlet (47.0% *vs* 13.7%).

Organized screening can reduce structural and economic barriers by expanding schedules, combining screening with other visits, such as the flu vaccination clinic, and making screening more convenient by offering passes or expanding insurance coverage[77]. Muliira and D'Souza[87] found improved participation rates from 11% to 91% with a patient navigator. Navigators were more effective in patients from minority groups. Selby *et al*[88] reported an adherence rate to diagnostic colonoscopy by FIT-positive subjects of more than 83% due to a combination of strategies, including insurance coverage that defines this procedure as preventive and telephone contact to schedule colonoscopy directly[87]. Eliminating economic barriers resulted in a substantial increase (ranging from 7% to 50%, depending on background rates of use) in population coverage, in particular among the low-income, least-educated subjects[68,89,90]

Organized screening programs can continuously monitor screening performance and clinical outcomes[91] and design interventions to address gaps. There are numerous examples of quality assurance programs related to the performance of colonoscopy, based on training and accreditation of endoscopy services[92-96]. Kaminski *et al*[94] tested a program to train endoscopy managers at low-performing facilities. They demonstrated improvements in the adenoma detection rate of the trained operator and the facility as a whole. In addition, they have shown that improved adenoma detection rates are associated with a decreased risk of interval cancer and cancer death[95,96].

Screening programs have reduced incidence, mortality, and surgery for CRC at the population level, but screening rates remain low in several countries[68,97,98]. Most screening in the United States occurs in the opportunistic setting. Organized CRC screening is more common in Europe than in the United States[97]. Opportunistic screening currently occurs in Latvia[99], Greece[99], and Bosnia-Herzegovina[100], while information on screening is lacking in Belarus, Slovakia, Liechtenstein, and Romania[98].

Similarly, most countries in Africa, Central America, South America, and the Middle East do not have organized screening programs[67], mainly due to the limited number of resources and the type of health system organization. Currently, organized screening is recommended in regions with the highest incidence of CRC (> 30 per 100000)[67,101]. Programs target individuals at average risk, aged 50 years to 75 years, and preferably apply the FIT test. Several East Asian countries have organized screening programs in place, including Japan, Korea, China, Hong Kong, Taiwan, and Bangkok[98,102]. In Asia the management of CRC screening is even more complex, as additional challenges are added, such as the lack of awareness of the usefulness of screening by some governments, government reluctance allocate money for building relevant infrastructure, inadequate manpower (too few surgeons and endoscopists relative to the population), and the issue of ethnicity[103-105]. In the case of multiethnic countries such as Malaysia, the risk of CRC is very different among Chinese, Malaysians, and Indians[106,107], with the incidence per 100000 population higher among Chinese and lower among Indians[106]. Therefore, it is difficult to reach consensus on the implementation of a national screening program in these regions[103].

CRC SCREENING AND CORONAVIRUS DISEASE 2019

In the United States, CRC screening is primarily based on colonoscopy, while in Europe most countries screen through FIT[108]. In Europe, a positive FIT must be followed by a colonoscopy within 1 mo[108]. Zorzi *et al*[109] reported that a delay of 9 mo after a positive FIT was associated with worse outcomes in terms of CRC risk and CRC progression. The same conclusion was reached by Lee *et al*[110] using data from the Taiwan Nationwide Screening Program while considering a 6-mo delay for colonoscopy after a positive FIT[110]. The coronavirus disease 2019 (COVID-19) pandemic beginning in March 2020 has overwhelmed the global healthcare system capacity and impacted the management of patients with cancer and other chronic diseases[111-113]. In response to the pandemic and to prevent COVID-19 infections and the spread of the virus in hospitals, there were global policy decisions like lockdowns. There was also redistribution of both human and material resources in the hospital setting[114,115]. This resulted in a drastic reduction of all non-essential services. Non-emergency visits, screenings, and elective surgeries were cancelled[116].

CRC management was severely affected by the pandemic. CRC screening activity decreased by up to 85%-95%. Care delivery was disrupted, and after resumption of activities, patients often refused colonoscopy for fear of being exposed to severe acute respiratory syndrome coronavirus 2, while planning processes were hampered by the need for viral testing prior to the procedure[117]. Delays in screening and surveillance resulted in the progression of precursor lesions and detection of tumors at a more advanced stage[108].

Meijer *et al*[118] reported a reduction in patients with stage I and II CRC from 29.5% and 26.6% to 20.0% and 25.5%, respectively, after the onset of the COVID-19 pandemic. They also noted an increase in patients with stage III and IV from 22.2% and 19.0% to 26.8% and 26.2%, respectively[118]. These changes were attributed to delays in CRC screening and diagnosis caused by the COVID-19 pandemic[112].

As a result, the mode of presentation of malignancy was also affected by the pandemic and the reduction in screening practices. Shinkwin *et al*[119] reported an increase in emergency presentations from 28.6% to 36.0%. Estimates suggested

that there would be approximately 10000 excess deaths from breast cancer and CRC in the United States alone due to pandemic-related treatment interruptions[120], while 18800 people in the United States may experience delays in CRC diagnosis[121]. Similarly, population data in the United Kingdom suggest an increase in preventable cancer deaths due to COVID-19, with up to 16.6% of deaths due to CRC in the 5 years after diagnosis[122].

EARLY CRC

While overall CRC incidence rates have remained stable or declined in many high-income countries, incidence of early-onset CRC (generally defined as CRC that is diagnosed in individuals younger than 50 years) has recently been increasing worldwide, especially in the United States, Europe, Canada, Australia, New Zealand as well as in some countries in Asia [123]. Although there is still little certainty, early-onset CRC appears to be associated with the westernization of lifestyle [124]. Among early onset-CRC, about 30% of patients have mutations that cause inherited cancer predisposition syndromes, and 20% have familial CRC.

The average annual percent changes in early-onset CRC incidence were 4.0% in New Zealand, 2.8% in Canada and Australia, and 2.2% in the United States during 2008-2012[125]. In the United States, the age-adjusted early-onset CRC incidence per 100000 people was 5.9 cases in 2000 and 8.4 cases in 2017. Increases in early-onset CRC have also been documented in most European countries. Early-onset CRC incidence (per 100000 people) increased from 0.8 to 2.3 cases in individuals aged 20-29 years during 1990-2016, from 2.8 to 6.4 cases in those aged 30-39 during 2006-2016, and from 15.5 to 19.2 cases in those aged 40-49 during 2005-2016[126,127]. The average annual percent changes in early-onset CRC incidence were 7.9% in individuals aged 20-29, 4.9% in those aged 30-39, and 1.6% in those aged 40-49 during 2004-2016 [127]. Taken together, early-onset CRC now represents a significant cancer burden among younger adults.

The increase of early-onset CRC incidence in the United States was initially largely driven by rectal cancer[126]. Since 2012 early-onset CRC incidence has increased similarly for colon and rectum with the annual percent change of approximately 1.8%[12]. The rise in early-onset CRC incidence appeared more prominent for colon cancer than for rectal cancer in Europe[127]. Within the next decade, the incidence rates of colon and rectal cancer are estimated to increase by 90% and 124%, respectively, among adults aged 20-34 years and 27% and 46% for those aged 35-49 years[128].

Patients with early-onset CRC are more likely to have synchronous and metachronous lesions and generally show a more advanced stage of disease because of lack of screening, poor consideration of symptoms, and reluctance to seek medical attention delay diagnosis[129,130]. Early-onset CRCs more frequently exhibit unfavorable histopathologic features, such as poor differentiation, perineural invasion, venous invasion, and mucinous and/or signet cell morphology [131,132].

Current population-based screening strategies need to be adapted. Therefore, the Multi-Society Task Force on CRC has recommended starting screening at age 45 years[133]. Early-onset CRC presents a challenge because most young adults diagnosed with CRC have no obvious risk factors and are classified as medium risk by current algorithms. Furthermore, because age and family history of cancer remain the cornerstones of CRC screening and risk stratification algorithms, empirical data supporting the effectiveness of screening young adults are lacking[134]. In fact, most of the landmark studies on screening involve patients over 50 years of age. However, half of all patients with early-onset CRC are younger than 45-years-old. Therefore, lowering the screening age will provide little or no benefit to these patients[135].

Ladabaum[136] reported a very interesting analysis on early onset-CRC, participation rates, and costs. By advancing the age of CRC screening participation in the United States by 5 years, it is estimated that 29400 cases and 11100 deaths from CRC could be averted in the next 5 years, at an incremental cost of about $10 billion and requiring nearly 11 million additional colonoscopies[136]. In comparison, achieving the goal of 80% screening participation at age 50 and above has been estimated to avert two-and-a-half times as many CRC cases and three times as many CRC deaths at an incremental cost of about one-third and requiring 13% more colonoscopies. The author then poses a crucial question: can the new recommendation be introduced without compromising efforts to achieve high screening participation rates in older or higher-risk people and higher FIT follow-up rates[136]?

CONCLUSION

In conclusion, despite significant advancements in medical technology, increased public awareness, and robust efforts to implement CRC screening programs, it is evident that the effectiveness of such initiatives still falls short of their intended goals. We delved into the intricate web of challenges and limitations that contribute to the persistent ineffectiveness of current CRC screening methodologies. The multifaceted nature of CRC, its biological heterogeneity, and the dynamic progression of the disease pose substantial hurdles to early detection and prevention. The limitations in sensitivity and specificity of screening tests, coupled with factors such as patient compliance, societal disparities, and healthcare accessibility issues, create a complex landscape that undermines the potential benefits of CRC screening. Missed lesions, overdiagnosis, interval cancers, and the failure to effectively address serrated lesions are all facets of the overarching problem of inadequate sensitivity and specificity of current screening methods. The invasive nature of certain procedures, the associated risks, and the psychological and emotional factors that deter patient participation, the delay in screening processes brought about by COVID-19, and the growing importance of early diagnosis of CRC further compound the challenge.

However, amidst these challenges, there remains room for optimism. Scientific research continues to advance our understanding of the intricate mechanisms underlying CRC, leading to the development of novel screening approaches and more personalized interventions. The integration of artificial intelligence, machine learning, and risk stratification models holds promise in refining screening algorithms and identifying high-risk populations that demand tailored approaches. Moreover, collaborations between medical professionals, researchers, policymakers, and the public are fundamental to surmounting the existing barriers. Public health campaigns, culturally sensitive education, and improved patient-physician communication have the potential to bolster compliance and participation rates. In the quest to enhance the effectiveness of CRC screening, it is crucial to acknowledge that there is no one-size-fits-all solution. A multifaceted strategy encompassing technological innovation, targeted interventions, policy changes, and patient empowerment is imperative. Only through persistent dedication to research, education, and patient-centered care can the medical community hope to meaningfully impact the trajectory of CRC and ultimately save lives.

Screening and early diagnosis not only reduce mortality and improve patient prognosis but also reduces health care costs. The positive cost impact in the lower cancer stages (stages I-II) can be explained by less invasive surgery, shorter hospital stays, fewer emergency admissions and outpatient visits, and less use of chemotherapy and biologic drugs. On the other hand, in patients with advanced CRC disease, we have to consider the costs of surgical reinterventions for recurrence or distant metastases, the high-cost drugs such as monoclonal antibodies and immunotherapy, the costs of radiotherapy, radiofrequency, transarterial chemoembolization, and all the techniques used to control a disease that has gotten out of control. However, we also have to consider the costs of absences from work for the patient and family members, costs of caregivers, colostomy supplies, home care and hospice admissions, *etc*. Clearly, spending more before results in a significant cost reduction afterwards.

FOOTNOTES

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REVIEW

Relationship among Parkinson's disease, constipation, microbes, and microbiological therapy

Xin-Yang Yuan, Yu-Sen Chen, Zhou Liu

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Abstract

This comprehensive review elucidates the complex interplay between gut microbiota and constipation in Parkinson's disease (PD), a prevalent non-motor symptom contributing significantly to patients' morbidity. A marked alteration in the gut microbiota, predominantly an increase in the abundance of *Proteobacteria* and *Bacteroidetes*, is observed in PD-related constipation. Conventional treatments, although safe, have failed to effectively alleviate symptoms, thereby necessitating the development of novel therapeutic strategies. Microbiological interventions such as prebiotics, probiotics, and fecal microbiota transplantation (FMT) hold therapeutic potential. While prebiotics improve bowel movements, probiotics are effective in enhancing stool consistency and alleviating abdominal discomfort. FMT shows potential for significantly alleviating constipation symptoms by restoring gut microbiota balance in patients with PD. Despite promising developments, the causal relationship between changes in gut microbiota and PD-related constipation remains elusive, highlighting the need for further research in this expanding field.

Key Words: Parkinson disease; Constipation; Gut microbiota; Prebiotics; Probiotics; Fecal microbiota transplantation

Core Tip: This comprehensive review explores the intricate relationship between gut microbiota and constipation, a prevalent non-motor symptom observed in Parkinson's disease (PD). Notably, we discuss the significant alterations in gut microbiota, particularly the increase in the abundance of *Proteobacteria* and *Bacteroidetes*, associated with PD-related constipation. Although currently available treatments are safe, their effectiveness in providing symptom relief remains suboptimal, necessitating the development of innovative therapeutic approaches. This review delves into the potential of therapies based on microbiological interventions such as prebiotics, probiotics, and fecal microbiota transplantation, in alleviating these symptoms.

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder with an increasing incidence worldwide[1]. The doubling of PD cases between 1990 and 2016 is expected to result in more than 12 million patients globally by the year 2050[2,3]. PD is characterized by both motor symptoms (*e.g.*, bradykinesia, resting tremor, and rigidity) and non-motor symptoms (*e.g.*, constipation, depression, impaired olfaction, and rapid eye movement sleep behavior disorder)[4]. Constipation is considered one of the most common precursor symptoms of PD and persists throughout the clinical stages of the disease, with its prevalence increasing as the disease progresses[5,6]. For patients with PD, constipation significantly reduces their ability to carry out daily activities and their overall quality of life[7]. Hence, effective therapeutic approaches to control PD-related constipation are urgently required. The pathological mechanisms of PD-related constipation remain unknown, but they may be associated with recto-anal dysfunction or smooth muscle dystonia in the gastrointestinal tract[8,9]. The role of intestinal microorganisms has attracted increasing research attention in recent years. Accumulating evidence reveals a relationship between gut microbiota and PD-related constipation[10-12]. Consequently, traditional treatment options are shifting toward microecological interventions[13-16]. This review summarizes currently available evidence supporting the roles of gut microbiota in the pathogenesis and treatment of PD-related constipation.

MICROBIOTA-GUT-BRAIN AXIS

The role of intestinal microbes in the central nervous system (CNS) has garnered increasing interest recently. The gut microbiota is a complex ecological community comprising hundreds of millions of microbes that live in the gut and regulates both normal physiology and disease susceptibility through its collective metabolic activities and host interactions[17]. A growing body of research linking PD to the microbiota-gut-brain axis suggests that gut microbiota and microbial metabolites have an important role in PD pathogenesis by influencing neuroinflammation, barrier function, and neurotransmitter activity[18,19]. The microbiota-gut-brain axis includes the autonomic nervous system, the enteric nervous system (ENS), the hypothalamic-pituitary-adrenal axis, and the intestinal microbes[18]. The gut microbiota and the brain can communicate directly through various signaling molecules or indirectly through the gut-brain axis; similarly, the brain can influence the microbes directly or indirectly through alterations to the gut microbiota environment[20].

BRAAK'S HYPOTHESIS

The pathological hallmarks of PD are loss of dopaminergic neurons together with abnormal accumulation of α-synuclein (α-syn) in the substantia nigra and the striatum[21]. Braak *et al*[22] and Hawkes *et al*[23] noticed α-synuclein-containing inclusion bodies in the intestines of patients with sporadic PD and hypothesized that the pathology of Lewy body in PD might begin in the gastrointestinal tract and then spread to the brain through the vagal nerve. Human α-syn fibrils were injected into the gut tissue of healthy rodents and transported through the vagus nerve to the dorsal motor nucleus of the vagal nucleus in the brainstem. These results provide the first direct experimental proof that α-syn can propagate from the gut to the brain[24]. Vagotomy has protective effects on the subsequent development of PD, as it can attenuate the pathological spread of α-syn, dopaminergic neuronal degeneration, and motor dysfunction. The vagus nerve is an important route for the transmission of pathological α-syn into the CNS[25-28]. These findings demonstrate that a-syn detection in the ENS could provide an opportunity to identify early PD neuropathology before the disease spreads to other regions and motor symptoms become evident. Shannon *et al*[29] reported a-syn detection in the neurites of the colonic submucosa in colonic biopsies collected 2-5 years before motor symptom onset in patients with PD[29]. This evidence suggests that a-syn detection in colonic mucosal biopsy samples could serve as a presymptomatic biomarker for PD. Additional evidence revealing a-syn accumulation in colonic biopsies for up to 8 years before motor symptom

manifestation further supports the potential of enteric a-syn as a diagnostic biomarker for PD[30]. Pouclet *et al*[31] performed a comparative analysis of a-syn deposition using biopsy samples collected from the rectum, descending colon, and ascending colon of 26 patients with PD and 9 control subjects. The authors discovered that 23%, 42%, and 65% of patients with PD had a-syn deposition in the rectum, descending colon, and ascending colon, respectively, while control subjects had no a-syn deposition. These findings indicate that enteric α-syn detection has the potential to be used as a sensitive, PD-specific, and clinically useful biomarker for early PD detection.

CONSTIPATION IN PD

Constipation, a prevalent non-motor symptom of PD, has been observed in as many as 90% of patients and is a notable early manifestation and risk factor for PD[32-34]. It is nearly three times more prevalent in patients with PD than in healthy individuals[8,35]. Research indicates that the severity of PD-related constipation helps diagnose the PD stage, with 67% sensitivity and 90% specificity[36]. A Taiwanese study revealed that constipation severity correlates with the probability of PD development[37]. A meta-analysis supported this finding, indicating a 2.27-times higher risk of PD in individuals with constipation[33]. Constipation has a significant 76.56% effect on PD and is mediated by gut microbial changes, as a result of altered gut conditions caused by constipation[12,38]. These changes may result in intestinal inflammation and PD symptoms[38]. Causes of PD-related constipation include delayed colon transit and outlet obstruction[8, 39]. The clinical course of PD worsens with constipation, resulting in evident severe motor and non-motor symptoms[7, 40]. The severity and frequency of constipation also increase as PD advances[41,42]. A unique correlation between gut health and cognitive function has been documented in patients with PD[43]. Studies from Spain suggest a link between constipation and cognitive decline in PD[44]. The presence and severity of constipation are associated with rapidly progressive dementia and reduced subcutaneous fat [45,46].

Evidence suggests an association between gastrointestinal dysfunction and PD medication[47]. Compared to patients with PD who have a normal colonic transit, those with a slow colonic transit require a considerably higher levodopa equivalent daily dose[48]. This indicates that slow colonic transit may delay peak plasma concentration and cause a reduction in the clinical efficacy of levodopa. Long-term PD-related constipation can lead to an abnormal overgrowth of bacterial decarboxylases in the gut[49]. Du *et al*[11] reported a significant increase in the abundance of the order *Lactobacillales* in the intestines of patients with PD-related constipation. Levodopa plasma availability has a negative association with *Lactobacillus* abundance[50], particularly as several bacterial species of the genus *Lactobacillus* contain genes encoding tyrosine decarboxylase[51]. This enzyme can convert levodopa, a common drug used for PD treatment, into dopamine, affecting blood dopamine levels and potentially causing motor fluctuations. This may necessitate more frequent administration of levodopa and decarboxylase inhibitor treatments[51]. Complex interactions occur between anti-PD medications and gastrointestinal symptoms[52]. Healthy rats treated with PD medication for 14 days exhibited significantly reduced gut motility and altered microbiota composition, including increased abundance of *Bifidobacterium* and *Lactobacillus* and decreased abundance of the families *Prevotellaceae* and *Lachnospiraceae*[50]. Alterations in microbiota composition may lead to microbial metabolite changes, leading to constipation. A comprehensive meta-analysis demonstrated that pramipexole administration increased constipation risk relative to placebo[53]. Evidence suggests that constipation marginally increased after 1 year in patients with PD on dopaminergic medication, particularly levodopa[54]. Another randomized, double-blind trial showed that pramipexole extended release led to a higher constipation likelihood versus placebo in patients with early PD[55]. A high levodopa equivalent dose increases constipation risk, which nearly doubles with the combination of levodopa and a dopamine agonist[56].

Slow colon transit

Approximately 80% of patients with PD exhibit a slow colon transit, often twice as long as that recorded in healthy control subjects[39,57,58]. This delayed motility is a sign of impaired peristalsis, which depends on the ENS, a network of two plexuses (myenteric and submucosal) within the gut walls[59]. A significant number of these plexus neurons express vasoactive intestinal peptide (VIP) and nitric oxide synthase — both being crucial for muscle relaxation and vasodilation [60]. PD-associated Lewy bodies are present in VIPergic neurons of the ENS, implying that a slower intestinal transit could primarily result from impaired reflex relaxation caused by the loss of inhibitory motor neurons[61]. Evidence indicates Lewy body-containing neurons in the sympathetic ganglia are immunoreactive to tyrosine hydroxylase, implying that the slow transit could be directly linked to the involvement of colonic myenteric plexus in the PD course [62]. Additionally, the loss of dopaminergic neurons in the ENS likely contributes to slow-transit constipation. Studies have found that dopamine inhibits the release of acetylcholine and slows intestinal motility through presynaptic D2 receptors[63]. Age-related loss of excitatory cholinergic neurons in the colon may also be a factor for the slow colonic transit in PD[64,65]. The type of constipation influences the risk of PD development, and people with slow-transit constipation have a very high likelihood of developing PD[66]. Therefore, individuals aged over 65 years with newly diagnosed slow-transit constipation should be considered for PD screening[66].

Outlet obstruction

More than 60% of patients with PD experience pelvic floor dyssynergia, an uncoordinated action of defecation muscles leading to outlet obstruction[67]. Normal defecation requires the relaxation of pelvic floor and sphincter muscles and a swift return of muscle activity post-defecation. The increase in intra-abdominal pressure, aided by the contraction of glottic, diaphragmatic, and abdominal wall muscles, acts synergistically with the inhibition of pelvic floor and external anal sphincter muscles[68]. In patients with PD, constipation often correlates with a paradoxical contraction of the

puborectalis muscle. This abnormal muscle behavior results in defecation obstruction, a decrease in the anorectal angle, and paradoxical perineum ascent[39,69]. PD-related constipation is indicative of significantly weaker gastrointestinal tract function, with slow transit suggesting colonic ENS involvement and outlet obstruction (dystonia) suggesting direct muscle involvement in PD[39]. The severity and duration of PD are closely associated with the degree of constipation[70].

GUT MICROBIOTA AND PD

In the context of gut microbiota and PD, functional gut changes in a PD mouse model appear well before the onset of motor symptoms, suggesting a potential gut origin for PD[71]. Alteration in gut function could influence PD progression by modifying gut microbiota composition[72]. Several studies have proposed that gut microbiota alteration could trigger PD development[73,74] and incite immunological activation[75]. Persistent immune responses in the gut can increase intestinal permeability, allowing microbial products and inflammatory mediators to escape from the gut, thereby stimulating systemic immune responses[76]. This proinflammatory immune activity and related conditions can elevate levels of α-synuclein (α-syn) in the gut[77]. Pathologic levels of α-syn can propagate in a prion-like manner from the gut to the brain through the vagus nerve[27,78,79]. One study suggested that oral administration of *Proteus mirabilis* stimulates α-synuclein aggregation in the brain and colon, resulting in PD symptoms[80]. Another research indicated that the abundance of specific bacterial families could identify patients with PD[36].

GUT MICROBIOTA AND PD-RELATED CONSTIPATION

Mechanism of action between gut microbiota and PD-related constipation

Current evidence suggests a delayed colon transit and outlet obstruction, both linked to alpha-synuclein-related neurodegeneration in the ENS, are primary factors for PD-related constipation[36,81]. However, emerging research points out to the imbalance in gut flora as a significant player in the development and progression of PD-related constipation[82]. Studies have found that excessive pre-synaptic α-synuclein production in the colonic myenteric ganglia could cause early defecation impairment[83]. This finding is supported by the fact that transgenic mice overexpressing α-synuclein show impaired colonic transit[84,85]. Moreover, α-synuclein overexpression in the CNS can alter gut function[86,87]. Notably, transplantation of PD microbiota into humanized mice worsened motor symptoms and intestinal dysfunction, implying that α-synuclein overexpression and microbiota imbalance both contribute to disease progression[72]. Research also suggests that gut microbiota may significantly influence gut motor function[88,89]. This finding was confirmed in a study in which aryl hydrocarbon receptor expression induced by the gut microbiota in enteric neurons affected gut motility [90]. In a mouse model of PD induced by rotenone, gut microbiota was seen to influence gastrointestinal dysfunction, indicating its possible role in PD[91]. Distinct differences in gut microbiome between patients with PD and individuals without PD have been identified[92]. A study of 197 patients with PD demonstrated that higher microbial diversity in the gut correlated positively with stool firmness, implying a link between higher microbial diversity and constipation[93]. Furthermore, most PD studies have reported a decrease in the abundance of the families *Prevotellaceae* and *Lachnospiraceae*, accompanied by an increase in the abundance of the family *Verrucomicrobiaceae* (including the genus *Akkermansia*)[94-97]. This suggests a complex interplay between gut microbiota and PD-related constipation.

Studies reveal that gut microbiota dysbiosis may reduce stool water content, and *Prevotella* enterotypes increases the stool water content[98,99]. Indeed, patients with *Prevotella*-enriched enterotypes showed less severe constipation[100]. Hydrogen sulfide secreted by *Prevotella*, known for protecting dopaminergic neurons, may decrease in concentration in patients with PD who have reduced *Prevotella* enterotypes, leading to constipation because of increased hydrogen sulfide absorption[101]. Hydrogen sulfide can inhibit colonic contractility by affecting cholinergic and tachykinergic excitatory pathways mediated by neurons[102]. *Prevotellaceae* and *Lachnospiraceae*, which produce short-chain fatty acids (SCFAs), can promote gastrointestinal peristalsis[103]. A correlation was found between the genus *Akkermansia*, particularly *Akkermansia muciniphila*, and colon transit time[104]. Uncontrolled growth of *Akkermansia muciniphila* may degrade the mucus layer, leading to drier or harder stools[105,106]. A study on 52 patients with PD found that Enterobacteriaceae, abundant in the colon of patients with PD, negatively correlated with stool frequency[107]. Enterobacteriaceae produce Curli, an amyloid protein that can promote the aggregation of α-syn in the intestine and brain[80,108]. Gut-restricted amyloid inhibitor treatment in mice alleviated motor and constipation-like symptoms[108]. Both commensal and pathogenic bacterial metabolites can influence gut functions[93,109] (Figure 1). SCFAs, glucagon-like peptide 1 (GLP-1), and peptide tyrosine tyrosine (PYY) can modulate gut sympathetic activity and gastrointestinal motility, highlighting the link between gut microbiota and neuronal function[110]. Additionally, SCFAs activate G-protein-coupled receptors on enteroendocrine cells, mediating GLP-1 and PYY secretion[111]. *In vitro* studies showed that SCFAs stimulate colonic contractions through an enteric reflex involving local sensory and cholinergic nerves[112] and regulate colonic motility through enteric neurons[113]. Changes in the cholinergic phenotype caused by butyrate have a prokinetic effect on colonic motility[99,113]. Alterations in dopamine, 5-HT4 receptors, and β3-adrenoceptors likely lead to colonic dysmotility and constipation in patients with PD[114]. The β3-adrenoceptor in colonic interstitial cells of Cajal inhibits colonic motility by inhibiting pacemaker potential[115]. Dopamine inhibits gastrointestinal motility by activating D1 receptors[116,117], while 5-HT promotes gut motility primarily through the 5-HT4 and 5-HT3 receptors[118,119]. SCFAs can activate 5-HT4 receptors of intrinsic sensory neurons, triggering a peristaltic colonic reflex[120]. Butyrate, which modulates gastrointestinal motility by stimulating 5-HT3 receptors of the vagal sensory fibers[121,122], negatively

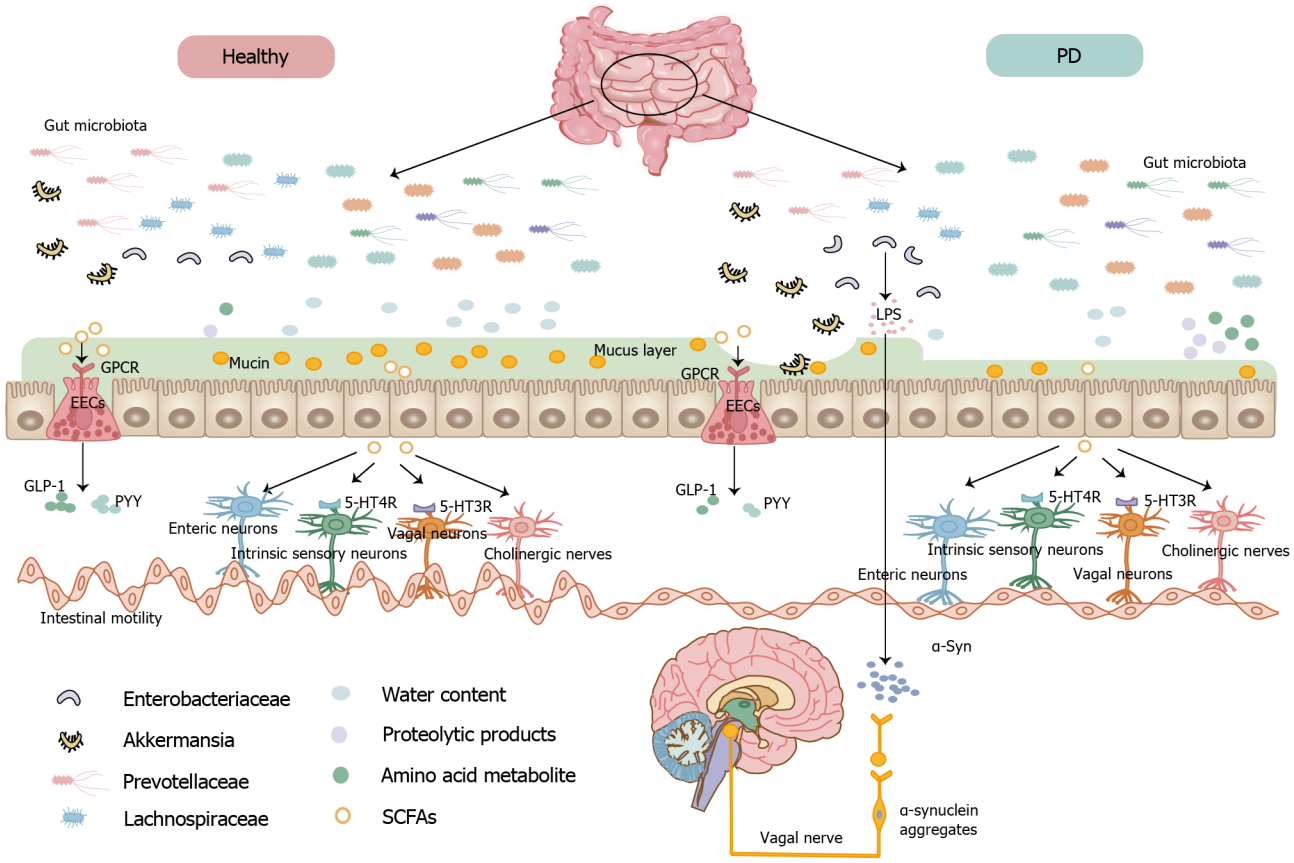

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Figure 1 Changes in microbiota composition and metabolites have been associated with the pathogenic mechanisms of Parkinson's disease-related constipation. Microbiota in patients with Parkinson's disease exhibited a shift in colonic microbiota metabolism away from carbohydrate fermentation and toward proteolysis, resulting in decreased short-chain fatty acids (SCFAs) production and increased proteolytic metabolite levels. Reduced SCFAs production causes a delay in colon transit time. Enhanced proteolytic fermentation has been linked to increased colon transit time. GLP-1: Glucagon-like peptide 1; PD: Parkinson's disease; PYY: Peptide tyrosine tyrosine; α-syn: α-synuclein; 5-HT4R: 5-HT4 receptors; 5-HT3R: 5-HT3 receptors; EECs: Enteroendocrine cells; GPCR: G protein-coupled receptors; LPS: Lipopolysaccharide; SCFAs: Short-chain fatty acids.

correlates with constipation severity[123] and increases mucin secretion[124]. Mucin acts as a lubricant, protecting the mucosa and aiding stool excretion[125]. Acetic acid is positively associated with defecation frequency in patients with PD [126].

A study identified higher levels of the harmful amino acid metabolite p-cresol sulfate in the cerebrospinal fluid of patients with PD[127]. The protein degradation byproducts p-cresol and phenylacetylglutamine are also found elevated in the serum of patients with PD, with strong associations with stool consistency and constipation[93]. Glycerolipids, sphingolipids, and sterol lipids are positively associated with constipation in patients with PD[123]. Additionally, constipation positively correlated with pantothenic acid, D-ribose, L-lactic acid, D-alanine, and xanthine in the Luxembourg Parkinson's Study[128]. In summary, the altered microbiota composition in PD-related constipation might lead to changes in microbial metabolites, especially SCFAs, suggesting the potential for manipulating SCFAs as a novel therapeutic strategy in PD-related constipation. Correlations between PD-related constipation, microorganisms, and their metabolites are summarized in Table 1.

Gut microbiota in PD-related constipation

Research indicates that the primary microorganisms in patients with PD-related constipation are those belonging to Proteobacteria and Bacteroidetes[14]. According to a study, the most prevalent bacteria in the fecal microbiota of patients with PD-related constipation were from the phylum *Bacteroidetes*, genus *Bacteroides*, order *Bacteroidales*, class *Bacteroidia*, and family *Bacteroidaceae*. The study also noted a significantly higher abundance of *Bacteroides* and a considerably lower abundance of *Faecalibacterium* in patients with PD-related constipation than in healthy controls[129]. Additionally, Du *et al* [11] reported that *Bifidobacteriales*, *Lactobacillales*, *Bacillales*, *Peptostreptococcales Tissierellales*, *Desulfovibrionales*, and *Coriobacteriales* were the most abundant microorganisms in the gut of patients with PD-related constipation. These patients also exhibited significantly higher levels of *Bacillus*, *Alistipes*, *Bifidobacterium*, *Romboutsia*, *Adlercreutzia*, *Desulfovibrio*, *Butyricicoccus*, *Bilophila*, *Intestinibacter*, *Holdemania*,*UCG_002 Actinomyces*, *Lachnospiraceae_UCG_008*, *Gordonibacter*, *Raoultibacter*, *Odoribacter*, *Oscillibacter*, *Eubacterium_nodatum*_group, and *uncultured* species than healthy individuals[11]. Interestingly, the gut microbiota of patients with chronic constipation is predominantly characterized by reduced abundance of *bifidobacteria* and *lactobacilli* and increased abundance of *Bacteroidetes*[130-133].

Table 1 Correlation between Parkinson's disease-related constipation and microorganisms and their metabolites

	Positive	Negative	Ref.
Microbial diversity	Alpha diversity		[93,100]
Gut microbiota	*Dorea, Oscillospira, Ruminococcus, Lactobacillus plantarum subgroup, Bifidobacterium, Verrucomicrobiaceae, Bradyrhizobiaceae*	*Faecalibacterium, Roseburia, Enterobacteriaceae* cluster, *Atopobium* cluster	[36,93,100,107,128]
Metabolites	p-cresol and its sulfated form, phenylacetylglutamine, xanthine, D-alanine, L-lactic acid, D-ribose, pantothenic acid. glycerolipids, sphingolipids, sterol lipids	Butyrate, acetic acid	[93,128,123,126]
Enterotype	*Firmicutes*	*Prevotella*	[100]

MICROBIAL TREATMENT FOR PD-RELATED CONSTIPATION

The current treatments for PD-related constipation mainly include prokinetics and laxatives. While these traditional therapies can be safe and effective, they are often limited in fully relieving clinical symptoms, indicating a need for more effective treatments[134,135]. Recent insights into the association between gut microflora and PD-related constipation have led to research exploring how altering gut microflora through prebiotics, probiotics, and fecal microbiota transplantation (FMT) might provide a cure. These interventions could supplement traditional treatments for PD-related constipation.

Prebiotics

Prebiotics are selectively utilized substrates that confer health benefits to host microorganisms[136]. Reports suggest that prebiotic fibers can alleviate constipation and improve bowel movements[137]. In particular, diets rich in insoluble fiber improved constipation in patients with PD[138], and a study reported that psyllium is useful in treating constipation in patients with PD, noting that it increased stool frequency and weight, with, on average, three bowel movements per week [139].

Probiotics

Probiotics are live microorganisms that confer health benefits to the host when administered in sufficient amounts and are thought to be another potential treatment for PD-related constipation. They can strengthen the gut barrier and restore normal intestinal microbiota[140], suggesting its potential as a novel treatment strategy for PD-related constipation[141,142]. Initial studies have shown promising results; For instance, patients with PD who took Lactobacillus casei Shirota for 5 weeks showed improved stool consistency[16], and those who took probiotics containing *Lactobacillus acidophilus* and *Bifidobacterium infantis* for 3 months experienced reduced abdominal pain and bloating[10]. Further research showed an increase in the number of complete bowel movements in patients with PD-related constipation after drinking fermented milk containing multiple probiotic strains and prebiotic fiber for 4 weeks[143]. A subsequent study reported that taking a multi-strain probiotic combined with prebiotic fiber for 8 weeks improved whole-gut transit time and the frequency of bowel opening in patients with PD-related constipation[144]. Additionally, a randomized controlled trial of 72 patients with PD-related constipation showed that multi-strain probiotics significantly improved weekly spontaneous bowel movements frequency and quality of life scores associated with constipation[15]. Du *et al*[11] reported that multi-strain probiotics effectively improved constipation symptoms and stool consistency in patients with PD, even altering the composition of their gut microbiota.

Fecal microbiota transplantation

FMT is a novel treatment approach that alleviates constipation by restoring the intestinal microenvironment. This method is based on the premise that alterations in the microbiome may affect gut motility through the production of different microbial-derived metabolites, and correcting these disruptions might improve the clinical symptoms[145]. FMT has shown promising results in treating PD-related constipation, as evidenced by increased abundance of *Firmicutes* and decreased abundance of *Proteobacteria* and *Bacteroidetes* in treated patients, leading to effective relief of constipation and tremors[14]. More recent studies support the beneficial role of FMT in improving PD-related constipation symptoms[13]. One study highlighted that FMT significantly reduced Bacteroidetes and increased *Prevotella* and *Blautia* in patients with PD-related constipation. Surprisingly, after FMT, the abundance of several other bacterial groups also increased at different times, accompanied by significant decreases in the patients' Wexner constipation scores and resolution of their constipation symptoms[129]. Such findings underline the therapeutic potential of FMT in rebuilding the gut microbiota of patients with PD-related constipation. Microbial alterations in PD-Related constipation after microbial treatments are summarized in Table 2.

CONCLUSION

In prodromal PD, abnormalities related to α-syn can be detected in the colon. Subsequently, α-syn spreads from the gut to the brain through the vagus nerve, which may lead to the development of PD. Constipation is considered one of the

Table 2 Microbial alterations in Parkinson's disease-related constipation after microbial treatments

Microbial treatments	Study design	Participant	Duration	Microbial alterations		Results	Ref.
				Increased	Decreased		
Probiotics	Randomized controlled clinical trial	46	12 wk	*g_Christensenella_sp._Marseille-P2437*	*g_Eubacterium_oxidoreducens_group*, *g_Eubacterium_hallii_group*, *s_Odoribacter_sp._N54.MGS-14* and *Prevotellaceae*	The probiotics group increased the average number of complete bowel movements per week as compared to the control group. The improvement rate of constipation in the probiotics group was significantly higher than that in the control group	[18]
FMT	Case report	1	3 d	*Firmicutes*	*Proteobacteria, Bacteroidetes*	After FMT, patients successfully defecated within 5 min and maintained daily unobstructed defecation until the end of follow-up	[14]
FMT	A prospective, single-center study	11	1 d	*Blautia, Prevotella*	*Bacteroidetes*	The PAC-QOL and Wexner constipation scores both decreased significantly	[129]

FMT: Fecal microbiota transplantation; PAC-QOL: Patient assessment of constipation quality of life.

precursor symptoms of PD, potentially stemming from α-syn pathology in the ENS. The exact mechanisms driving PD-related constipation are still largely unknown, with potential causes ranging from outlet obstruction to delayed colon transit. Current evidence shows a correlation between PD-related constipation and changes in gut microbiota, suggesting a complex interplay between the gut microbiome and PD-related constipation. However, whether the onset of PD-related constipation precedes intestinal dysbiosis or vice versa is still unknown. Despite the unclear cause-effect relationship, studies indicate that gut microbiota dysbiosis can exacerbate constipation and that restoring the gut microbiota can mitigate these symptoms, suggesting gut microbiota as a potential therapeutic target for PD-related constipation. Microbiological intervention treatments for PD-related constipation, including prebiotics, probiotics, and FMT, can prove beneficial and possibly more effective than traditional treatments.

This review covered longitudinal studies on gut dysbiosis in PD-related constipation. However, it has a few weaknesses. The limited number of studies may not have accurately captured the full longitudinal changes in the microbiota associated with PD-related constipation. Furthermore, there is a scarcity of clinical studies examining intestinal flora specifically in PD-related constipation, making it difficult to infer the particular microbial taxa linked to this condition. In addition, as most studies have been conducted at the phylum and genus levels, further research at the species and strain levels could provide greater mechanistic insights. Therefore, future studies should focus on identifying specific bacterial species that promote PD-related constipation development. Finally, pinpointing the causative microbes could enable targeted microbial therapies for PD-related constipation in the future. However, more rigorous clinical studies are needed to elucidate the precise microbiota compositional and functional changes underlying PD-related constipation before such therapeutic approaches can be applied. However, this is a nascent field of research with various limitations and challenges and hence requires future extensive research.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Effectiveness of antibiotic prophylaxis for acute esophageal variceal bleeding in patients with band ligation: A large observational study

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Abstract

BACKGROUND

Esophageal variceal bleeding is a severe complication associated with liver cirrhosis and typically necessitates endoscopic hemostasis. The current standard treatment is endoscopic variceal ligation (EVL), and Western guidelines recommend antibiotic prophylaxis following hemostasis. However, given the improvements in prognosis for variceal bleeding due to advancements in the management of bleeding and treatments of liver cirrhosis and the global concerns regarding the emergence of multidrug-resistant bacteria, there is a need to reassess the use of routine antibiotic prophylaxis after hemostasis.

AIM

To evaluate the effectiveness of antibiotic prophylaxis in patients treated for EVL.

METHODS

We conducted a 13-year observational study using the Tokushukai medical database across 46 hospitals. Patients were divided into the prophylaxis group (received antibiotics on admission or the next day) and the non-prophylaxis group (did not receive antibiotics within one day of admission). The primary outcome was composed of 6-wk mortality, 4-wk rebleeding, and 4-wk spontaneous bacterial peritonitis (SBP). The secondary outcomes were each individual result and in-hospital mortality. A logistic regression with inverse probability of treatment weighting was used. A subgroup analysis was conducted based on the Child-Pugh classification to determine its influence on the primary outcome measures, while sensitivity analyses for antibiotic type and duration were also performed.

RESULTS

Among 980 patients, 790 were included (prophylaxis: 232, non-prophylaxis: 558). Most patients were males under the age of 65 years with a median Child-Pugh score of 8. The composite primary outcomes occurred in 11.2% of patients in the prophylaxis group and 9.5% in the non-prophylaxis group. No significant differences in outcomes were observed between the groups (adjusted odds ratio, 1.11; 95% confidence interval, 0.61-1.99; P = 0.74). Individual outcomes such as 6-wk mortality, 4-wk rebleeding, 4-wk onset of SBP, and in-hospital mortality were not significantly different between the groups. The primary outcome did not differ between the Child-Pugh subgroups. Similar results were observed in the sensitivity analyses.

CONCLUSION

No significant benefit to antibiotic prophylaxis for esophageal variceal bleeding treated with EVL was detected in this study. Global reassessment of routine antibiotic prophylaxis is imperative.

Key Words: Esophageal varices; Endoscopic hemostasis; Antibiotic prophylaxis; Liver cirrhosis; Inverse probability of treatment weighting

Core Tip: Esophageal variceal bleeding, a serious condition linked to liver cirrhosis, often requires endoscopic treatment. While western guidelines suggest using antibiotics after endoscopic treatment, data from multiple Japanese medical centers indicates that these prophylactic antibiotics are not associated with 6-wk mortality. Based on advances in cirrhosis treatment and the appropriate use of antibiotics, the necessity of routine prophylaxis must be reassessed.

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INTRODUCTION

Esophageal variceal bleeding is a life-threatening complication in patients with liver cirrhosis[1], and endoscopic hemostasis is recommended as the first line of treatment[2]. However, even after hemostasis, there is a risk for infection, such as spontaneous bacterial peritonitis (SBP)[3], and rebleeding triggered by these infections[4,5], which are believed to contribute to increased mortality. While current Japanese guidelines do not specifically address antibiotic prophylaxis[6], western guidelines advocate prophylaxis for all patients[7-10].

The rationale for this recommendation lies in several studies conducted prior to the early 2000s that reported high mortality and an infection incidence of approximately 30% after upper gastrointestinal bleeding in patients with cirrhosis [11,12]. However, since the late 2000s, both mortality and infection incidence following upper gastrointestinal bleeding have improved, to less than 10%[13,14]. This marked improvement can be attributed to the shift in the recommended hemostatic method from endoscopic injection sclerotherapy (EIS) to endoscopic variceal ligation (EVL) and advancements in the treatment of liver cirrhosis[15]. These findings have prompted a reconsideration of the current practice of universal antibiotic prophylaxis across all clinical scenarios. Recent reports suggest that such prophylaxis may not always be necessary in modern medical settings[16-19]. Yet, these assertions are primarily from single-center observational studies; no multi-center study has been conducted. Furthermore, the inappropriate use of antibiotics, which has been identified as a cause of the emergence of multidrug-resistant bacteria, is a global issue[20].

Therefore, we aim to reassess the effectiveness of antibiotic prophylaxis in patients with esophageal variceal bleeding treated with EVL using data from several centers in Japan over a 13-year period. It is crucial to conduct research in regions such as Japan, where the guidelines do not recommend antibiotic prophylaxis after hemostasis.

MATERIALS AND METHODS

Data source

We conducted a retrospective cohort study using data from the Tokushukai medical database[21]. The Tokushukai group is a large hospital group in Japan that manages more than 70 hospitals nationwide. 50 hospitals are part of the Diagnosis Procedure Combination (DPC) system. The DPC system is a comprehensive payment system used in Japan that is specifically designed for acute care[22,23]. The Tokushukai Medical Database primarily comprises administrative claims data (specifically, DPC inpatient data) and electronic health records, including inpatient and outpatient blood test results.

The DPC inpatient data includes patient age; sex; admission and discharge dates; discharge status; main diagnosis; comorbidities at admission; post-admission complications recorded by the attending physician using the 2003 version of the International Classification of Diseases, 10th revision (ICD-10) codes; types of surgery (coded with original codes and text data in Japanese); and daily records of drugs and procedures. A distinguishing feature of this database is its capacity to access individual patient medical records. If necessary, additional details can be retrieved directly from these records.

Patient selection

This study included adult patients (aged ≥ 18 years) with esophageal variceal bleeding (ICD-10 code, I850) who underwent emergency EVL on the day of admission between January 2010 and December 2022. We excluded patients with the following criteria: (1) Death occurring on the day of admission or the following day; (2) discharge on the day of admission or the following day; (3) use of a mechanical ventilator on the day of admission or the following day; (4) use of continuous renal replacement therapy (CRRT) on the day of admission or the following day; (5) interventional radiology (IVR) or a surgical procedure on the day of admission or the following day; and (6) the presence of symptoms of infection, defined as having a fever of ≥ 38 ℃ or obtaining a blood culture, on the day of admission or the following day.

Exposure

The patients were divided into prophylaxis and non-prophylaxis groups. The prophylaxis group included patients who received antibiotics on the day of admission or the following day. The non-prophylaxis group included patients who did not receive antibiotics on the day of admission or the following day. The types of antibiotics considered in this study are detailed in Table 1, and the duration of administration was assumed to be at least one day.

Variables and outcomes

The variables included age, sex, the Barthel Index[24], the Child-Pugh Score and classification[25,26], the Charlson Comorbidity Index[27], maintenance hemodialysis, hepatic cancer, malignancy history, alcohol-related disease, and past varix rupture history. We also collected data regarding the use of antiplatelets, anticoagulants, nonsteroidal anti-inflammatory drugs, corticosteroids, and acid blockers prescribed on the day of admission or the following day or as part of the regular medications of the patient. The antiplatelet drugs used included aspirin, ticlopidine, clopidogrel, prasugrel, and ticagrelor. The anticoagulants prescribed included warfarin, dabigatran, edoxaban, rivaroxaban, apixaban, and heparin. Laboratory data collected on the day of admission included total bilirubin (mg/dL), aspartate aminotransferase (U/L), alanine aminotransferase (U/L), albumin (g/dL), white blood cells (/μL), hemoglobin (g/dL), platelets (10^3/μL), C-reactive protein (mg/dL), prothrombin time percentage (PT, %), activated partial thromboplastin time (APTT, sec), and estimated glomerular filtration rate (eGFR, mL/min/1.73 m^2). Additionally, the shock index[28,29] was evaluated based on the vital signs at the hospital visit. The use of vasopressors and red blood cell transfusion volume on the day of admission were also obtained. Age was classified into four categories: < 65, 65-4, 75-84, and ≥ 85 years. The Barthel Index was categorized into three groups: 0 (worst disability), 1-99, and 100 (full ability). Albumin and PT% were categorized according to the Child-Pugh score, while APTT was categorized into < 40, 40-60, and ≥ 60 sec groups. All variables, excluding the Child-Pugh classification, were used as confounders in the analysis.

The primary outcome was a composite of 6-wk mortality, 4-wk rebleeding, and 4-wk onset of SBP. We defined rebleeding as cases when patients underwent endoscopic hemostasis procedures, such as EVL, EIS, or endoscopic clip hemostasis, two or more days after admission. To ensure outcome accuracy, all hemostatic procedures were verified by an endoscopy specialist using electronic medical records. Hemostatic procedures not associated with active bleeding but instead performed for future bleeding prevention, such as EVL or EIS on other varices and argon plasma coagulation, were excluded. SBP was defined as a polymorphonuclear cell count of 250/μL or greater[7,30], resulting from an ascites puncture performed during hospitalization. The secondary outcomes were the individual assessments of 6-wk mortality, 4-wk rebleeding, and 4-wk onset of SBP each assessed individually and in-hospital mortality. Also included were the 4-wk onset of clostridium difficile infection (CDI) and the length of hospital stay. CDI was defined as a diagnosis of ICD-10 code A047 on the second day of hospitalization or later and patients who were administered metronidazole or oral vancomycin.

Statistical methods

Continuous variables are reported as median and interquartile range (IQR), and categorical variables are reported as numbers and percentage. We determined the average treatment effect on the treated-based inverse probability of treatment weighting (IPTW) for the prophylaxis and non-prophylaxis groups. This method minimizes the effects of selection bias and imbalances in patient backgrounds between groups[31,32]. We estimated the propensity scores using logistic regression with prophylaxis as the dependent variable and all covariates as independent variables. Balances in baseline variables were also examined using standardized MD (SMD), and absolute values < 10% were considered balanced[33]. We used logistic regression to evaluate odds ratios (ORs) with 95% confidence intervals (CIs) to assess the

Table 1 List of antibiotics included in the study

ATC code	Type of antibiotics
J01AA	Tetracyclines
J01BA	Amphenicols
J01BB	Macrolides, lincosamides and streptogramins
J01CA	Penicillins with extended spectrum
J01CE	Beta-lactamase sensitive penicillins
J01CF	Beta-lactamase resistant penicillins
J01CR	Combinations of penicillins, including beta-lactamase inhibitors
J01DB	First-generation cephalosporins
J01DC	Second-generation cephalosporins
J01DD	Third-generation cephalosporins
J01DE	Fourth-generation cephalosporins
J01DF	Monobactams
J01DH	Carbapenems
J01EA	Trimethoprim and derivatives
J01EB	Short-acting sulfonamides
J01EC	Intermediate-acting sulfonamides
J01ED	Long-acting sulfonamides
J01EE	Combinations of sulfonamides
J01FA	Macrolides
J01FF	Lincosamides
J01FG	Streptogramins
J01GA	Aminoglycoside antibacterials
J01GB	Other aminoglycosides
J01MA	Fluoroquinolones
J01MB	Other quinolone antibacterials
J01XA	Glycopeptide antibacterials
J01XB	Polymyxins
J01XC	Steroid antibacterials
J01XD	Imidazole derivatives
J01XE	Nitrofuran derivatives
J01XX	Other antibacterials

ATC: Anatomical Therapeutic Chemical.

outcomes for categorical variables. The length of hospital stay was evaluated using negative binomial regression with rate ratios and 95%CI. The two-sided significance level for all tests was set at $P < 0.05$.

For the subgroup analysis, we evaluated the interaction effect between antibiotic prophylaxis and the Child-Pugh classification on the primary composite outcome. We employed logistic regression with IPTW, consistent with our primary analysis approach, using a dataset derived from multiple imputation data.

We conducted several sensitivity analyses to determine the robustness of our inferences. First, we performed both propensity score matching (PSM) to evaluate the robustness of the results. For PSM, we used the same propensity scores estimated for IPTW. A one-to-one PSM was conducted utilizing the nearest neighbor method without replacement. The caliper width was set at 20% of the standard deviation of the propensity scores on the logit scale. Second, considering the absence of a clear consensus on the duration of antibiotic prophylaxis, we narrowed the exposure period to those who received antibiotics for 2 d, 3 d, and 4 or more days. For these analyses, the exposure timing, definition of the control group, and analysis methods were identical to those used in the main analysis. Third, there is no consensus regarding the

appropriate type of antibiotic for prophylaxis. Therefore, to investigate the potential differences in outcomes due to the type of antibiotic used, we conducted a similar analysis with only third-generation cephalosporins that have a relatively large amount of evidence as the exposure[8].

In this study, we handled missing data by making a missing at-random assumption and conducting multiple imputations. These multiple imputations were conducted using chained equations with 100 imputed datasets and 200 iterations (maxit = 200) for each dataset. The imputation models included all the variables of interest and relevant auxiliary variables. Pooled estimates were obtained by combining the results across the imputed datasets, according to Rubin's rules[33,34].

All analyses were performed using R software (version 4.2.3; R Foundation for Statistical Computing, Vienna, Austria).

Sample size calculation

Based on previous reports, we assumed the incidence of the composite outcome to be 15%[11], and the antibiotic administration rate to be > 30%[16], expecting an unexposed to exposure ratio of approximately 2:1. We set a clinically meaningful risk ratio of 0.5 that was clinically meaningful for the composite outcomes associated with antibiotic prophylaxis[11,35]. With an α error of 0.05 and a power of 80%, using Kelsey's equation, the required sample size was calculated to be 687 cases. Our sample size became larger than the predefined sample size, as described in the Results section.

Ethics

This study was conducted in accordance with the Declaration of Helsinki. It was reviewed and approved by the Institutional Review Board of the Future Medical Research Centre Ethical Committee (Approval Number: No. TGE02100-02). Due to the observational nature of the study, where patient data were accessed from hospital medical records without taking biological samples from patients, informed patient consent was deemed not necessary. Instead, an opt-out method was used and provided on the website of each hospital. This study is based on the Strengthening the Reporting of Observational Studies in Epidemiology guidelines.

RESULTS

A total of 980 patients from 46 hospitals who met the inclusion criteria were considered for inclusion in this study (Table 2). After applying the exclusion criteria, 790 patients were included in the analyses (Figure 1). The patients were divided into the prophylaxis (n = 232) and non-prophylaxis (n = 558) groups. Antibiotic prophylaxis was administered in 29.4% of patients. Most patients were male, under 65 years of age, and had a moderate level of functional independence (Table 3). The prevalence of alcohol-related diseases, varix rupture history, and β-blocker usage were higher in the prophylaxis group. Other variables, including the Child-Pugh score and the Charlson Comorbidity Index, were similar between the groups. The antibiotics used in the prophylaxis group included four carbapenems, 32 first-generation cephalosporins, 51 s-generation cephalosporins, 106 third-generation cephalosporins, 14 beta-lactamase inhibitor combinations, 22 macrolides, and three lincosamides (Table 4). The mean duration of administration was 4.59 d.

We ensured that the baseline conditions for the analysis were appropriately met. Figure 2 illustrates the overlap of the propensity scores for each group within one of the imputed datasets. The average C-statistic across the imputed datasets was 0.64. A comparison of patient characteristics before and after IPTW, as indicated by SMD, is outlined in Table 3 and Figure 3. Upon the application of IPTW, a balanced equivalence in the baseline characteristics was achieved between the groups.

Table 5 presents the outcomes before and after adjustment using IPTW. Before the application of IPTW, the composite outcome was 11.2% in the prophylaxis group and 9.5% in the non-prophylaxis group; the 6-wk mortality was 6.9% in the prophylaxis group and 6.6% in the non-prophylaxis group; the 4-wk rebleeding was 3.9% in the prophylaxis group and 2.9% in the non-prophylaxis group; the 4-wk onset of SBP was 2.2% in the prophylaxis group and 1.8% in the non-prophylaxis group; and the in-hospital mortality was 6.0% in the prophylaxis group and 6.1% in the non-prophylaxis group. There was one case of CDI in each group (0.4% in the prophylaxis group and 0.2% in the non-prophylaxis group). The median length of hospital stay was 8 d (IQR: 5-15 d) in the prophylaxis group and 9 d (IQR: 6-15 d) in the non-prophylaxis group.

Upon adjustment with IPTW, no significant differences regarding the composite outcome (adjusted OR, 1.11; 95%CI, 0.61-1.99; P = 0.74), 6-wk mortality (adjusted OR, 0.97; 95%CI, 0.47-1.98; P = 0.93), 4-wk rebleeding(adjusted OR, 1.21; 95%CI, 0.45-3.24; P = 0.71), 4-wk onset of SBP (adjusted OR, 1.20; 95%CI, 0.32-4.46; P = 0.78), or in-hospital mortality (adjusted OR, 0.89; 95%CI, 0.42-1.87; P = 0.75) were observed between the groups. The length of hospital stay did not significantly differ between the groups (adjusted rate ratio, 1.06; 95%CI, 0.94-1.19; P = 0.34).

In the subgroup analysis, there was no significant interaction between antibiotic prophylaxis and the Child-Pugh classification in relation to the composite outcome (P for interaction = 0.32) (Table 6). The sensitivity analyses of the PSM results and antibiotic duration were consistent with the main analysis (Table 7, Figures 4 and 5).

DISCUSSION

This long-term observational study involving data from 46 acute care hospitals across Japan explored the effectiveness of antibiotic prophylaxis for esophageal variceal bleeding treated with EVL. No benefits of antibiotic prophylaxis in terms of

Table 2 Distribution of facilities and cases across regions in Japan

Region	Number of facilities	Number of cases
Hokkaido	3	34
Tohoku	2	5
Kanto	14	319
Chubu	5	32
Kansai	11	359
Chugoku/Shikoku	1	2
Kyushu/Okinawa	10	229

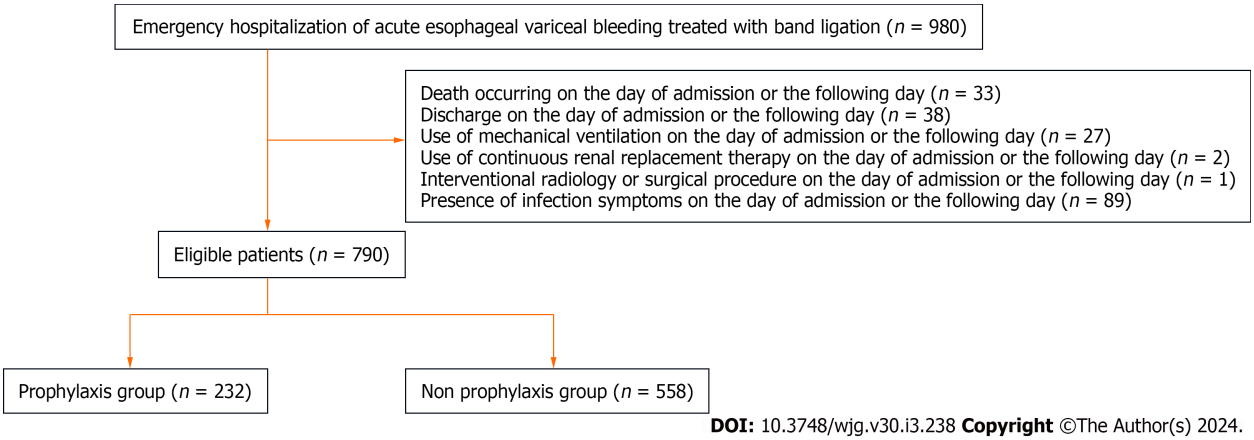

Figure 1 Patient flow.

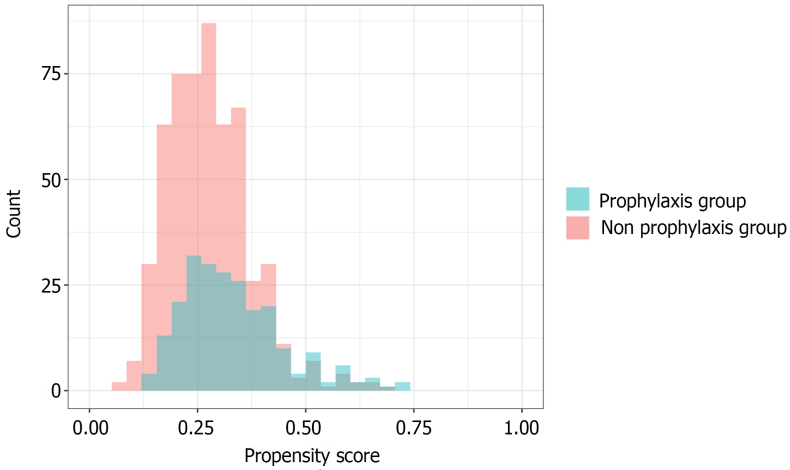

Figure 2 Overlap of the propensity score of each group.

composite outcomes, individual outcomes, or length of hospital stay were identified. The effectiveness of prophylactic antibiotics in terms of composite outcomes were not significantly affected by the Child-Pugh classification.

Our findings underscore the diminishing role of universal prophylactic antibiotic administration in modern medical settings, aligning more closely with post-2010 results rather than older data. In previous randomized controlled trials (RCTs) regarding variceal bleeding that were conducted until the early 2000s, early mortality ranged from 4.2%-24%, rebleeding from 12.5%-20.8%, and the incidence of infections from 15-27.5%[35-39]. In contrast, only one RCT reported after 2010 reported early mortality and rebleeding rates of 3% and 8.5%, though the infection incidence was not assessed [13]. In this study, the 6-wk mortality was 6.7%, 4-wk rebleeding was 3.2%, and 4-wk onset of SBP was 1.9%, highlighting the improving treatment outcomes. Although a 2022 systematic review advocated for the benefits of antibiotic prophylaxis[15], it included one RCT published after 2010. The majority of studies reported after 2010 are single-center observa-

Table 3 Patient characteristics, missing data, and comparison of standardized mean differences, *n* (%)

	Before Imputation and IPTW				After imputation and IPTW
	Prophylactic groups	Non-prophylactic groups			
Variables	*n* = 232	*n* = 558	Missing (%)	SMD	SMD
Age, yr			0	0.18	0.01
< 65	143 (61.6)	322 (57.7)			
65-74	46 (19.8)	144 (25.8)			
75-84	39 (16.8)	75 (13.4)			
≥ 85	4 (1.7)	17 (3.0)			
Sex, male (%)	181 (78.0)	417 (74.7)	0	0.08	< 0.01
Barthel index (%)			17.3	0.10	< 0.01
100 (full activity)	83 (40.5)	186 (41.5)			
1-99	63 (30.7)	152 (33.9)			
0 (worst disability)	59(28.8)	110 (24.6)			
Child-Pugh score, median (IQR)	8 (7-10)	8 (7-10)	12.9	0.05	0.03
Child-Pugh classification (%)			10.1	0.06	0.02
A	42 (19.4)	93 (18.8)			
B	110 (50.9)	266 (53.7)			
C	64 (29.6)	136 (27.5)			
Presence of ascites	67 (31.0)	171 (34.5)			
Comorbidities					
Charlson Comorbidity Index, median (IQR)	4 (4-5)	4 (4-5)	0	< 0.01	0.01
Maintenance hemodialysis	3 (1.3)	8 (1.4)	0	0.01	< 0.01
Hepatic cancer	38 (16.4)	112 (20.1)	0	0.10	< 0.01
Malignant tumor history	29 (12.5)	65 (11.6)	0	0.03	< 0.01
Alcohol-related disease	127 (54.7)	246 (44.1)	0	0.21	< 0.01
Past varix rupture history	63 (27.2)	127 (22.8)	0	0.10	< 0.01
Medications					
Antiplatelet use	3 (1.3)	8 (1.4)	0	0.01	< 0.01
Anticoagulant use	5 (2.2)	8 (1.4)	0	0.05	< 0.01
NSAIDs use	5 (2.2)	13 (2.3)	0	0.01	< 0.01
Corticosteroid use	0 (0)	2 (0.4)	0	0.09	< 0.01
Acid blocker use	214 (91.8)	486 (87.1)	0	0.15	< 0.01
β blocker use	26 (11.2)	26 (4.7)	0	0.24	< 0.01
Laboratory data					
Total bilirubin, mg/dL, median (IQR)	1.6 (1-2.9)	1.4 (0.9-2.4)	3.2	0.13	0.02
Aspartate aminotransferase, U/L, median (IQR)	54.5 (32.2-94.8)	47 (31-83)	2.2	0.09	0.01
Alanine aminotransferase, U/L, median (IQR)	30.5 (20-47)	27 (19-42)	2.2	0.08	< 0.01
Albumin			4.6	0.11	0.02
> 3.5 g/dL	37 (16.5)	72 (13.6)			

2.8–3.5 g/dL	97 (43.3)	256 (48.3)			
< 2.8 g/dL	90 (40)	202 (38.1)			
White blood cell, /μL, median (IQR)	7720 (5900-10700)	7300 (5200-10400)	1.8	0.15	0.01
Hemoglobin, g/dL, median (IQR)	9 (7.3-10.5)	8.5 (6.9-10.2)	1.8	0.12	< 0.01
Platelet, 10^3/μL, median (IQR)	99 (72-139)	103 (75-144)	1.8	< 0.01	0.02
C-reactive protein, mg/dL, median (IQR)	0.3 (0.1-0.7)	0.3 (0.1-0.8)	4.7	0.03	0.04
Prothrombin time			5.9	0.02	< 0.01
> 70%	48 (21.5)	113 (21.7)			
40%-70%	138 (61.9)	324 (62.3)			
< 40%	37 (16.6)	83 (16.0)			
Activated partial thromboplastin time			11.4	0.08	0.01
≤ 40 s	185 (87.3)	436 (89.3)			
40-60 s	23 (10.8)	47 (9.6)			
> 60 s	4 (1.9)	5 (1.0)			
eGFR < 30 mL/min/1.73 m^2	19 (8.3)	40 (7.3)	1.9	0.03	< 0.01
Shock index > 1	94 (41.4)	197 (36.5)	3	0.10	< 0.01
Vasopressor use	7 (3.0)	19 (3.4)	0	0.02	< 0.01
RBC transfusion, Unit, median (IQR)	4 (0-4)	2.5 (0-4)	0	0.09	< 0.01

IPTW: Inverse probability of treatment weighting; SMD: Standardized mean difference; IQR: Interquartile range; eGFR: Glomerular filtration rate; RBC: Red blood cell.

Table 4 Antibiotic use in prophylaxis group

Antibiotic class	Number of patients
Carbapenems	4
First-Generation Cephalosporins	32
Second-Generation Cephalosporins	51
Third-Generation Cephalosporins	106
Beta-Lactamase Inhibitor Combinations	14
Macrolides	22
Lincosamides	3

tional studies, indicating a lack of strong evidence supporting the routine use of antibiotics prophylaxis in contemporary settings.

The outcomes of our study can be understood through several underlying factors. The predominant role of EVL in hemostasis may have played a significant role in our findings. EVL results in fewer complications compared to EIS and offers superior control over bleeding[7-10,40]. While several previous RCTs incorporated EIS into their hemostatic protocols[35-39], both the current investigation and the most recent RCT focused exclusively on EVL[13]. This shift in technique may have contributed to a reduced incidence of complications, such as infections, suggesting that the need for antibiotic prophylaxis may be less pronounced when EVL is conducted. Additionally, the exclusion criteria of this study provides context. Severe patients, including those requiring mechanical ventilation, CRRT, IVR, or surgery, may have an inherent increased need for antibiotics. By design, our study did not include these patients. When patients that have effectively undergone hemostasis using EVL are included and critically ill patients are excluded, prophylactic antibiotics may not be as crucial as previously reported.

In recent years, a study evaluating the effectiveness of antibiotic prophylaxis for patients with cirrhosis presenting with upper gastrointestinal bleeding was conducted in Japan using a large-scale database[41]. In this study, the rate of antibiotic prophylaxis was 11.5%. Although the target was upper gastrointestinal bleeding and not esophageal variceal bleeding, it is evident that prophylactic antibiotics are not typically administered to patients with cirrhosis in Japan. Similar to our findings, their study did not demonstrate the utility of prophylactic antibiotic administration[41]. It may be necessary to reconsider the use of antibiotic prophylaxis for patients with cirrhosis in current medical settings.

Table 5 Crude and inverse probability of treatment weighting outcomes, *n* (%)

Outcomes	Before imputation and IPTW			After imputation and IPTW	
	Prophylaxis group (*n* = 232)	Non-prophylaxis group (*n* = 558)	Odds ratio (95%CI)	Odds ratio (95%CI)	*P* value
Composite outcome	26 (11.2)	53 (9.5)	1.20 (0.72-1.96)	1.11 (0.61-1.99)	0.74
6-wk mortality	16 (6.9)	37 (6.6)	1.04 (0.55-1.88)	0.97 (0.47-1.98)	0.93
4-wk rebleeding	9 (3.9)	16 (2.9)	1.37 (0.57-3.08)	1.21 (0.45-3.24)	0.71
4-wk onset of SBP	5 (2.2)	10 (1.8)	1.21 (0.37-3.44)	1.20 (0.32-4.46)	0.78
In-hospital mortality	14 (6.0)	34 (6.1)	0.99 (0.50-1.84)	0.89 (0.42-1.87)	0.75
			Rate ratio (95%CI)	Rate ratio (95%CI)	
Length of hospital, median (IQR)	8 (5-15)	9 (6-15)	1.01 (0.90-1.14)	1.06 (0.94-1.19)	0.34

IPTW: Inverse probability of treatment weighting; CI: Confidence interval; IQR: Interquartile range.

Table 6 Outcomes of subgroup analysis

Child-Pugh classification	Odds ratio (95%CI)	*P* value	*P* for interaction
A	0.87 (0.22–3.34)	0.84	0.32
B	0.79 (0.46–1.38)	0.41	
C	1.91 (1.20–3.02)	0.01	

Table 7 Outcomes of sensitivity analysis

Analysis method	Odds ratio (95%CI)	*P* value
IPTW	1.11 (0.61-1.99)	0.74
Propensity score matching	1.12 (0.62-2.03)	0.71
Duration of antibiotics		
2 d or more	1.14 (0.59-2.20)	0.70
3 d or more	1.05 (0.50-2.21)	0.91
4 d or more	0.96 (0.43-2.18)	0.93
Third-generation cephalosporins only	1.57 (0.64-3.87)	0.33

CI: Confidence interval; IPTW: Inverse probability of treatment weighting.

Our study's findings, revealing no significant benefit of antibiotic prophylaxis in patients undergoing EVL for esophageal variceal bleeding, add to the critical discourse on the necessity of routine prophylactic antibiotics in an era marked by escalating antibiotic resistance. The burgeoning concern for multidrug-resistant organisms (MDROs), highlighted in recent studies, is a pressing global health issue[42-44]. While our study did not directly address the intricate challenge of MDROs' emergence, the results imply that indiscriminate antibiotic use might not offer additional advantages and may, in fact, exacerbate the threat of antibiotic resistance. Consequently, our findings support a prudent reevaluation of antibiotic prophylaxis practices, especially in clinical environments where MDRO prevalence is high, and the risk of fostering resistance is a significant worry.

Patient groups for whom prophylactic antibiotic administration is beneficial must be identified. In our study, we demonstrated only the average effect across the population, showing that antibiotic prophylaxis is not effective. Previous reports have indicated differences in effectiveness based on the severity of the Child-Pugh classification. Although we conducted a subgroup analysis evaluating the interaction effect between antibiotic prophylaxis and the Child-Pugh classification on the primary composite outcome, we did not observe any significant results. Machine learning models are currently being used to identify heterogenous effects of antibiotic prophylaxis[45-47]. Using such methods, patients who would benefit from antibiotic prophylaxis must be identified.

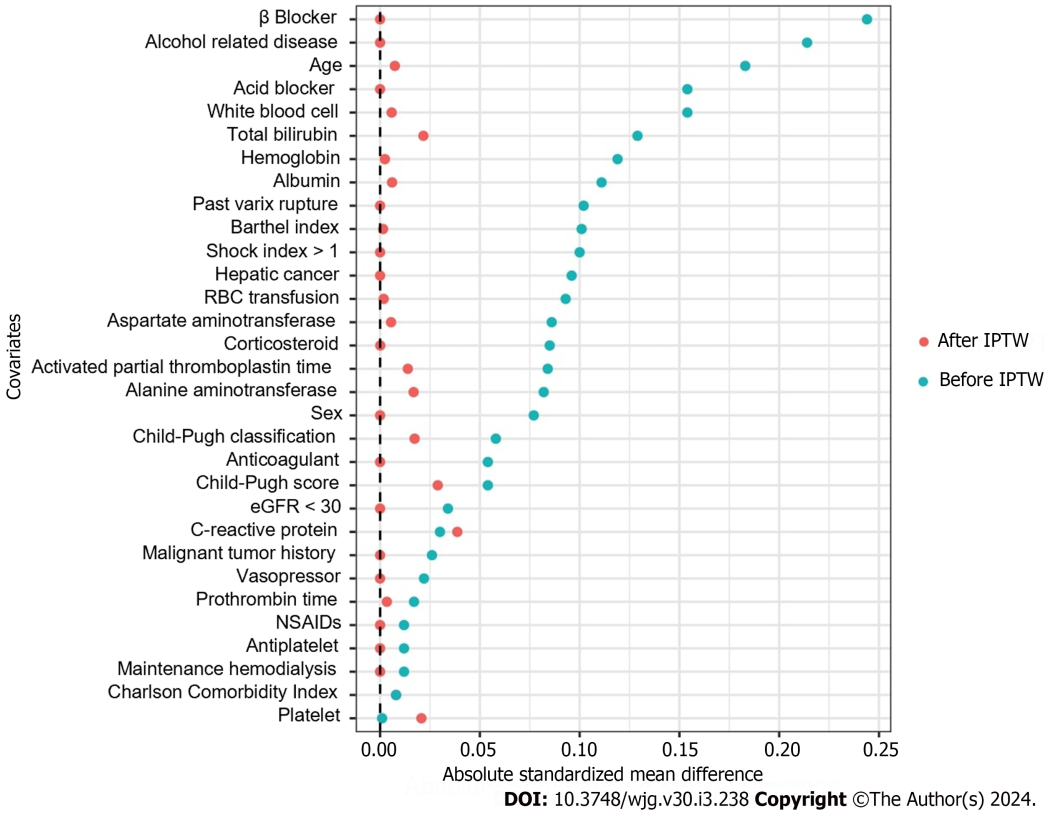

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Figure 3 Comparison of standardized mean difference before and after inverse probability of treatment weighting. IPTW: Inverse probability of treatment weighting; RBC: Red blood cell; eGFR: Glomerular filtration rate; NSAIDs: Nonsteroidal anti-inflammatory drugs.

Our study has several strengths. First, focusing on esophageal varix bleeding, this study was conducted on an unprecedented scale and comprised a wide sample of patients from multiple hospitals throughout various regions in Japan, bolstering the generalizability of our results. Second, the Tokushukai medical database offered us unique access to detailed blood test data, vital signs, and the ability to review electronic medical records in-depth. This enabled us to conduct a study with heightened precision.

Limitations

However, this study is not without limitations. First, due to the observational nature of this study, potential unmeasured confounding factors may be present. Second, the study is based on data from Japanese individuals, which limits the ability to generalize these findings to other populations or races. Third, the study encompasses only hemostasis information resulting from EVL. Patients who received treatment solely through EIS, balloon tamponade, or pharmacological interventions, such as somatostatin and vasopressors, were excluded. Fourth, treatment approaches differ notably between Japan and other countries. In Japan, a transjugular intrahepatic portosystemic shunt is not covered by insurance; therefore, no patients in our study received this treatment.

CONCLUSION

Our extensive multicenter observational study did not find a significant benefit to antibiotic prophylaxis for esophageal variceal bleeding treated with EVL. These results suggest that the recommendation for routine prophylactic antibiotic administration may not be universally essential. With growing concerns regarding the misuse of antibiotics and the consequential emergence of multidrug-resistant bacteria combined with advances in the management of esophageal variceal bleeding and liver cirrhosis treatment, there is a compelling need for a global reassessment of the necessity of routine antibiotic prophylaxis.

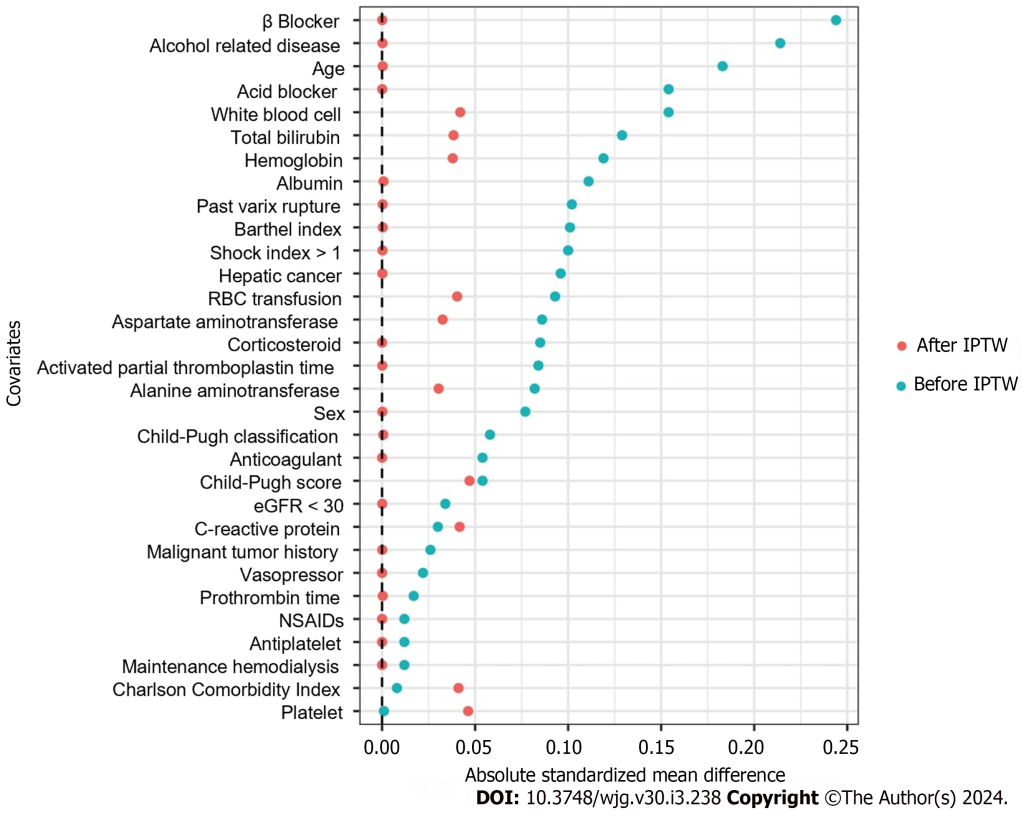

Figure 4 Comparison of standardized mean difference before and after propensity score matching. RBC: Red blood cell; eGFR: Glomerular filtration rate; NSAIDs: Nonsteroidal anti-inflammatory drugs; PTW: Inverse probability of treatment weighting.

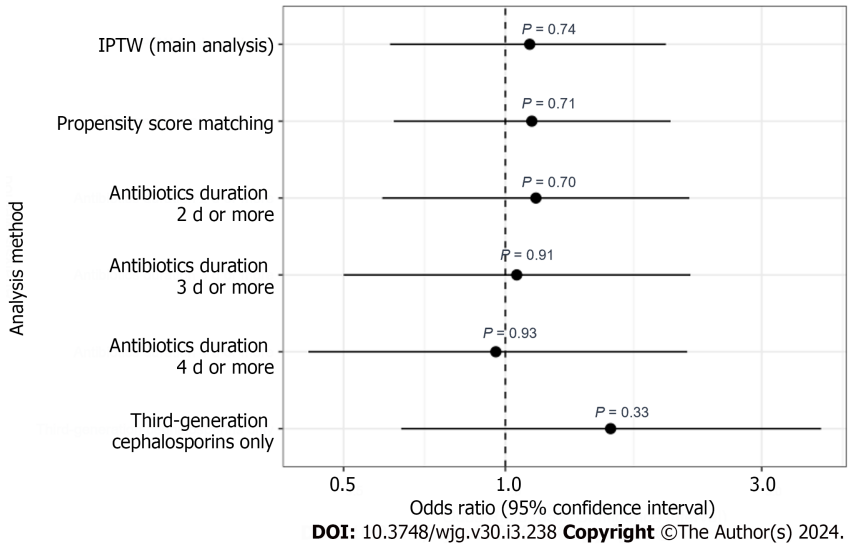

Figure 5 Forest plot of sensitivity analysis. IPTW: Inverse probability of treatment weighting.

ARTICLE HIGHLIGHTS

Research background

Esophageal variceal bleeding is a critical complication of liver cirrhosis, typically managed with endoscopic variceal ligation (EVL). While current Western guidelines advocate antibiotic prophylaxis post-EVL, the evolving landscape of cirrhosis management and the rise of multidrug-resistant bacteria necessitate a reevaluation of this practice.

Research motivation

This study was motivated by the need to reassess the effectiveness of routine antibiotic prophylaxis following EVL in the context of improved cirrhosis treatments and increasing concerns regarding antibiotic resistance. Understanding the real-

world impact of prophylaxis on patient outcomes may result in a more effective and judicious use of antibiotics.

Research objectives

The primary objective was to evaluate the effectiveness of antibiotic prophylaxis in patients undergoing EVL for esophageal variceal bleeding using data from multiple Japanese medical centers. The study aimed to provide evidence that could influence future guideline recommendations and clinical practice.

Research methods

A 13-year observational study was conducted, using the Tokushukai medical database that includes data from 46 hospitals. Patients were categorized into prophylaxis and non-prophylaxis groups, with outcomes measured in terms of mortality, rebleeding, and spontaneous bacterial peritonitis. Logistic regression, inverse probability of treatment weighting, subgroup, and sensitivity analyses were conducted.

Research results

The study included 790 patients, and the primary outcomes were not significantly different between the prophylaxis and non-prophylaxis groups. These findings persisted across various subgroups and sensitivity analyses, suggesting that routine antibiotic prophylaxis post-EVL may not be beneficial.

Research conclusions

These findings challenge the current standard of prescribing antibiotics following EVL for esophageal variceal bleeding. They highlight the need for a global reassessment of this practice, considering the minimal impact on patient outcomes and the broader context of antibiotic resistance.

Research perspectives

Future research should focus on personalized approaches to antibiotic use in cirrhosis-related procedures, considering patient-specific factors and broader public health concerns. Further studies should also explore alternative strategies for managing complications in patients with liver cirrhosis.

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FOOTNOTES

Author contributions: Ichita C contributed to the planning, data gathering, literature review, writing, and editing of the manuscript; Shimizu S and Goto T are experts in epidemiological statistics and were responsible for the causal inference and analysis methods in this study; Haruki U is an expert in portal hypertension and provided appropriate advice in this field, whereas Itoh N is an expert in infectious diseases and offered appropriate guidance on antibiotics; Iwagami M is a leading expert in the use of the Tokushukai medical database and provided appropriate advice on its utilization; Sasaki A is an expert in endoscopy and provided valuable advice on endoscopic hemostasis; all the authors reviewed the various drafts of the manuscript and have approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the Future Medical Research Centre Ethical Committee (Approval No. TGE02100-02).

Informed consent statement: Owing to the observational nature of the study, where patient data were accessed from hospital medical records without taking biological samples from patients, informed patient consent was not deemed to be necessary. Instead, an opt-out method directed at patients was employed on the website of each hospital.

Conflict-of-interest statement: All other authors have nothing to disclose.

Data sharing statement: Due to privacy and ethical considerations, the data supporting the findings of this study is not publicly available. However, the study protocol and analysis code can be made available upon contacting the corresponding author. As for the participant data, ethical approval is required for access. The corresponding author can facilitate this process upon receipt of an appropriate request. After ethical approval, data sharing will be possible. The process of accessing the data will be carried out in accordance with ethical guidelines, ensuring respect for participant privacy and confidentiality.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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ORIGINAL ARTICLE

Basic Study

SLC6A14 promotes ulcerative colitis progression by facilitating NLRP3 inflammasome-mediated pyroptosis

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Abstract

BACKGROUND

Ulcerative colitis (UC) is an inflammatory condition with frequent relapse and recurrence. Evidence suggests the involvement of SLC6A14 in UC pathogenesis, but the central regulator remains unknown.

AIM

To explore the role of SLC6A14 in UC-associated pyroptosis.

METHODS

Quantitative real-time polymerase chain reaction (qRT-PCR), immunoblotting, and immunohistochemical were used to assess SLC6A14 in human UC tissues. Lipopolysaccharide (LPS) was used to induce inflammation in FHC and NCM460 cells and model enteritis, and SLC6A14 levels were assessed. Pyroptosis markers were quantified using enzyme-linked immunosorbent assay, Western blotting, and qRT-PCR, and EdU incubation, CCK-8 assays and flow cytometry were used to examine proliferation and apoptosis. Mouse models of UC were used for verification.

RESULTS

SLC6A14 was increased and correlated with NLRP3 in UC tissues. LPS-induced FHC and NCM460 cells showed increased SLC6A14 levels. Reducing SLC6A14 increased cell proliferation and suppressed apoptosis. Reducing SLC6A14 decreased pyroptosis-associated proteins (ASC, IL-1β, IL-18, NLRP3). NLRP3 overexpression counteracted the effects of sh-SLC6A14 on LPS-induced FHC and

NCM460 cell pyroptosis. SLC6A14 improved the mucosa in mice with dextran sulfate sodium-induced colitis.

CONCLUSION
SLC6A14 promotes UC pyroptosis by regulating NLRP3, suggesting the therapeutic potential of modulating the SLC6A14/NLRP3 axis.

Key Words: Ulcerative colitis; SLC6A1; NLRP3; Pyroptosis; Inflammasome

Core Tip: Ulcerative colitis (UC) is an inflammatory condition associated with frequent relapse and recurrence. Dysregulation of intestinal epithelial cells (IECs) and mucosal barrier impairment contribute to sustained inflammation in UC. Hence, an in-depth exploration of the triggers and mechanisms of IEC death could result in efficacious therapeutic options for UC patients. Here, we demonstrated the close involvement of SLC6A14 in promoting pyroptosis in the context of UC by upregulating NLRP3 expression. These findings indicate the potential of targeting SLC6A14/NLRP3 axis-mediated pyroptosis as a promising therapeutic strategy for treating UC. Our research provides valuable insights into the mechanisms driving UC pathogenesis and offers a possible direction for developing innovative treatments to alleviate the impact of this chronic inflammatory disorder.

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INTRODUCTION

Ulcerative colitis (UC) is an inflammatory condition associated with frequent relapse and recurrence. Its prevalence is increasing in developing nations as a result of increased consumption of Western diets[1,2]. UC is typified by ulceration and inflammation of the colonic and rectal mucosa, leading to symptoms such as pain, diarrhea, and bleeding[3,4]. UC has a complex etiology involving genetic, environmental, and immunological elements[5]. Dysregulation of intestinal epithelial cells (IECs) and mucosal barrier impairment contribute to sustained inflammation[6,7]. The death of IECs disrupts the balance between intestinal microorganisms and the host, the regulation of mucosal immunity, nutrient absorption, and the integrity of the mucosal barrier, culminating in recurrent, prolonged colitis[8,9]. Hence, an in-depth exploration of the triggers and mechanisms of IEC death could result in efficacious therapeutic options for UC patients.

Recent investigations underscore the vital role of NLRP3, the NLR family pyrin domain containing 3, inflammasome in UC[10,11]. This is activated in conditions of tissue damage, pathogen infection, and oxidative stress[12-14]. NLRP3 interacts with ASC through its N-terminal PYD domain, upregulating pro-IL-1β and pro-IL-18 and thus augmenting inflammation[15,16]. Consequently, NLRP3, in conjunction with IL-1β, IL-18, and ASC, has emerged as a prospective therapeutic target for ameliorating pyroptosis and mitigating inflammatory responses in UC.

The solute carrier (SLC) transporter family has been recognized as a significant regulator of ferroptosis and diverse cancers[17]. SLC6A14, SLC family 6 member 14, belongs to the SLC transporter family and may contribute to UC[18,19]. In this study, we investigated SLC6A14, which is upregulated in various colonic disorders, including UC, to evaluate its role in modulating IEC pyroptosis within the context of UC by using human IECs and murine models. Our findings revealed robust SLC6A14 expression in UC patients, experimental colitis models, and pyroptosis-induced cell models. Importantly, a positive correlation between SLC6A14 and NLRP3 expression was identified. Downregulating SLC6A14 reduced the levels of pyroptosis-associated proteins, including NLRP3, and the production of IL-18 and IL-1β. Mechanistically, SLC6A14 was a positive regulator of NLRP3, a central figure in inflammasome-driven pyroptosis. The promotion of pyroptosis by SLC6A14 by targeting NLRP3 was verified in LPS-treated IECs and a UC mouse model, indicating the potential of SLC6A14 in the treatment of UC.

MATERIALS AND METHODS

Subjects and samples

Colorectal mucosal biopsies were procured from two groups: healthy individuals (n = 29) and patients diagnosed with UC (n = 55). These biopsies were obtained during endoscopic examinations conducted at the Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital [Lunshen (Yan)2022-380]. The study protocol was granted ethics approval by the Ethics Committee of the same institution and was performed in compliance with the Declaration of Helsinki (2013 revision). Prior to the extraction of the tissue samples, written informed consent was provided by each

participant. The collected tissue specimens were promptly cryopreserved using liquid nitrogen for subsequent analysis.

Histopathological assessment

Hematoxylin and eosin (HE) staining was performed using established protocols. In summary, murine and human colonic samples were initially fixed (4% paraformaldehyde) and embedded in paraffin before being sectioned (5 μm). The sections were stained with hematoxylin to visualize the nuclei, and eosin was used to reveal cytoplasmic structures.

Immunohistochemical staining

Immunohistochemical (IHC) staining was performed as previously described using a DAB kit (Gene Tech, Shanghai, China). In summary, colonic tissue sections were treated overnight at 4 °C with an anti-SLC6A14 antibody (Abcam Cat# ab254786, RRID:AB_3073883) at a 1:200 dilution. Then, a subsequent incubation was conducted with an appropriate HRP-conjugated secondary antibody for 1 h at 37 °C. The reactions were visualized after incubation with DAB (brown) with hematoxylin counterstaining (purple). The prepared tissue sections were subsequently observed and imaged by phase-contrast microscopy (Leica, Germany). An evaluation of these images was performed by two independent blinded pathologists, and the slides were assessed by multiplying the staining intensity (ranging from grades 0 to 5, with 0 indicating negative and 5 indicating strong positivity) by the corresponding positivity score (ranging from 0 to 5, where 0% to 100% was indicated by the scores).

Cell culture

The normal colon epithelial cell lines FHC and NCM460 were sourced from the National Collection of Authenticated Cell Cultures (Shanghai, China). FHC and NCM460 cells were grown in RPMI-1640 medium (Gibco, United States) with 10% fetal bovine serum (Gibco). To establish a cellular model of colitis, the cells were incubated with 10 ng/mL lipopolysaccharide (LPS) for 6 h[20,21]. The cells were grown in 6-well plates at 37 °C and 5% CO_2.

Plasmid construction and cell transfection

To downregulate SLC6A14, shRNA sequences targeting SLC6A14 and the negative control (NC) shRNA were obtained from GenePharma (Shanghai, China). Additionally, the NLRP3 overexpression plasmid pcDNA4.0-NLRP3 and the empty pcDNA4.0 plasmid were procured from Synbio Technologies Co. Ltd.™ (Suzhou, China). Cells were grown to 70% confluence and transfected with the plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, United States) according to the provided directions. RNA was isolated after 48 h, and protein was isolated after 72 h.

Western blotting

SLC6A14 protein levels in cells and patient biopsy tissues were analyzed by Western blotting. Proteins were extracted from the samples using RIPA buffer containing protease and phosphatase inhibitors, and concentrations were assessed using a BCA kit (Thermo Fisher). Aliquots (30 μg) were separated on 10%-12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Bedford, MA, United States). Primary antibodies against SLC6A14 (Thermo Fisher Scientific Cat# PA5-87998, RRID:AB_2804576, 1:1500), NLRP3 (Abcam Cat# ab263899, RRID:AB_2889890, 1:1000), ASC (Abcam Cat# ab283684, RRID:AB_3073880, 1:800), pro-IL-18 (Proteintech Cat# 10663-1-AP, RRID:AB_2123636, 1:1000), IL-1β (Abcam Cat# ab254360, RRID:AB_2936299, 1:1000), IL-18 (Abcam Cat# ab207324, RRID:AB_3073881, 1:1000), and GAPDH (Abcam Cat# ab9485, RRID:AB_307275, 1:1000) were used to probe the blots overnight at 4 °C, after which the blots were incubated with secondary antibodies for 60 min at room temperature. Bands were visualized using the ECL Western Blotting Detection System (Amersham, United Kingdom) and quantified using ImageJ. The internal reference was GAPDH. Three separate experiments were conducted.

Quantitative real-time polymerase chain reaction

RNA was extracted from the samples using TRIzol reagent (Invitrogen, United States). cDNA was reverse transcribed using the Bestar qPCR RT kit (DBI Bioscience, #2220, Germany). To examine SLC6A14, the following primer sequences were used: forward primer 5′-TGCACCTGCTACCAGTCAAG-3′ and reverse primer 5′-GTCCATGGTTCACTCCCTCG-3′. The GAPDH primers were forward, 5′-GACAGTCAGCCGCATCTTCT-3′ and reverse, 5′-GCGCCCAATACGACCAAATC-3′. To assess SLC6A14 expression, Bestar qPCR MasterMix (DBI Bioscience, #2043) was used. GAPDH was used as the internal reference. The $2^{-\Delta\Delta Ct}$ method was used to quantify gene expression according to established procedures[22].

Cell viability assessment

Cell proliferation was assessed using CCK-8 assays (Dojindo Laboratories, Japan). Cells (2×10^3/well) were plated in 96-well plates and grown at 37 °C for 2 h. Absorbances at 450 nm were measured every 24 h for 72 h.

EdU assay

Cellular proliferation was assessed using an EdU assay kit (Ribobio, China). Cells were seeded on confocal plates at 10×10^5 cells per well. Next, the cultures were treated with 50 μM EdU solution for 120 min at 37 °C, after which they were fixed (4% formaldehyde, 30 min) and permeabilized (0.1% Triton X-100, 20 min) before the nuclei were stained with Hoechst and the samples were evaluated by fluorescence microscopy.

Apoptosis assessment

Apoptosis was assessed using flow cytometry. FHC and NCM460 cells were transfected and grown to 90% confluence in 6-well plates. The cells were harvested and treated with 10 μL of reagent from the Annexin V-FITC/PI apoptosis kit (Lianke Biotech, China) (10-15 min, room temperature, away from light). The cells were evaluated on a FACSCalibur flow cytometer (BD Biosciences, United States).

Enzyme-linked immunosorbent assay

Culture supernatants and standards were incubated in precoated enzyme-linked immunosorbent assay (ELISA) plates for 2 h at 37 °C. This was followed by two washes and probing with an HRP-conjugated secondary antibody (1 h, 37 °C). After further rinsing, the plates were incubated with the chromogenic solution (10-15 min, room temperature, away from light). After the addition of the stop solution, the absorbances at 450 nm were measured. The specific antibodies used were anti-human IL-1β (Abcam, ab214025), anti-human IL-18 (Abcam, ab215539), anti-mouse IL-1β (Abcam, ab197742), and anti-mouse IL-18 (Abcam, ab216165). All assays were performed in triplicate.

Animal selection and experimental design

Kunming (KM) mice (male, 20 ± 2 g) were sourced from the Experimental Animal Center of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, China. All animal experiments were performed with the approval of the Animal Care and Use Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital (Lunshen (Yan)2022-380). The animals were maintained at a temperature of 22-24 °C with 20% humidity and a 12-h light/dark cycle. They were provided unrestricted access to standard feed and water. After one week of acclimatization, 48 mice were randomly assigned to four groups, with each group containing 12 mice. In Group 1, the mice were provided regular drinking water. Groups 2 to 4 were given drinking water containing 3% dextran sulfate sodium (DSS) (MW 36 kDa to 50 kDa, MP Biomedicals LLC, Santa Ana, CA, United States) for seven days to induce UC. Group 3 received 100 μL of control lentivirus (LV-sh-CTRL, obtained from GenePharma, Suzhou, China) *via* tail vein injection twice per week, while Group 4 was injected with 100 μL of the SLC6A14 knockdown lentivirus (LV-sh-SLC6A14, obtained from GenePharma) *via* tail vein injection twice per week. The mice were euthanized using 1% sodium pentobarbital (administered *via* intraperitoneal injection) and sacrificed after a 14-d period. Colon lengths were measured, and the colons were immediately rinsed with chilled physiological saline. Colon samples were collected; some samples were immediately fixed (10% formalin), while other samples were frozen at -80 °C for further analysis.

Assessment of the disease activity index

The animals were subjected to daily evaluations to gauge UC severity, and body weights, observable rectal bleeding, and stool consistency were assessed. A comprehensive disease activity index (DAI) score was assessed using an established method to quantify disease severity. The DAI score was determined by combining three individual scores: the weight loss rate score, the stool trait score, and the occult blood score. These three scores were added together and then divided by three to yield the final DAI score. This systematic approach provides an accurate representation of the overall disease severity experienced by the mice.

Colon histopathology

Colon lengths were measured, and a sample (0.5 cm) was collected and fixed (10% formalin, 24 h). Following fixation, the tissue was paraffin-embedded and sectioned (5-μm sections) before undergoing HE staining. Each sample was meticulously evaluated at a magnification of 100×.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad, United States). The data are presented as the mean ± SD. Group differences were analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni post hoc test. For nonparametric data, the Kruskal-Wallis test was used, and continuous variables were analyzed using one-way ANOVA. P values < 0.05 were considered statistically significant.

RESULTS

SLC6A14 is increased in UC

To examine the role of SLC6A14 as a diagnostic biomarker for UC, we examined SLC6A14 protein expression. Histopathological analysis revealed that UC samples showed typical colonic inflammation, which was particularly noticeable in UC biopsies compared to healthy adjacent tissue. IHC analysis of UC samples revealed increased SLC6A14 protein levels, which led to higher histological scores (Figure 1A and E). Additionally, a comprehensive investigation was performed using a dataset containing 55 UC tissues and 29 normal tissues, and SLC6A14 mRNA levels were quantified by quantitative real-time polymerase chain reaction. Significant increases in the mRNA expression of SLC6A14 were observed in UC tissues relative to control tissues (P < 0.01, Figure 1B). Notably, these expression levels were further confirmed by Western blot analysis (Figure 1C and D).

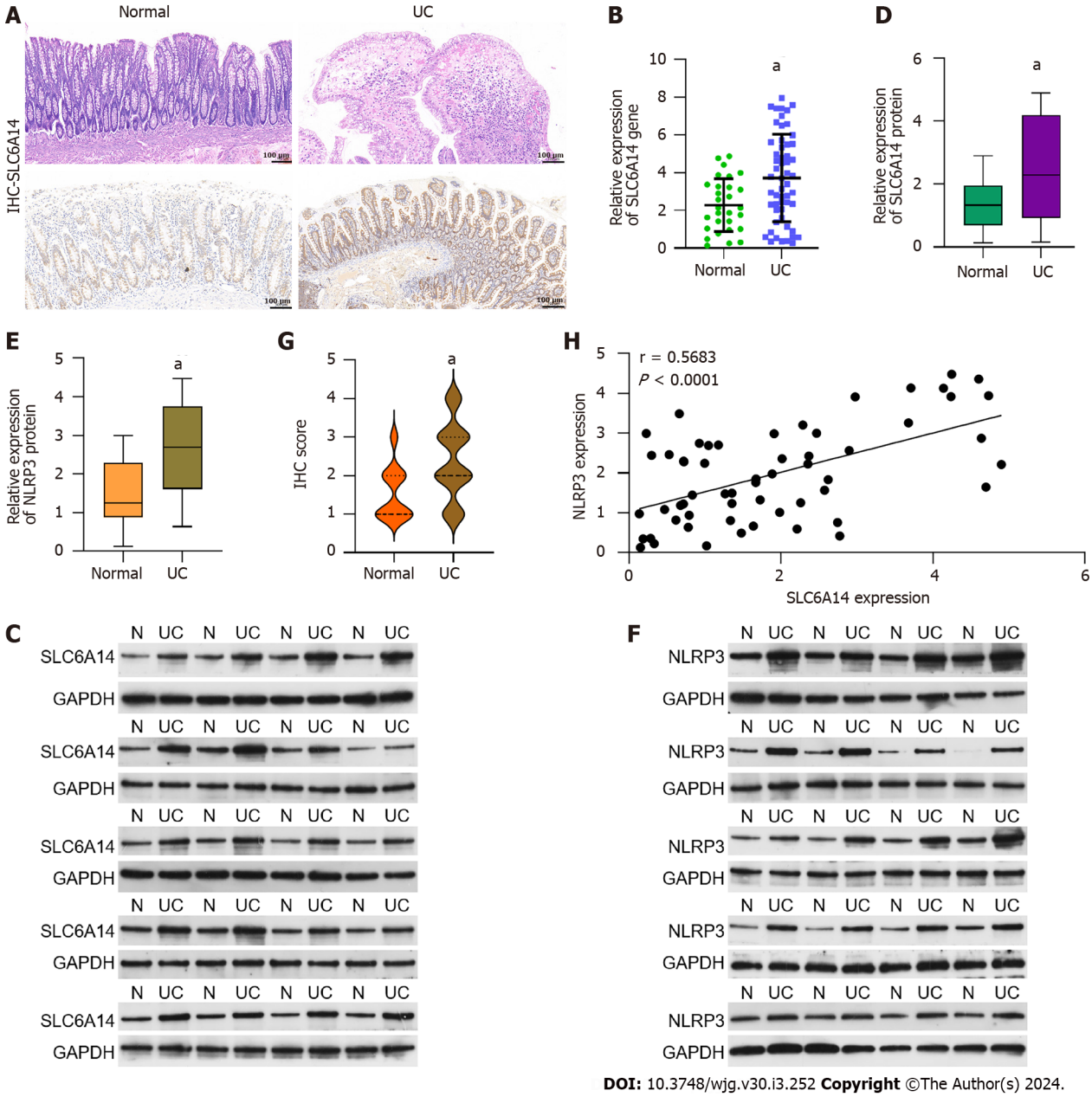

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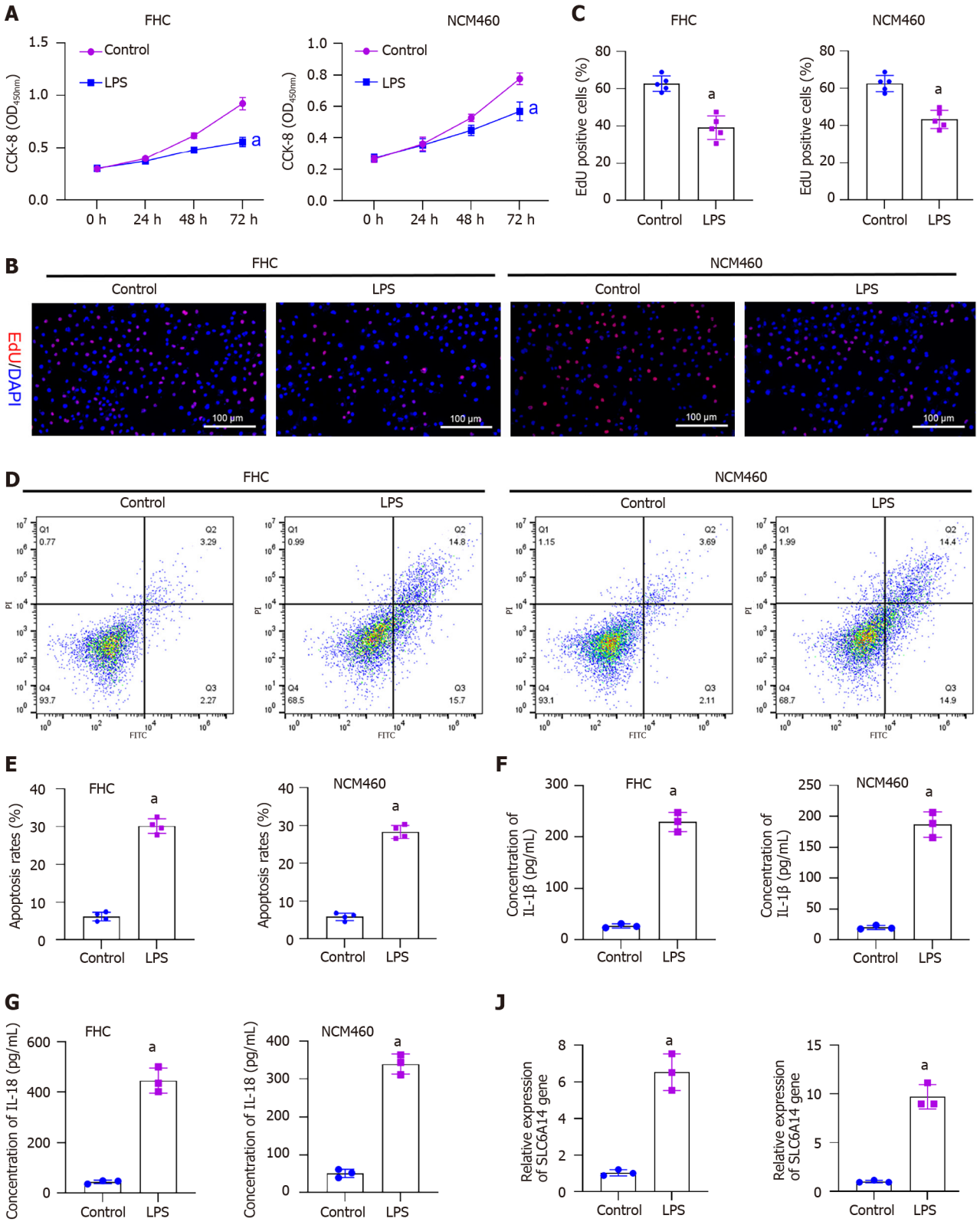
Figure 1 SLC6A14 in ulcerative colitis tissues. A: Hematoxylin and eosin staining and immunohistochemical (IHC) analysis of SLC6A14 expression in normal and ulcerative colitis (UC) tissues (magnification, × 200; scale bar = 100 μm); B: SLC6A14 mRNA levels were assessed by real-time polymerase chain reaction in UC (n = 55) and normal tissues (n = 29); C and D: SLC6A14 protein levels in UC (n = 55) and normal tissue samples (n = 29); E: Quantification of the IHC scores for SLC6A14 expression; F and G: NLRP3 protein levels in UC (n = 55) and normal tissue samples (n = 29); H: Association between SLC6A14 and NLRP3 levels in human colonic tissues. The data represent the means ± SD. $^{a}P < 0.01$ *vs* the controls. UC: Ulcerative colitis.

SLC6A14 contributes to pyroptosis

To explore the correlation between SLC6A14 and pyroptosis, we examined NLRP3 protein levels in the tissues of UC patients. The results showed substantial upregulation of the NLRP3 protein in UC tissues compared to their normal counterparts (Figure 1F and G). Moreover, our investigation revealed a positive correlation between SLC6A14 expression and NLRP3 expression (Figure 1H). These findings indicate a pronounced increase in SLC6A14 expression in the context of UC.

LPS induces pyroptosis in IECs

To mimic UC-induced inflammation, we treated FHC and NCM460 cells, which are normal IECs, with 10 ng/mL LPS. This stimulation reduced cell proliferation (Figure 2A-C). In parallel, flow cytometry indicated an increase in cell death following LPS exposure (Figure 2D and E). Moreover, LPS increased inflammatory factor levels in the cells. Pyroptosis has been shown to enhance inflammation, and increased levels of IL-1β and IL-18 were observed after LPS exposure (Figure 2F and G). Subsequently, we used Western blot analysis to assess key effector proteins associated with pyroptotic pathways, including ASC, pro-IL-1β, pro-IL-18, IL-1β, IL-18, and NLRP3. Increases in these proteins were observed after LPS stimulation of FHC cells (Figure 2H and Supplementary Figure 1). Notably, SLC6A14 levels in LPS-treated IECs were


A
FHC
NCM460
Control
LPS
CCK-8 (OD450nm)
0 h
24 h
48 h
72 h
a
C
EdU positive cells (%)
B
EdU/DAPI
100 μm
D
Q1
Q2
Q3
Q4
PI
FITC
E
Apoptosis rates (%)
F
Concentration of IL-1β (pg/mL)
G
Concentration of IL-18 (pg/mL)
J
Relative expression of SLC6A14 gene

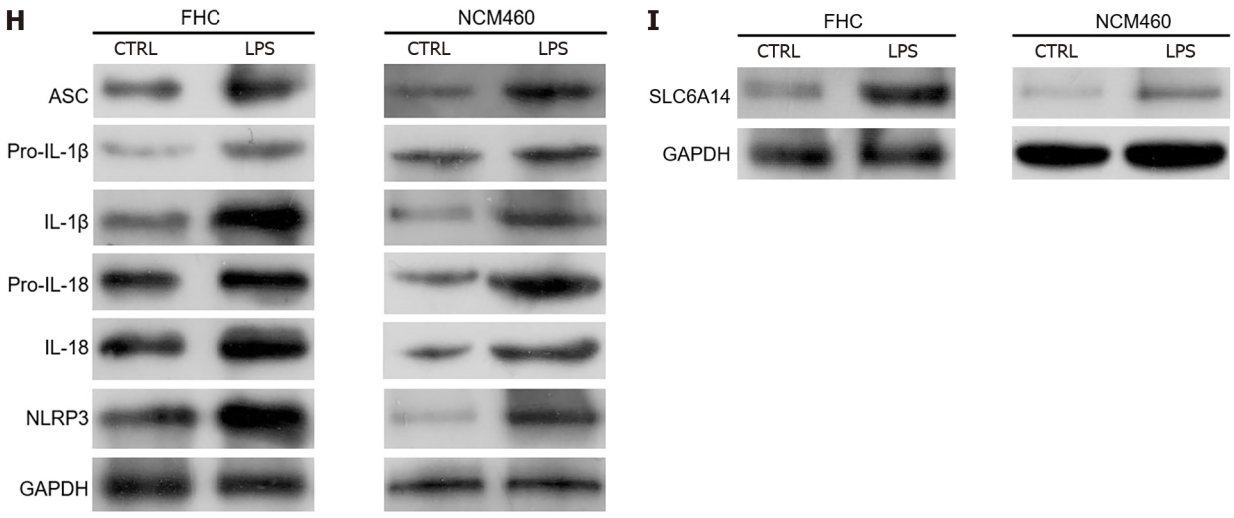


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Figure 2 Induction of pyroptosis by lipopolysaccharide in FHC and NCM460 intestinal epithelial cells. A: CCK-8 assay showing the impact of lipopolysaccharide (LPS) on FHC and NCM460 cell proliferation; B: EdU assay showing the proliferation of LPS-treated and untreated FHC and NCM460 cells; C: Quantification of EdU-positive cells; D and E: Flow cytometric analysis of apoptosis in cells with and without LPS treatment; F and G: Enzyme-linked immunosorbent assay analysis of the effects of LPS on IL-1β and IL-18 secretion; H: Western blot analysis showing the levels of pyroptosis-associated proteins in LPS-treated and untreated cells; I and J: Western blot analysis showing SLC6A14 levels in LPS-treated and control cells. $^{a}P < 0.01$ *vs* controls. LPS: Lipopolysaccharide.

markedly increased relative to those in the controls (Figure 2I and J). These findings suggest that LPS induces pyroptosis in FHC and NCM460 cells, underscoring the potential involvement of SLC6A14 in this process.

Suppressing SLC6A14 enhances proliferation and reduces apoptosis in LPS-induced epithelial cells

Given the close association between proinflammatory cytokine production and intestinal inflammation, we aimed to determine whether decreasing SLC6A14 expression could mitigate inflammation in the LPS-stimulated epithelial cell model. We introduced the sh-SLC6A14 plasmid into FHC and NCM460 cells to knockdown SLC6A14 expression. Subsequently, the cells were subjected to LPS stimulation (10 ng/mL) for 24 h. As shown in Figure 3A and B, there was a significant reduction in SLC6A14 expression in the LPS + sh-SLC6A14 group. This observation demonstrated the efficacy of the synthetic sh-SLC6A14 vector. Subsequently, we revealed that SLC6A14 downregulation increased cell proliferation (Figure 3C-E). Moreover, flow cytometry showed that suppressing SLC6A14 expression mitigated apoptosis induced by LPS (Figure 3F and G). Collectively, these findings suggested that suppressing SLC6A14 expression enhanced proliferation and reduced apoptosis in FHC and NCM460 cells subjected to LPS stimulation.

SLC6A14 enhances LPS-induced inflammatory cytokine secretion

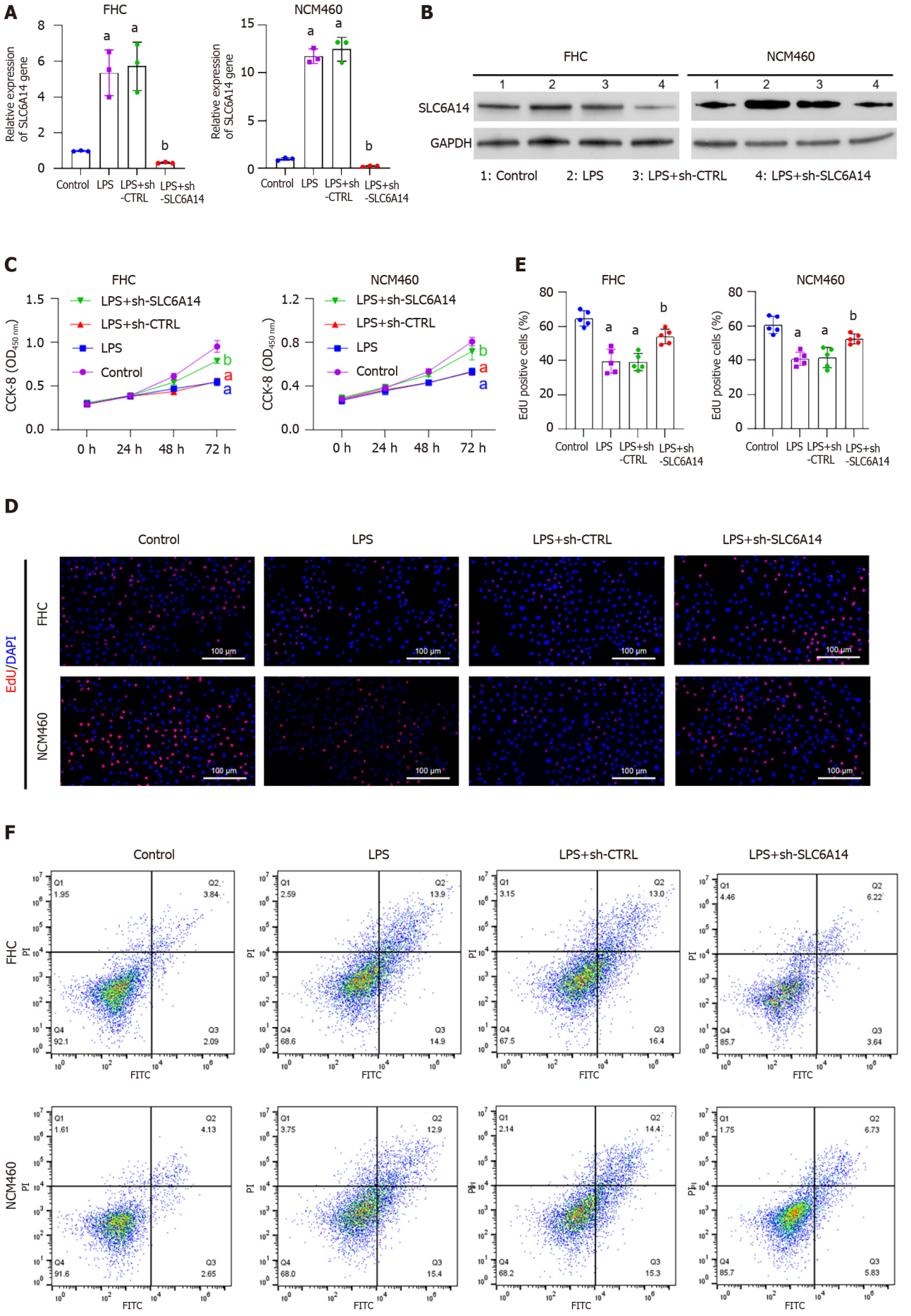
Our subsequent experiments revealed that SLC6A14 could promote the secretion of the pyroptosis-associated proteins IL-1β and IL-18 by LPS-induced IECs (Figure 4A). In addition, Western blotting showed that reducing SLC6A14 expression inhibited key pyroptotic effector proteins (Figures 4B and Supplementary Figure 2). These findings suggest that SLC6A14 can amplify LPS-induced inflammatory cytokine secretion.

NLRP3 overexpression counteracts the inhibitory effect of SLC6A14 knockdown on LPS-induced pyroptosis

The EdU and CCK-8 assay results showed that upregulating NLRP3 counteracted the inhibitory effects of SLC6A14 knockdown induced by the sh-SLC6A14 plasmids on the proliferation of LPS-treated FHC and NCM460 cells (Figure 5A-C). Flow cytometry further revealed that NLRP3 upregulation mitigated the impact of SLC6A14 downregulation on apoptosis in LPS-induced FHC cells (Figure 5D and E). Moreover, ELISA revealed that NLRP3 overexpression reversed the SLC6A14-induced suppression of IL-18 and IL-1β production in both cell lines after LPS treatment (Figure 6A). Finally, Western blotting showed that increasing NLRP3 expression increased the levels of ASC, IL-1β, IL-18, and NLRP3. This effect abrogated the inhibitory effect of SLC6A14 downregulation of these protein levels (Figure 6B and Supplementary Figure 3). Taken together, these results demonstrate the involvement of SLC6A14 in NLRP3-mediated pyroptosis.

SLC6A14 downregulation attenuates DSS-induced UC in vivo

The colons of UC mice were markedly shorter than those of the controls. The downregulation of SLC6A14 mitigated the reduction in colon length induced by DSS (Figure 7A). DSS-treated animals and those that received a combination of DSS and the control vector exhibited greater fluctuations in weight relative to the controls. Furthermore, animals in the DSS and DSS plus vector groups experienced more substantial weight loss relative to those in the DSS plus SLC6A14 vector group (Figure 7B). An increase in the DAI was observed in the DSS-treated groups, as indicated by markedly increased DAI scores compared with those in the controls. Notably, lower DAI scores were observed in the DSS plus SLC6A14 vector group relative to the DSS-only group (Figure 7C). Additionally, histopathological analysis revealed that downreg-


A
FHC
NCM460
Relative expression of SLC6A14 gene
Control
LPS
LPS+sh -CTRL
LPS+sh -SLC6A14
B
FHC
NCM460
SLC6A14
GAPDH
1: Control
2: LPS
3: LPS+sh-CTRL
4: LPS+sh-SLC6A14
C
FHC
NCM460
LPS+sh-SLC6A14
LPS+sh-CTRL
LPS
Control
CCK-8 (OD450 nm)
0 h
24 h
48 h
72 h
E
FHC
NCM460
EdU positive cells (%)
D
Control
LPS
LPS+sh-CTRL
LPS+sh-SLC6A14
EdU/DAPI
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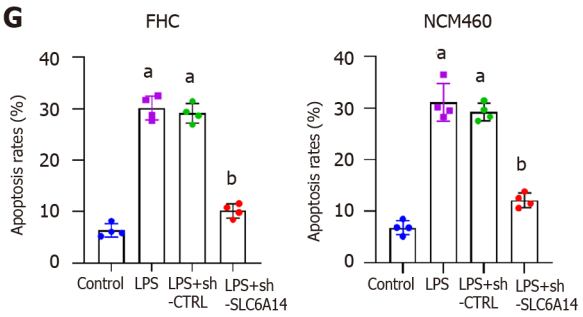

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Figure 3 Downregulating SLC6A14 promotes proliferation and inhibits apoptosis in lipopolysaccharide-treated intestinal epithelial cells. A and B: The expression of SLC6A14 in lipopolysaccharide (LPS)-, LPS+shCTRL-, and LPS+sh-SLC6A14-treated FHC and NCM460 cells was assessed by quantitative real-time polymerase chain reaction (A) and Western blotting (B); C: Cell viability was determined by CCK-8 assays; D and E: An EdU assay was used to detect cell proliferation, and the scale bar represents 100 μm; F and G: Flow cytometry showing apoptosis in FHC and NCM460 cells, followed by quantitative analyses. $^{a}P < 0.01$ *vs* controls; $^{b}P < 0.01$ *vs* LPS+sh-CTRL. LPS: Lipopolysaccharide.

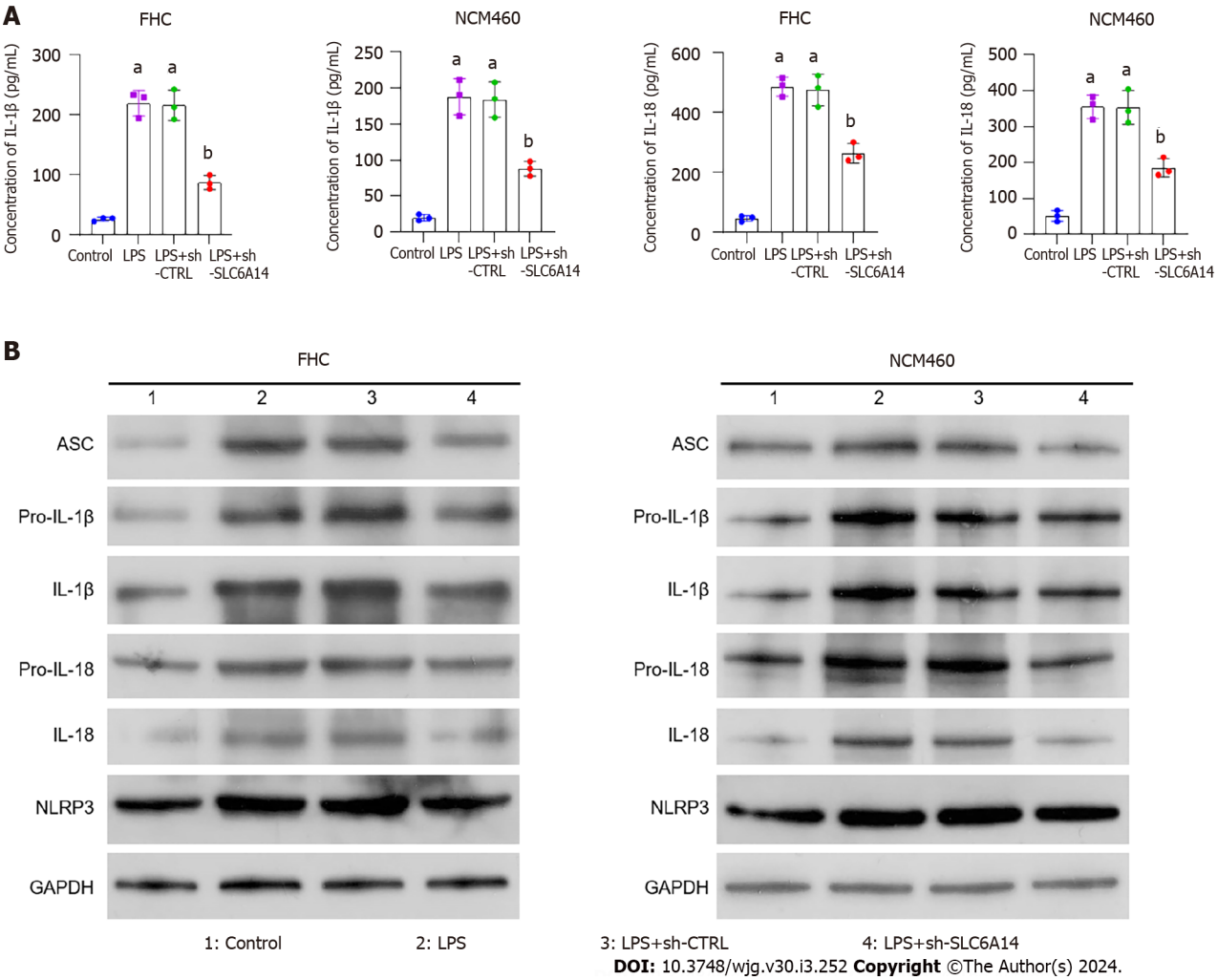

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Figure 4 SLC6A14 enhances lipopolysaccharide-induced inflammatory cytokine secretion. A: Enzyme-linked immunosorbent assay analysis of IL-1 β and IL-18; B: Western analysis of the levels of pyroptosis-associated proteins. The data are means ± SD ($n = 5$). $^{a}P < 0.01$ *vs* controls; $^{b}P < 0.01$ *vs* LPS+sh-CTRL. LPS: Lipopolysaccharide.

ulating SLC6A14 significantly alleviated colonic inflammation induced by DSS and reduced epithelial crypt numbers, mucosal barrier disruption, and inflammatory cell infiltration (Figure 7D). Intriguingly, DSS dramatically induced SLC6A14 expression, while SLC6A14 expression was markedly decreased in DSS plus SLC6A14 knockdown mice compared to other DSS-treated mice (Figure 7E and F). It was also found that SLC6A14 downregulation reduced cytokine production. Thus, our results revealed that downregulating SLC6A14 ameliorated pyroptosis induced by activation of the

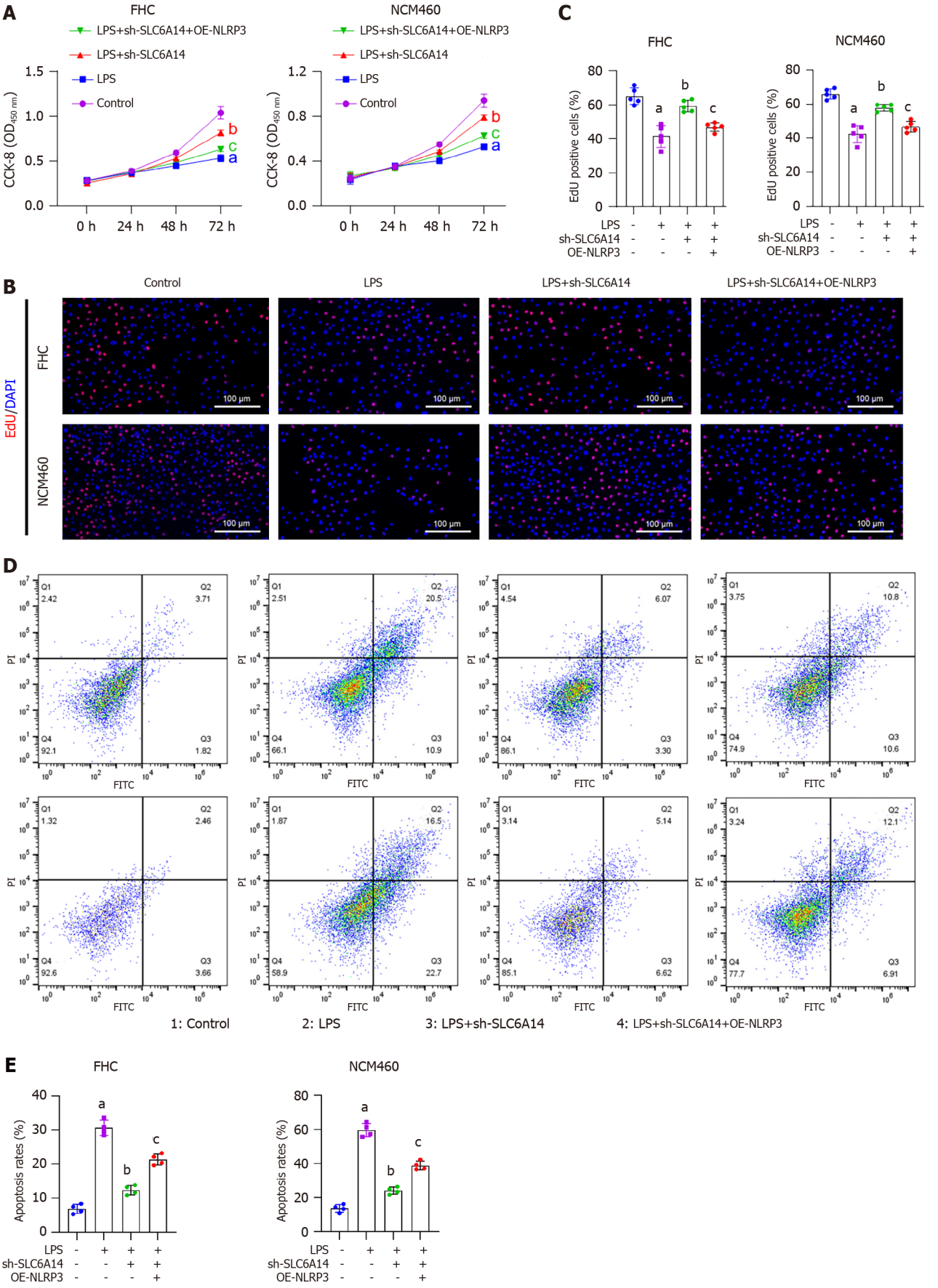

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Figure 5 NLRP3 overexpression reverses the suppressive effect of SLC6A14 knockdown on lipopolysaccharide-induced FHC cell pyroptosis. A: CCK-8 assays were performed to determine whether NLRP3 overexpression counteracted the inhibitory effect of SLC6A14 knockdown on

lipopolysaccharide (LPS)-induced intestinal epithelial cell (IEC) proliferation; B and C: EdU staining was performed to assess whether NLRP3 overexpression could reverse SLC6A14-mediated promotion of proliferation in LPS-stimulated epithelial cell models; D: Flow cytometry was used to investigate whether NLRP3 overexpression could reverse the proapoptotic effects of SLC6A14 silencing on LPS-treated IECs; E: Quantification of apoptosis. [a]*P* < 0.01 *vs* controls; [b]*P* < 0.01 *vs* LPS; [c]*P* < 0.01 *vs* LPS+sh-SLC6A14. LPS: Lipopolysaccharide; OE: Overexpression.

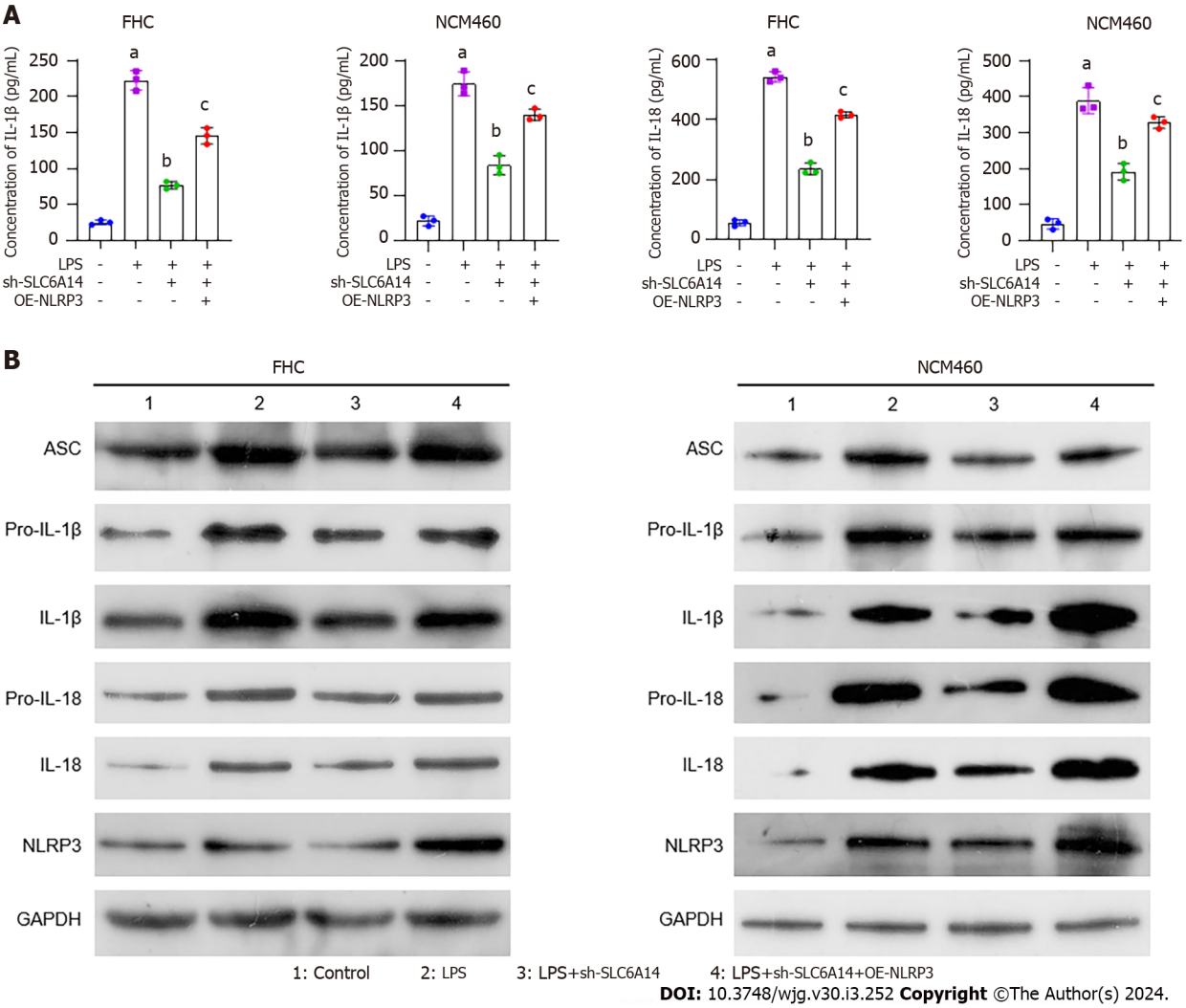

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Figure 6 NLRP3 overexpression reverses the inhibitory effect of SLC6A14 knockdown on lipopolysaccharide-induced FHC cell pyroptosis. A: Effects of NLRP3 overexpression on IL-1β and IL-18 production facilitated by SLC6A14 in lipopolysaccharide (LPS)-treated intestinal epithelial cells (IECs), as shown by enzyme-linked immunosorbent assay; B: Western analysis of the effects of NLRP3 overexpression on pyroptosis-associated protein levels mediated by SLC6A14 in IECs after LPS treatment. [a]*P* < 0.01 *vs* controls; [b]*P* < 0.01 *vs* LPS; [c]*P* < 0.01 *vs* LPS+sh-SLC6A14. LPS: Lipopolysaccharide; OE: Overexpression.

NLRP3 inflammasome after DSS treatment (Figure 7G). Additionally, SLC6A14 transfection reduced NLRP3 levels in the colons of mice following DSS treatment (Figures 7H and Supplementary Figure 4). In summary, our findings suggest that the downregulation of SLC6A14 effectively protected colon tissue integrity and mitigated the morphological changes caused by DSS, suggesting its potential for treating UC.

DISCUSSION

UC is associated with chronic inflammation in the colonic mucosa, and new forms of treatment are urgently needed[23-25]. Recent studies focusing on the pathogenesis of UC highlight the involvement of an aberrant immune response and dysregulated inflammation in UC development[26,27]. Invading pathogens and threats are identified by PAMPs and DAMPs associated with the innate immune response[28,29]. Intracellular inflammasome complexes respond to PAMPs and DAMPs, triggering inflammation[30]. NLRP1, NLRP3, NLRC4, and AIM2 are well-documented inflammasome components and receptors. There is strong evidence of the involvement of the NLRP3 inflammasome in inflammatory

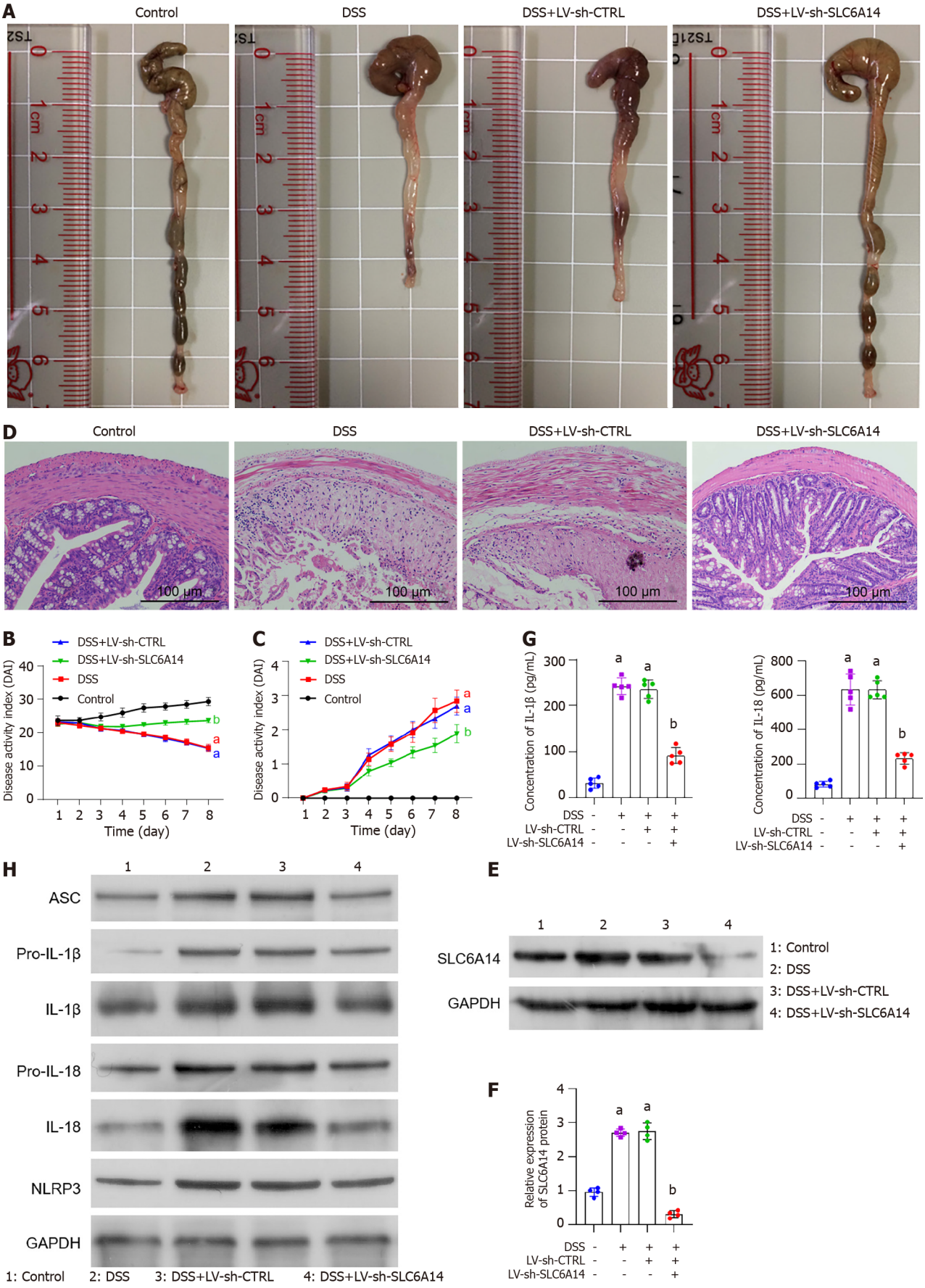

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Figure 7 SLC6A14 reduces pyroptosis induced by NLRP3 activation in ulcerative colitis mouse models. A: Measurement of colon length; B: Assessment of body weight changes; C: Disease-activity index; D: Histology (magnification 200 ×, scale bar = 100 μm); E and F: Western blot analysis of SLC6A14;

G: Effects of SLC6A14 on IL-1β and IL-18 production in the ulcerative colitis mouse model, as measured by enzyme-linked immunosorbent assay; H: Western blot analysis of the levels of pyroptosis-associated proteins. The data are means ± SD. $^{a}P < 0.01$ *vs* controls, $^{b}P < 0.01$ *vs* DSS+LV-sh-CTRL. DSS: Dextran sulfate sodium.

bowel diseases (IBDs), including Crohn's disease and UC[31,32].

Consistently, recent strategies to suppress chronic inflammation have targeted pyroptosis, offering a novel approach to managing IBD. For instance, L38 exerted positive therapeutic effects on a DSS-induced UC mouse model by inhibiting NLRP3 inflammasome activation and pyroptosis[33]. Similarly, mesalazine and corticosteroids have been shown to attenuate pyroptosis in IECs[34]. These approaches highlight the potential of targeting pyroptosis to alleviate inflammation in the context of IBD. There is a close link between UC progression and increased levels of IL-1β and IL-18, suggesting that overproduction of these cytokines by cells such as macrophages can worsen this condition[35]. However, NLRP3 activation stimulates IL-18 production and enhances intestinal barrier integrity. Intriguingly, the administration of exogenous recombinant IL-18 has been shown to alleviate the inflammatory symptoms of UC resulting from DSS administration[36]. This dual function of NLRP3 emphasizes the importance of selectively targeting macrophages rather than IECs for the effective management of UC[37].

Given the complex and sometimes contradictory aspects associated with NLRP3 activation in different cell types in the context of colitis, it is evident that further research is needed to elucidate the role of pyroptosis in UC. This would help guide the development of targeted therapeutic strategies to modulate the inflammatory response and effectively treat the disease. In our study, we treated IECs with LPS to create a model of intestinal inflammation. By analyzing the levels of key pyroptosis-associated factors, we observed the upregulation of these markers, indicating that LPS effectively induced pyroptosis in colonic epithelial cells. Additionally, our investigation of SLC6A14 expression in the context of LPS-induced colonic epithelial cells revealed the significant upregulation of SLC6A14. This finding is consistent with recent microarray expression data[38].

The SLC transporter family, which includes proteins such as SLC7A11, SLC3A2, and SLC25A28, is linked with a variety of metabolic disorders, especially those of the liver. SLC6A14 in particular has been shown to be upregulated in different colonic diseases, including ulcerative colitis[18]. Studies using microarrays of colonic tissue from UC patients and normal tissue showed a noticeable increase in SLC6A14 mRNA expression in UC cases[39]. SLC6A14 is an efficient transporter of amino acids that is associated with various intracellular activities. Leucine, which is one of its substrates, is critical for activating the mTOR signaling pathway in tumor cells. Moreover, SLC6A14 contributes to cellular glutathione synthesis by using glycine as a substrate. Multiple studies have showed the critical involvement of SLC6A14 in UC. Zhang *et al*[40] suggested that SLC6A14 was a biomarker of UC in tissue biopsies and might offer a novel target for gene therapy in UC. Similarly, Li *et al*[41] identified a potential regulatory pathway involving NEAT1-miR-342-3p/miR-650-SLC6A14 in UC. More recently, Chen *et al*[18] demonstrated that knockdown of SLC6A14 blocked ferroptosis and that SLC6A14 promoted ferroptosis in epithelial cells through C/EBPβ-PAK6 signaling in UC. In the context of our study, we showed that downregulating SLC6A14 effectively blocked NLRP3 activation, resulting in notable alleviation of colitis. This suggests a potential therapeutic strategy for managing colitis by targeting SLC6A14 to modulate the NLRP3 pathway and pyroptosis. These findings highlight the complexity of SLC6A14 in inflammatory conditions and UC.

We also examined the role of SLC6A14 in UC pathogenesis using a UC mouse model. These mice showed severe damage to colonic tissue, increased oxidative stress and cytokine production, and NLRP3 inflammasome activation. These findings indicate that DSS-induced UC led to the activation of the inflammatory caspase-mediated NLRP3 inflammasome. NLRP3 activation increased cytokine production and induced pyroptosis. Notably, our findings suggest that DSS induced pyroptosis, which further aggravated colon tissue damage. Our investigations revealed that downregulating SLC6A14 inhibited IEC pyroptosis in UC, which was mediated by the NLRP3 pathway. Interestingly, our results also indicated a positive association between the increase in SLC6A14 expression and pyroptosis in UC tissue samples. This evidence suggests that SLC6A14 actively promotes pyroptosis in the context of UC by regulating the NLRP3 pathway. Consequently, our findings highlight SLC6A14 as a prospective new therapeutic target that could be used to mitigate cellular damage during the course of UC. Our study contributes to our knowledge of UC pathogenesis and identifies a potential strategy for therapeutic intervention.

CONCLUSION

In summary, the results demonstrated the close involvement of SLC6A14 in promoting pyroptosis in the context of UC by upregulating NLRP3 expression. These findings indicate the potential of targeting SLC6A14/NLRP3 axis-mediated pyroptosis as a promising therapeutic strategy for treating UC. Our research provides valuable insights into the mechanisms driving UC pathogenesis and offers a possible direction for developing innovative treatments to alleviate the impact of this chronic inflammatory disorder.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is an inflammatory condition associated with frequent relapse and recurrence. Dysregulation of intestinal epithelial cells (IECs) and mucosal barrier impairment contribute to sustained inflammation in UC. Hence, an in-depth exploration of the triggers and mechanisms of IEC death could result in efficacious therapeutic options for UC patients.

Research motivation

Evidence suggests the involvement of SLC6A14 in UC pathogenesis, but the central regulator remains unknown.

Research objectives

We aimed to explore the role of SLC6A14 in UC-associated pyroptosis.

Research methods

Quantitative real-time polymerase chain reaction (qRT-PCR), immunoblotting, and IHC assessed SLC6A14 in human UC tissues. LPS induced FHC and NCM460 cell inflammation, modeling enteritis; SLC6A14 levels were assessed. Pyroptosis markers were quantified using enzyme-linked immunosorbent assay, Western blotting, and qRT-PCR, while EdU incubation, CCK-8 assay and flow cytometry examined proliferation and apoptosis, respectively. Mouse models of UC were used for verification.

Research results

SLC6A14 was elevated, correlating with NLRP3 in UC tissues. LPS-induced FHC and NCM460 cells showed increased SLC6A14. Reduced SLC6A14 boosted cell proliferation, suppressed apoptosis. Lower SLC6A14 Led to decreased pyroptosis-associated proteins (ASC, IL-1β, IL-18, NLRP3). NLRP3 overexpression counteracted sh-SLC6A14 effects on LPS-induced FHC and NCM460 cell pyroptosis. SLC6A14 improved murine dextran sulfate sodium colitis mucosa.

Research conclusions

SLC6A14 promotes UC pyroptosis *via* NLRP3 upregulation, indicating therapeutic potential through SLC6A14/NLRP3 axis modulation.

Research perspectives

We demonstrated the close involvement of SLC6A14 in promoting pyroptosis in the context of UC by upregulating NLRP3 expression. These findings underline the potential significance of targeting the SLC6A14/NLRP3 axis-mediated pyroptosis as a promising therapeutic strategy for addressing UC.

FOOTNOTES

Co-first authors: Qing Gu and Huan Xia.

Co-corresponding authors: He-Ping Chen and Li Zhang.

Author contributions: Gu Q and Xia H contributed equally to this work; Chen HP and Zhang L conceived and designed the study and provided administrative support; Gu Q, Xia H and Zhang L performed the experiments and analyzed data; Gu Q, Song YQ, Duan J and Chen HP analyzed and interpreted the data; Xia H, Chen Y, Zhang Y and Zhang L wrote the manuscript; all authors read and approved the final manuscript.

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Institutional animal care and use committee statement: All animal experiments were performed following the approval of the Animal Care and Use Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital [Lunshen (Yan)2022-380].

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Data sharing statement: No additional data are available.

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P-Editor: Yu HG

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ORIGINAL ARTICLE

Basic Study

Calcium/calcimimetic *via* calcium-sensing receptor ameliorates cholera toxin-induced secretory diarrhea in mice

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Abstract

BACKGROUND

Enterotoxins produce diarrhea through direct epithelial action and indirectly by activating the enteric nervous system. Calcium-sensing receptor (CaSR) inhibits both actions. The latter has been well documented *in vitro* but not *in vivo*. The hypothesis to be tested was that activating CaSR inhibits diarrhea *in vivo*.

AIM

To determine whether CaSR agonists ameliorate secretory diarrhea evoked by cholera toxin (CTX) in mice.

METHODS

CTX was given orally to C57BL/6 mice to induce diarrhea. Calcium and calcimimetic R568 were used to activate CaSR. To maximize their local intestinal

actions, calcium was administered luminally *via* oral rehydration solution (ORS), whereas R568 was applied serosally using an intraperitoneal route. To verify that their actions resulted from the intestine, effects were also examined on *Cre-lox* intestine-specific CaSR knockouts. Diarrhea outcome was measured biochemically by monitoring changes in fecal Cl^- or clinically by assessing stool consistency and weight loss.

RESULTS

CTX induced secretory diarrhea, as evidenced by increases in fecal Cl^-, stool consistency, and weight loss following CTX exposure, but did not alter CaSR, neither in content nor in function. Accordingly, calcium and R568 were each able to ameliorate diarrhea when applied to diseased intestines. Intestinal CaSR involvement is suggested by gene knockout experiments where the anti-diarrheal actions of R568 were lost in intestinal epithelial CaSR knockouts (*villinCre/Casrflox/flox*) and neuronal CaSR knockouts (*nestinCre/Casrflox/flox*).

CONCLUSION

Treatment of acute secretory diarrheas remains a global challenge. Despite advances in diarrhea research, few have been made in the realm of diarrhea therapeutics. ORS therapy has remained the standard of care, although it does not halt the losses of intestinal fluid and ions caused by pathogens. There is no cost-effective therapeutic for diarrhea. This and other studies suggest that adding calcium to ORS or using calcimimetics to activate intestinal CaSR might represent a novel approach for treating secretory diarrheal diseases.

Key Words: Cholera; Enteric nervous system; Secretory diarrhea; Oral rehydration solution; Calcium-sensing receptor; Gene knockout

Core Tip: Treatment of diarrhea remains a global challenge. Enterotoxins induce diarrhea through direct epithelial action and indirectly by activating the enteric nervous system. Using *in vitro* models in isolated tissues, we have previously shown that calcium-sensing receptor (CaSR) inhibits both actions. In the present study, we use a mouse model of secretory diarrhea in conjunction with a tissue-specific knockout approach and demonstrate that calcium or calcimimetic *via* CaSR ameliorates cholera toxin-induced secretory diarrhea *in vivo*. This study suggests that adding calcium to oral rehydration solution or using calcimimetic to activate intestinal CaSR might represent a new approach for treating secretory diarrheal diseases.

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INTRODUCTION

Acute infectious diarrhea remains among the top causes of morbidity and deaths in children throughout the world[1,2]. According to the United Nations Children's Fund/World Health Organization[3], approximately 9 million children (about half the population of New York) under 5 years died in 2008. 40% of these deaths were due to two diseases: Pneumonia and diarrhea. Diarrhea remains the second leading cause of death in children younger than 5 years globally, accounting for one in every five child deaths - around 1.5 million a year - more than acquired immune deficiency syndrome, malaria, and measles combined. Importantly, most of the morbidity and mortality is not due to infection but dehydration. Accordingly, reducing the fluid loss from acute diarrhea offers a major opportunity for improving child health globally.

Enterotoxins induce diarrhea through direct epithelial action and indirectly by activating the enteric nervous system (ENS)[4]. For example, cholera induces diarrhea through the generation of cholera toxin (CTX) from *V. cholera*. CTX binds to the enterocyte, leading to ADP-ribosylation of the G_s α-subunit. This constitutively activates membrane-bound adenylyl cyclase in enterocytes and elevates intracellular cyclic adenosine monophosphate (cAMP). Increased cAMP stimulates protein kinase A and phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) channel, as well as the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), causing secretion of Cl^- and water. Elevated cAMP levels also inhibit Cl^- and water absorption mediated by Cl^-/HCO_3^- exchange and Na^+/H^+ exchange[4,5]. In addition to direct epithelial action, CTX elicits neuronal secretory reflexes by binding to mucosal enterochromaffin cells, leading to the production of 5-hydroxytryptamine, activation of ENS and release of neurotransmitters (*e.g.*, vasoactive intestinal peptide and acetylcholine) that stimulate and amplify fluid secretion, leading to dehydration and rapid body weight loss[4,6,7].

Importantly, the extracellular calcium-sensing receptor (CaSR)[8] is present on cells in both pathways[5,9,10] and, when activated *in vitro*, blocks both diarrhea-causing pathways evoked by CTX and other diarrhea-causing enterotoxins/secretagogues. For example, using a microperfused colonic crypt technique, it has been shown that calcium, calcimimetics or polyamines that activate CaSR can act on intestinal epithelium and reverse CTX/forskolin-induced fluid secretion

using a signal transduction pathway that promotes cyclic nucleotide destruction[11-13]. Using Ussing chambers, it has been shown that the effects of CTX/forskolin and CaSR agonists on electrolyte secretion by the intestine can also be attributed to opposing actions of enterotoxins/secretagogues and CaSR on ENS activity[14,15]. These results suggest that targeting intestinal CaSR might represent a previously undescribed novel approach for treating secretory diarrheal diseases[5,9,10,16-18]. However, all of the experiments that suggest CaSR modulates dual-pathway secretion by the intestine have been performed *in vitro* in isolated tissues. Neither the functionality of the CaSR receptors *in vivo* nor the anti-diarrheal potential of CaSR agonists in live animals have been documented, although the latter is necessary before clinical trials in humans are performed.

In this study, we tested the hypothesis that calcium/calcimimetic *via* CaSR ameliorates secretory diarrhea *in vivo* in mice. A CTX mouse model of secretory diarrhea was employed and the effects of CaSR agonists on biochemical (*i.e.*, changes in fecal Cl^-) and clinical outcomes (*i.e.*, changes in stool consistency and body weight loss) of secretory diarrheal disease were assessed. We selected the CTX mouse model because we had employed it as a model in previous *in vitro* studies. In addition, it has been widely used to provide proof of concept of whether an anti-diarrheal agent is therapeutic or not[19-22]. In addition to testing calcium, we also examined the effects of the calcimimetic R568, a pharmacological allosteric CaSR agonist[23]. To maximize their local intestinal actions, we delivered agonists as follows: Calcium was administered orally by adding it to oral rehydration solution (ORS) and R568 was applied serosally using an intraperitoneal route, as previously described[15]. To verify that their actions resulted from intestinal tissues and not a non-specific off-target action, the effects were also measured on intestine-specific CaSR knockouts. We show for the first time that targeting intestinal CaSR with calcium or calcimimetic is efficacious in reducing CTX-evoked secretory diarrhea *in vivo* in live animals and that this occurs through receptor-mediated reduction of both the neurally and non-neuronally mediated secretory responses. A portion of this work was presented in an abstract in the Global Health Forum of 5th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition[24].

MATERIALS AND METHODS

Animals

Experiments were performed using male/female C57BL/6 mice (wild type and *Casr* mutants). Mice lacking CaSR expression in intestinal epithelial cells ($^{villin}Cre/Casr^{flox/flox}$ mice) and mice lacking CaSR expression in intestinal neurons ($^{nestin}Cre/Casr^{flox/flox}$ mice) and their wild type littermates were bred and maintained in-house at the University of Florida Communicore Animal Facility. Mutant mice were generated as previously described[25,26]. Briefly, CaSR flox/flox mice were bred with transgenic mice expressing *Cre* recombinase under the control of the Villin 1 or Nestin promoter and genotyped prior to all experiments after approximately 20-30 generations. Mice were used at 5-10 wk of age and weight of 17-23 g in accordance with the Animal Welfare Act and the Public Health Policy on Humane Care. Animals were fed and maintained on regular chow (Harlan) with free access to water before the experiment. To maximally protect animal welfare, we used numbers of animals in each experiment group as minimal and small as scientifically or statistically allowed. Thus, depending on variation of the data obtained and/or availability of the animals tested, 5-11 animals were employed, although 2-3 animals were used in some dose-dependence studies. This was because these were the minimal numbers required for statistical significance using one-way ANOVA and $P < 0.05$ as determined in a pilot experiment. To minimize the effects of subjective bias in allocating animals, we treated controls, interventions, wild type, and mutants in the same manner on the same days by the same investigators. The animal protocols were designed to minimize pain or discomfort to the animals. After completion of the experiment, animals were sacrificed with standard CO_2 inhalation and by cervical dislocation. The use of animals as well as the protocols for CTX treatment and colon tissue isolation was approved by the Institutional Animal Care and Use Committee (IACUC# 201807567) at the University of Florida.

CTX mouse model of secretory diarrhea

Two protocols were used to induce diarrhea: Protocol 1 is long and was used to compare the effects of with *vs* without oral calcium, a poorly absorbed mineral agonist of CaSR[27-29]; whereas protocol 2 is short and was used to compare the effects of with *vs* without R568, a quickly absorbable small-molecule agonist of CaSR[30].

Protocol 1: Animals were first fasted overnight for 16 h before they were gavaged intragastrically (*i.g.*) with 200 μL 7% $NaHCO_3$ buffer containing 20 μg CTX or vehicle per mouse to induce diarrhea. After CTX gavage, animals were fasted for an additional 90 min before they were allowed access to regular chow to avoid food interference on toxin binding and action. Afterwards, animals were divided into two groups: Group 1 received drinking bottles containing ORS only whereas Group 2 received drinking bottles containing ORS supplemented with 5 mmol/L calcium. This calcium concentration was selected because it is the lowest concentration of calcium that generated maximal CaSR-activation effects[11,12]. Diarrhea was monitored and was either semi-quantitated clinically according to stool consistency [0, normal feces (solid); 1, moist feces (semi-solid); 2, mild diarrhea (loose); and 3, severe diarrhea (watery)[31]] or quantitated biochemically according to fecal Cl^- content. Degree of dehydration was measured by diarrhea-associated body weight loss[32]. In this study, the onset of diarrhea is defined as the appearance of the first diarrheic stool with stool consistency scored one or higher, as described[31].

Protocol 2: Animals were pretreated and treated as in protocol 1 except for the following: (1) Calcimimetic R568 (diluted in 100 μL normal saline) was administered serosally at the time when diarrhea was induced. R568 was administered serosally using an intraperitoneally (*i.p.*) rather than *per os* route to minimize the unwanted systemic effects while

maximizing the desired local intestinal action as described[15]. Neither anesthesia nor analgesia were used; (2) 1.5 h post CTX treatment, animals were allowed to drink ORS without calcium; and (3) Animals were sacrificed 3.5 h after CTX treatment before watery stool was seen. Pilot studies showed that diarrhea started to occur about 0.5 h post CTX gavage and reached a peak plateau about 1.5 h later[33], but no diarrheic stool was seen until 3.5 h post CTX treatment. Three and half-hours later, animals were killed, fluid accumulated in the intestine was removed and weighed, changes compared, and is expressed as mg/mg intestine.

Fecal Cl^- measurement

Feces were collected in pre-weighed Eppendorf tubes. To avoid variations from freeze-thaw cycle and bacterial overgrowth from storage, all fecal samples were collected, gently processed, and promptly measured as described[34]. In brief, following collection, samples were weighed and diluted appropriately in deionized water so that the Cl^- content in each sample fell within the linear range of the standard curve. Diluted samples were gently but thoroughly homogenized through pipetting before centrifugation at 14000 g for 10 min. Sonication was not used to minimize release of intracellular contents. The resulting supernatants were collected, and Cl^- contents measured with an ion-selective electrode by potentiometric titration (Model LIS-146CLCM-XS system; JENCO Electronics, Ltd, Taipei City, Taiwan). The results were calculated according to the standard curve, and are expressed as mole/L, where 1 L of feces ≈ 1 kg of feces. Previous studies have shown that these methods caused no or minimal variations in fecal Cl^-[34-36].

CaSR western blot

Isolation and preparation of intestinal homogenates and lysates and western blot analysis of CaSR protein were performed as previously described[12] with an affinity-purified mouse monoclonal antibody (5C10, ADD) raised against a 22-amino acid peptide corresponding to amino acid residues 214-235 of human CaSR (Abcam, Cambridge, MA, United States). CaSR protein signals were normalized against heat shock protein 90 (HSP90) as a loading control and are expressed as CaSR/HSP90 protein signal ratios[37].

Chemicals, antibodies, and solutions

CTX was obtained from Sigma (St Louis, MO, United States), and 5 mg/mL stock solutions were prepared in water. R568 was purchased from Tocris Bioscience (Ellisville, MI, United States), and 20 mg/mL stock solutions were prepared in 15% 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals International, Natick, MA, United States). Calcium chloride was from Sigma, and rabbit polyclonal antibody against HSP90 was from Santa Cruz Biotechnology (Dallas, TX, United States). ORS was prepared fresh containing (in mmol/L) 75 Na^+, 20 K^+, 65 Cl^-, 10 citrate and 75 glucose with total osmolarity of 245 mOsm/kg H_2O.

Statistical analysis

The statistical methods of this study were reviewed by Dr. Han-Zhi Gao, PhD, member of the Biostatistics Service from the Clinical and Translational Science Institute of the University of Florida. Data from all animals were included in the analysis. Values are given as means ± standard error of the mean. The normality of variables was checked; the data for intestinal fluid accumulations exhibited a skewed distribution and were therefore log transformed. After log transformation, the data became normally distributed. Statistical comparisons between two means were performed by Student's *t*-test, whereas comparisons among multiple means were by one-way ANOVA with Tukey's *post hoc* tests. Both tests were performed using Microsoft Excel 2016 for Windows or using GraphPad Prism version 6.07 for Windows (GraphPad Software, San Diego, CA, United States). $P < 0.05$ was considered significant.

RESULTS

Effects of calcimimetic

Our first set of experiments was performed with the calcimimetic R568 used in conjunction with intestinal CaSR-specific knockouts to demonstrate the functionality of intestinal CaSR *in vivo* and to demonstrate the intestinal specificity of the agent. In these experiments, diarrhea-provoking CTX or vehicle was given *i.g.* whereas the anti-diarrheal R568 or vehicle was administered *i.p.* to avoid interference between the two agents. For accurate quantification, diarrhea was induced such that all the secreted fluid was contained inside the intestinal lumen without loss outside of the body. Before they were sacrificed, animals did not exhibit any noticeable adverse effects. Data from all animals were included in the analysis.

CTX induces secretory diarrhea but does not alter CaSR expression in the intestine

In unstimulated vehicle-gavaged wild type mice, intestinal fluid accumulation was 0.20 ± 0.02 mg/mg intestine. Intragastric gavage of CTX caused diarrhea, as evidenced by increased intestinal fluid accumulation in a dose-dependent fashion (Figure 1). At the EC_{50} of approximately 0.5 mg/kg, the amount of intestinal fluid accumulation was 1.46 ± 0.15 mg/mg intestine, which is about a 7-fold increase in intestinal fluid accumulation compared to non-CTX controls (Table 1).

Many conditions like carcinogenesis reduce CaSR expression in the intestine[38]. To assess if this occurred to CTX-treated intestines, we examined CaSR protein expression by western blots (Figure 1). Although CTX caused diarrhea, the toxin did not alter intestinal CaSR expression. The CaSR/HSP90 protein signal ratios were control 0.50 ± 0.02 (5) *vs* CTX

Table 1 Intestinal fluid secretory responses to cholera toxin and R568 calcimimetic in calcium-sensing receptor wild type and mutant mouse intestine

Group	Intestinal fluid accumulation in mg fluid/mg intestine		
	Wild type	*villin*Cre/Casr*flox/flox*	*nestin*Cre/Casr*flox/flox*
Control	0.20 ± 0.02 (11)	0.59 ± 0.13 (7)[a]	0.17 ± 0.02 (6)
R568 (50 mg/kg)	0.15 ± 0.09 (5)	0.61 ± 0.11 (5)	0.15 ± 0.11 (5)
CTX (0.5 mg/kg)	1.46 ± 0.15 (10)[c]	0.80 ± 0.03 (5)[b]	0.55 ± 0.14 (6)[b]
CTX (0.5 mg/kg) + R568 (50 mg/kg)	0.92 ± 0.16 (6)[d]	0.82 ± 0.09 (5)	0.95 ± 0.29 (5)

[a]$P < 0.05$ *vs* wild type.
[b]$P < 0.05$ *vs* control.
[c]$P < 0.01$ *vs* control.
[d]$P < 0.05$ vs cholera toxin alone.
Data are means ± standard error of means (*n*). CTX: Cholera toxin.

0.53 ± 0.03 (5), $P > 0.05$.

R568 reverses CTX-induced diarrhea

Having known that CaSR protein expression was unaltered, we then studied CaSR function by assessing the ability of the calcimimetic R568 to reverse CTX-induced diarrhea *in vivo* (Figure 2). For this, the EC_{50} dose 0.5 mg/kg of CTX was *i.g.* gavaged to induce moderate diarrhea, while different doses of R568 were administered *i.p.* Three and half hours later, animals were sacrificed, intestines were removed, and intestinal fluid was measured (Figure 2A). Indeed, CTX induced diarrhea (Figure 2B). When R568 was applied to the CTX-induced diseased intestine, it ameliorated diarrhea and reduced CTX-induced fluid accumulation (Figure 2B) in a dose-dependent manner. Fluid accumulation was reduced by approximately 50% when the near maximal effective doses of 30-50 mg/kg of R568 were applied (Figure 2C) (ANOVA test; $P < 0.05$). In non-CTX vehicle gavaged mice, R568 generated no or only minimal inhibitory effects (Figure 2C) (ANOVA test; $P > 0.05$). These results indicate that CaSR function remains unaltered in diseased intestines, consistent with the *in vitro* findings[13,15].

R568 reverses CTX-induced diarrhea via intestinal CaSR

To show that intestinal CaSR was indeed targeted and was not simply due to a non-specific off-target action, additional studies on intestinal tissue-specific CaSR knockout mice (*i.e.*, $^{villin}Cre/Casr^{flox/flox}$ and $^{nestin}Cre/Casr^{flox/flox}$) were performed and effects compared (Table 1, Figure 2D and E). Under non-CTX vehicle-stimulated basal conditions, intestinal fluid accumulation was increased in $^{villin}Cre/Casr^{flox/flox}$ mice (0.59 ± 0.13 mg/mg intestine, $P < 0.05$ *vs* wild type mice) (Table 1), and was unchanged in $^{nestin}Cre/Casr^{flox/flox}$ mice (0.17 ± 0.02 mg/mg intestine, $P > 0.05$ *vs* wild type mice) (Table 1). Addition of CTX (0.5 mg/kg) caused diarrhea, as evidenced by significantly increased intestinal fluid accumulation of these mice (Table 1), although the diarrhea was less severe in knockouts than in wild type (Table 1) due to activation of compensatory mechanisms. Importantly, R568 administration at all tested concentrations did not inhibit CTX-induced diarrhea in neither $^{villin}Cre/Casr^{flox/flox}$ mice (ANOVA test; $P > 0.05$) (Figure 2D) nor $^{nestin}Cre/Casr^{flox/flox}$ mice (ANOVA test; $P > 0.05$) (Figure 2E), confirming that the anti-diarrheic action was indeed exerted largely, if not exclusively, *via* CaSR in the intestinal tissues. R568 alone had no effect in neither $^{villin}Cre/Casr^{flox/flox}$ mice (ANOVA test; $P > 0.05$) (Figure 2D) nor $^{nestin}Cre/Casr^{flox/flox}$ mice (ANOVA test; $P > 0.05$) (Figure 2E) despite the presence of diarrhea in $^{villin}Cre/Casr^{flox/flox}$ mice, further confirming that the CaSR is required for the calcimimetic to exert its anti-diarrheic action.

Effects of calcium

After performing the proof-of-concept studies using the calcimimetic R568 and verifying the functionality of intestinal CaSR, we tested whether targeting CaSR with calcium, an inexpensive widely available child-friendly mineral, was an anti-diarrheal *in vivo*. We added calcium to ORS and investigated whether it helped reduce the severity of diarrhea and enhance the rate of rehydration by ORS. In these experiments, animals were first *i.g.* gavaged with CTX or vehicle. Ninety minutes later, they were allowed to drink ORS with calcium or ORS alone, and development and progression of diarrhea was monitored both biochemically through changes in fecal Cl^- content and clinically by assessing changes in stool consistency and diarrhea-associated body weight loss (Figure 3A).

Adding calcium to ORS reduces CTX-induced Cl^- losses from the intestine

In secretory diarrhea, active Cl^- secretion and decreased Cl^- absorption is the primary driving force for water moving from the blood to the intestinal lumen[39]. Thus, to assess whether calcium supplemented ORS (ORS + Ca) is better than ORS alone in reducing intestinal Cl^- loss from diarrhea, we first measured and compared changes in fecal Cl^- concentration (Cl^-). Figure 3B shows the changes in fecal Cl^- at day 1, day 4, and day 6 post-CTX gavage. Day 1 represents the acute stage of diarrhea, day 4 the recovery stage, and day 6 post-recovery. Consistent with enterotoxin-induced intestinal Cl^- loss, mice in both groups displayed a significantly higher mean fecal Cl^- upon CTX exposure ($P < 0.01$, day 1 post CTX *vs*

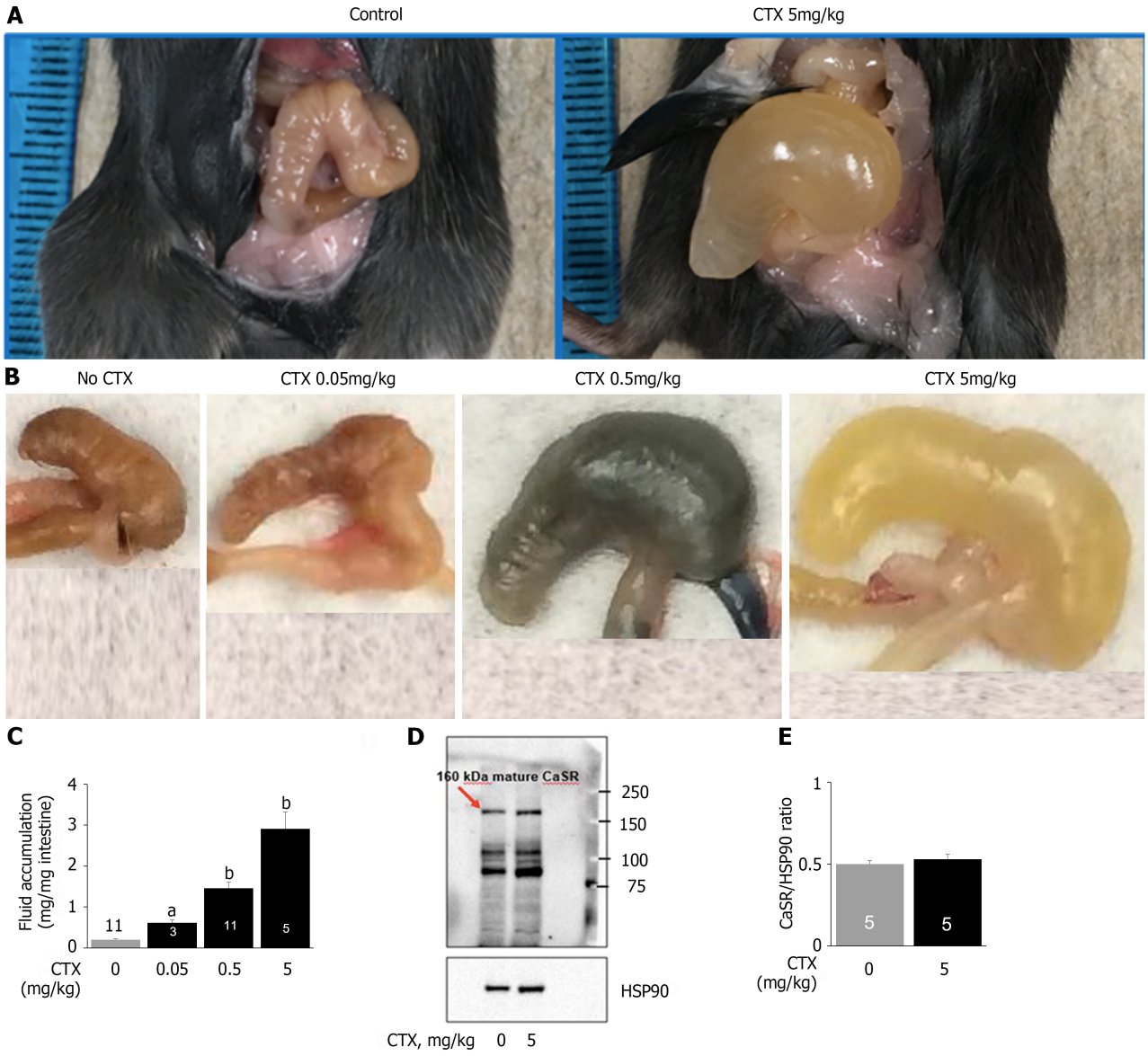

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Figure 1 Cholera toxin induces diarrhea but does not alter calcium-sensing receptor in the intestine. A: Representative images of fluid accumulation in the intestine; B: Representative images of fluid accumulation in the cecum; C: Quantifications of fluid accumulation in the intestine; D: Representative western blot for intestinal calcium-sensing receptor (CaSR); E: Quantification of intestinal CaSR western blot. Mice were intragastrically gavaged with vehicle or cholera toxin at the indicated doses to induce diarrhea/intestinal fluid secretion. Three and half-hours later, animals were killed, intestines removed, and fluid and CaSR protein quantitated. The CaSR protein signals were normalized by heat shock protein 90 as an internal control to correct protein loading differences. Shown are means ± standard error. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* no cholera toxin control. The blue color noted in intestines was Evans Blue dye that was used to monitor intestinal motility (data not shown; will be reported separately). CTX: Cholera toxin; HSP90: Heat shock protein 90.

day 1 Control). However, compared to the high fecal Cl^- in CTX:ORS-treated mice, a lower fecal Cl^- was noted in mice receiving CTX:(ORS + Ca) treatment (ANOVA test $P < 0.05$; Student's *t* test *P* values at day 1, 4 and 6 = 0.56, 0.07 and 0.08 *vs* respective non-Ca controls). Moreover, relative to mice on CTX:ORS treatment, mice on CTX:(ORS + Ca) recovered from diarrhea-associated Cl^- losses significantly faster. Thus, while the CTX:ORS group fecal Cl^- losses had remained significantly elevated above baseline until day 6 post-CTX gavage, in CTX:(ORS + Ca) group, a close to normal fecal Cl^- had been observed at day 4 post-CTX treatment (Figure 3B). Calcium had no or minimal effects on non-CTX vehicle-treated mice (one-way ANOVA test; $P > 0.05$). These results suggest that ORS + Ca is better than ORS alone in reducing diarrhea-associated intestinal Cl^- losses.

Adding calcium to ORS reduces severity and duration of CTX-induced diarrhea

Reducing intestinal Cl^- loss suggests the possibility of reducing diarrhea and dehydration. Thus, we compared the onset, severity and recovery of diarrhea/dehydration induced by CTX in ORS + Ca *vs* ORS groups. First comparison was made regarding the onset of diarrhea (*i.e.*, the time from CTX gavage to the appearance of the first diarrheic stool). Since it would take some time for calcium to produce a clinically visible anti-diarrheal action, calcium may or may not influence the onset of diarrhea. Before CTX gavage, all mice displayed normal solid stool. Following CTX gavage, mice receiving

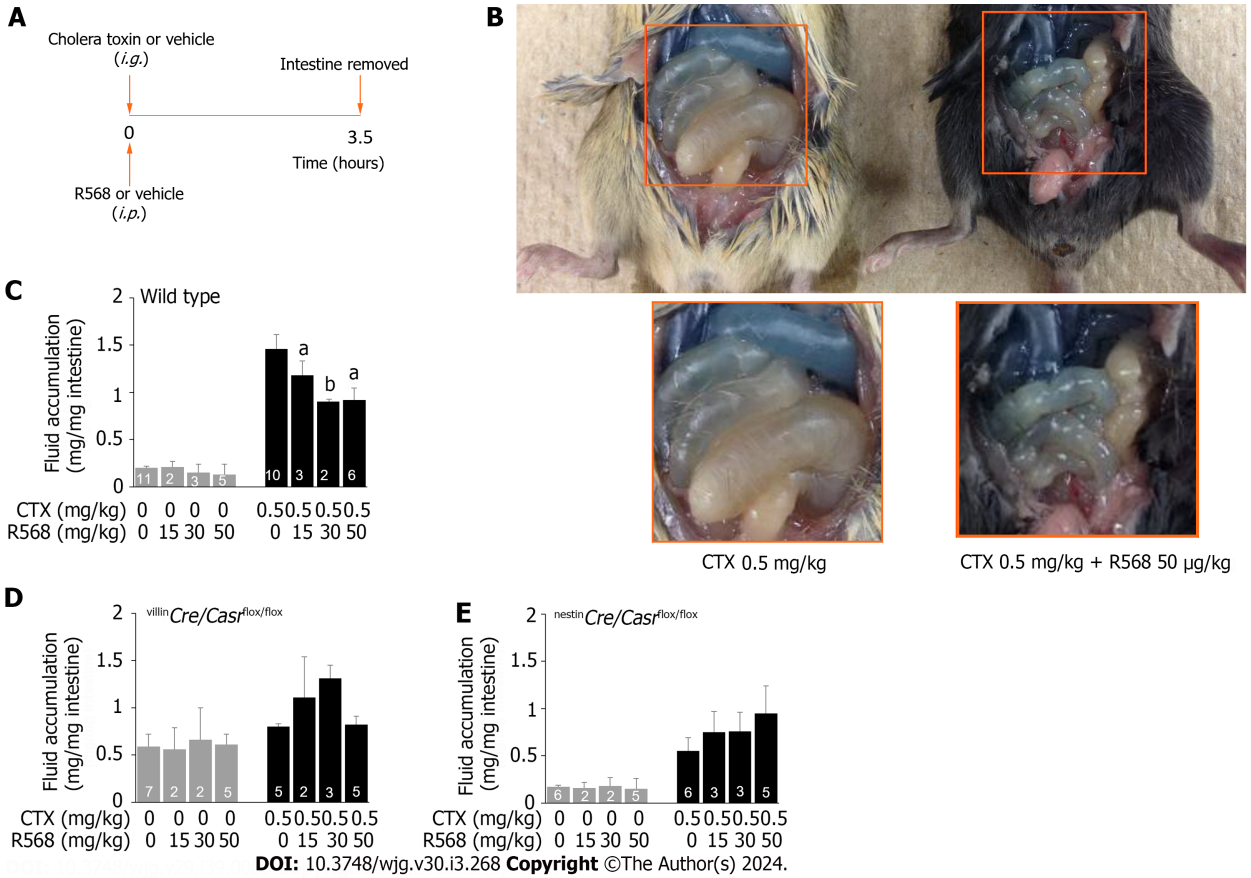

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Figure 2 Calcimimetic R568 reverses cholera toxin-induced diarrhea in wild type but not in intestine-specific calcium-sensing receptor knockouts. A: Experimental protocol; B: Representative images of R568 effect on fluid accumulation in the intestine of wild type mice; C: Summary of fluid accumulation in the intestine of wild type mice; D: Summary of fluid accumulation in the intestine of *villinCre/Casrflox/flox* mice; E: Summary of fluid accumulation in the intestine of *nestinCre/Casrflox/flox* mice. Mice were intragastrically gavaged with cholera toxin or vehicle while receiving R568 or vehicle intraperitoneally. Three and half-hours later, animals were killed, intestines removed, and fluid quantitated. Shown are means ± standard errors. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* without R568. CTX: Cholera toxin.

ORS had the first diarrheic stool at 4.5 ± 1.7 h, whereas mice receiving ORS + Ca developed diarrhea at 4.3 ± 1.8 h. No statistically significant difference was noted ($P = 0.69$).

We then compared the changes in stool consistency over time. Similarly, adding calcium reduced the stool consistency score in CTX-treated (ANOVA test; $P < 0.05$) but not in the non-CTX control (ANOVA test; $P > 0.05$). The result is summarized in Figure 3C. Specifically, in ORS group, CTX significantly increased stool consistency scores on day 1, day 2 and day 3 but not on day 4 compared to the non-CTX control, whereas in ORS + Ca group, CTX significantly increased stool consistency scores only on day 1 and day 2 but not on day 3 and day 4. Thus, while the ORS group stool consistency score remained significantly elevated above baseline until day 4 post-CTX gavage, in ORS + Ca group, a normal stool consistency score had been observed 1 d earlier at day 3 post-CTX treatment. These results suggest that ORS + Ca is better than ORS alone in reducing diarrhea.

Considering that the stool consistency scoring is only semi-quantitative and has large performance-dependent variations, we compared body weight changes before and after disease induction, a quantitative way of measuring diarrhea severity and degree of dehydration[32]. We chose to monitor body weight instead of monitoring 24-h stool volume because we had technical difficulties in accurately collecting stool and quantifying 24-h stool volume. Figure 3D shows body weight changes over time in mice in ORS + Ca group *vs* ORS only group along with their non-CTX controls. In response to CTX challenge, mice on both groups lost significant weight, particularly in day 1 and day 2. However, mice receiving ORS + Ca lost significantly less weight and recovered significantly sooner than mice receiving ORS only (ANOVA test; $P < 0.01$). Thus, while mice in the ORS group continued to lose weight to a statistically significant degree until after day 4 post-CTX, mice in ORS + Ca group had achieved a close to normal body weight at day 3 pos-CTX treatment (Figure 3D). The estimated time at which mice returned to their initial weight was 3.5 d in Ca-ORS group and 4.5 d in ORS only group, which is 22% faster in ORS + Ca group. These results suggest that adding calcium to ORS reduces the severity of dehydration, hastens its recovery, and accelerates the rate of rehydration by ORS.

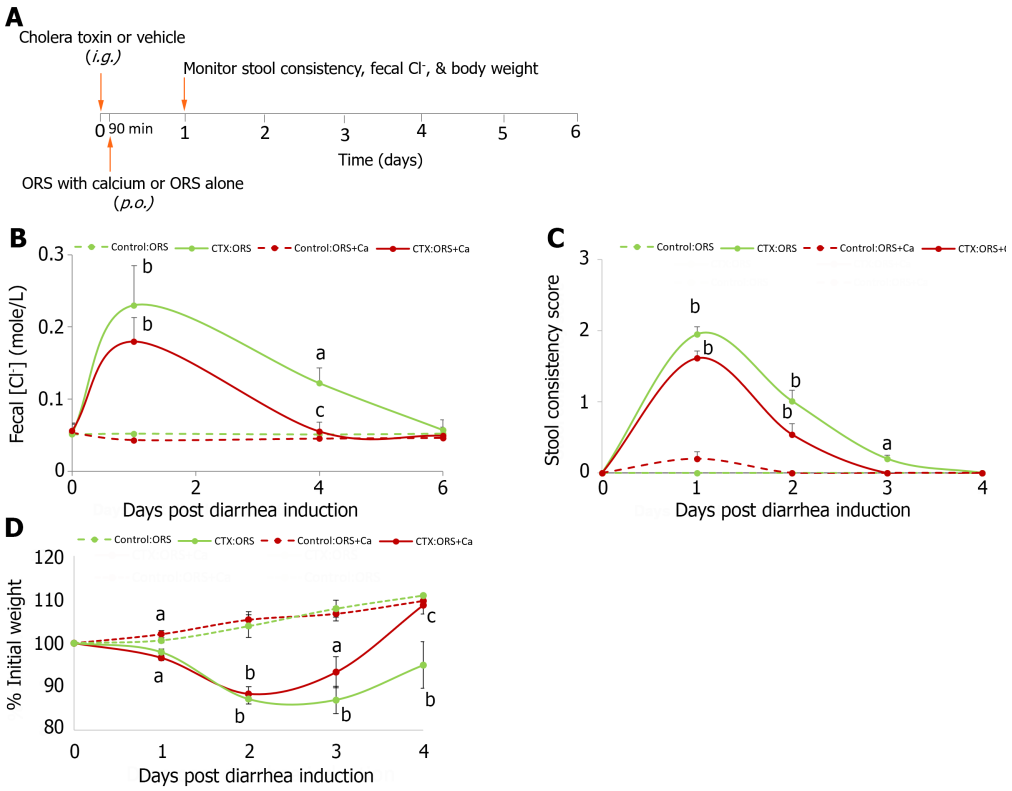

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Figure 3 Adding calcium to oral rehydration solution rescues cholera toxin-induced intestinal Cl^- loss and stool consistency, and promotes the rate of rehydration. A: Experimental protocol; B: Changes in fecal Cl^-; C: Changes in stool consistency score; D: Changes in % initial weight. At day 0, all animals were intragastrically gavaged with 20 μg/mouse of cholera toxin (CTX) to induce diarrhea. Ninety minutes later, they were allowed to drink oral rehydration solution (ORS), or ORS supplemented with 5 mmol/L calcium (ORS + Ca). Changes in fecal Cl^- (B), stool consistency score (C) and % initial weight (D) were monitored. Shown are means ± standard errors. n = 5-6 for each data point. No significant differences between groups were noted in initial body weights (in grams): Control:ORS *vs* ORS + Ca: 19.1 ± 0.6 *vs* 19.1 ± 0.6; CTX:ORS *vs* ORS + Ca: 19.9 ± 0.6 *vs* 19.5 ± 0.4. $^aP < 0.05$, $^bP < 0.01$ *vs* no cholera toxin controls; $^cP < 0.05$ *vs* oral rehydration solution control. ORS: Oral rehydration solution; CTX: Cholera toxin.

DISCUSSION

This first *in vivo* study proves that targeting intestinal CaSR with calcium or calcimimetic is efficacious in reducing CTX-evoked secretory diarrhea and that this occurs through receptor-mediated reduction of both the neurally (*i.e.*, Nestin-expressing enteric neuron) and non-neuronally (*i.e.*, Villin-expressing epithelial cell) mediated Cl^- secretory responses. A schematic diagram illustrating how CTX induces and calcium/calcimimetic inhibits these two Cl^- secretory responses is depicted in Figure 4.

We showed that CTX induced secretory diarrhea in mice as previously reported[19-22]. This was evidenced by increased fecal Cl^- and water content/stool consistency and weight loss following CTX induction. Importantly, although it altered intestinal fluid balance and caused diarrhea, CTX did not seem to alter CaSR content or function. Accordingly, when applied to diseased intestines, calcium and calcimimetic were each able to ameliorate diarrhea. Intestinal CaSR involvement is further supported by gene knockout experiments in which the anti-diarrheal activity of CaSR agonists observed in wild type mice was not noted in knockouts. Neither the $^{villin}Cre/Casr^{flox/flox}$ mice that lack epithelial CaSR nor the $^{nestin}Cre/Casr^{flox/flox}$ mice that lack neuronal CaSR experienced amelioration of diarrhea with CaSR agonists.

Interestingly, while both CaSR knockouts responded to CTX stimulation, their responses were less prominent than their wild types. The reason is unknown and is related to the downregulation of NKCC1 and CFTR in these animals[32]. NKCC1 and CFTR are two ion transporters required for the intestine to generate an effective diarrheal response to secretagogues[40,41].

Additionally, differences in the phenotype of two intestinal CaSR knockouts under basal conditions are noted. While $^{villin}Cre/Casr^{flox/flox}$ mice developed spontaneous diarrhea, as evidenced by mild but significant increased fluid accumulation in unstimulated intestines, $^{nestin}Cre/Casr^{flox/flox}$ mice did not, as there was no increase in fluid accumulation in unstimulated intestines (Table 1). The reason is unknown but may be related to the fasting condition used and differences in roles and functions these epithelial and neuronal CaSR receptors play in intestinal function. The primary function of the gastrointestinal (GI) tract is to digest food and absorb nutrients. To aid digestion, the GI tract secretes a large amount of fluid to mix the food components and lubricate the luminal surface. It is estimated that following the ingestion of a meal, intestinal secretion can be increased eightfold[42]. Upon completion of digestion and extraction of nutrients, intestinal secretion stops. While studies suggest that mechanical sensors in the ENS have a significant role in triggering the meal-evoked secretion[6], there is evidence that chemical sensors (*e.g.*, CaSR) on the epithelium and enteric neurons have a key

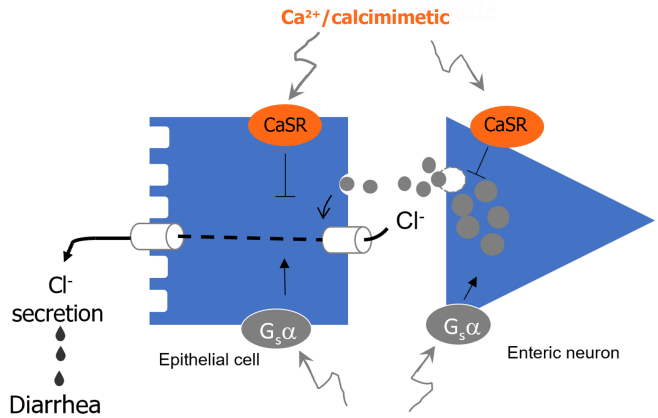

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Figure 4 Diagram illustrates how cholera toxin stimulates and calcium/calcimimetic inhibits the dual pathways of Cl^- secretion resulting in diarrhea. Cholera toxin (CTX) *via* G_s α-subunit stimulates transepithelial Cl^- secretion both neurally through Nestin-expressing enteric neurons releasing neurotransmitters and non-neuronally through direct action on Villin-expressing epithelial cells. The outcome is stimulating both Cl^- entry from blood side into intestinal epithelial cell and exit from epithelial cell into the intestinal lumen causing secretory diarrhea. On the other hand, Ca^{2+}/calcimimetic *via* calcium-sensing receptor (CaSR) inhibits both actions of CTX *via* direct epithelial action and indirectly *via* enteric neuron. The involvement of Nestin-expressing enteric neurons and Villin-expressing epithelial cells is evidenced in the two tissue-specific knockout mice whose Cl^- secretory responses to CTX and CaSR stimulation are compromised.

role in terminating this process. The latter do so through their ability to sense nutrients released during digestion[25] (also, a recent review by Tang *et al*[10]). Consistent with active regulation of intestinal secretion by CaSR, under the no-food-no-nutrient fasting condition used in the present study, neuronal CaSR would have been silent and, as a result, no intestinal phenotype would be expected in $^{nestin}Cre/Casr^{flox/flox}$ mice (Table 1).

The finding of spontaneous diarrhea in $^{villin}Cre/Casr^{flox/flox}$ mice is notable (Table 1). This indicates that unlike neuronal CaSR, epithelial CaSR does remain active, at least to some degree, under a no-food-no-nutrient fasting condition. This is not surprising given the multiple roles and functions that CaSR plays in GI biology[43]. In addition to its established function as a nutrient sensor regulating fluid secretion and absorption during food digestion, epithelial CaSR is a fundamental mechanism for sensing and regulating the ionic and nutrient compositions of extracellular milieu surrounding the epithelium of the entire GI tract[10]. Thus, at basal no-food no-nutrient fasting state, this epithelial CaSR may perform other tasks, for example, monitoring the Ca^{2+} surrounding the epithelium.

According to the present model of intestinal Ca^{2+} transport[44], the Ca^{2+} ion cycles between the leaking crypt, which secretes Ca^{2+} *via* a passive paracellular pathway, and the electrically tight villous/surface epithelium, which absorbs back Ca^{2+} *via* an active transcellular transport mechanism. Interestingly, under basal conditions, fluid also cycles in a similar fashion between the crypt, which secretes, and the villous/surface epithelium, which absorbs[45]. The purpose of this fluid cycling is to lubricate the luminal surface to prevent the crypt lumen from obstructing and to defend the crypt from invasion by lumen bacteria. It is likely that CaSR located on enterocyte apical and basolateral membranes constitutively sense Ca^{2+}, thereby monitoring and controlling these processes.

CONCLUSION

The most notable observation of the present study is that calcium and calcimimetic both significantly ameliorated CTX-induced secretory diarrhea. The latter has important therapeutic value. Treatment of acute secretory diarrheas remains a global challenge. Despite advances in diarrhea research, few advancements have been made in the realm of diarrhea therapeutics, and ORS therapy has remained the standard of care even though it does not stop the loss of intestinal fluid and ions caused by pathogens. There is no cost-effective therapeutic for diarrhea. This study suggests that adding calcium to ORS or using calcimimetic to activate intestinal CaSR might represent a novel approach for treating secretory diarrheal diseases. Limitations of this study include: (1) The present study was an animal but not human study; (2) No data on oral calcimimetic was obtained; and (3) Neither local nor systemic adverse effects were documented, despite the fact that some animals in study protocol 1 appeared to be sick, particularly at day 2 following the exposure of disease-causing CTX. Better designed animal studies and randomized clinical trials in humans are warranted.

ARTICLE HIGHLIGHTS

Research background

Treatment of diarrhea such as cholera remains a global challenge. Cholera toxin (CTX) produces diarrhea through direct epithelial action and indirectly by activating the enteric nervous system. Calcium-sensing receptor (CaSR) is present in

both tissues and, when activated, inhibits both actions. The latter has been well documented *in vitro* but not *in vivo*. Thus, the present study tested whether activating intestinal epithelial or neuronal CaSR inhibits diarrhea *in vivo*.

Research motivation

Acute infectious diarrhea remains among the top causes of morbidity and death in the world. Most of the morbidity and mortality is not due to infection but dehydration. Accordingly, how to effectively reduce the fluid loss from acute diarrhea offers a major opportunity for improving global health.

Research objectives

The objective of the present study was to determine whether CaSR agonists ameliorate secretory diarrhea evoked by CTX in wild type mice, epithelial-specific CaSR knockout mice ($^{villin}Cre/Casr^{flox/flox}$) and neuronal-specific CaSR knockout mice ($^{nestin}Cre/Casr^{flox/flox}$).

Research methods

To realize the objectives, CTX was administered orally to C57BL/6 mice to induce secretory diarrhea while calcium and calcimimetic R568 were employed to activate CaSR. To maximize their local intestinal actions, calcium was administered luminally *via* oral rehydration solution (ORS) whereas R568 was applied serosally using an intraperitoneal route. To verify that their actions resulted from the intestinal epithelium and enteric neurons, effects were also examined on two Cre-lox intestine-specific CaSR knockouts. Diarrhea outcome was measured biochemically by monitoring changes in fecal Cl^- or clinically by assessing stool consistency and weight loss.

Research results

CTX induced secretory diarrhea, as evidenced by increases in fecal Cl^-, stool consistency, and weight loss following CTX exposure. Calcium and R568 each ameliorated CTX-induced secretory diarrhea in wild type mice but not in either knockout mouse model.

Research conclusions

Based on the present study, we propose that activating intestinal epithelial or neuronal CaSR can inhibit secretory diarrhea *in vivo*. Adding calcium to ORS or using calcimimetic to activate intestinal CaSR might represent a novel approach for treating secretory diarrheal diseases in humans.

Research perspectives

Future research should be directed to conduct randomized clinical trials utilizing calcium or calcimimetics to treat cholera and other secretory diarrheal diseases in humans.

FOOTNOTES

Author contributions: Cheng SX conceptualized the study; Tang LQ, Fraebel J, and Cheng SX designed the study and analyzed the data; Tang LQ, Fraebel J, Jin S, and Winesett SP performed the experiments; Tang LQ and Cheng SX drafted the manuscript; Harrell J and Chang WH edited the manuscript; Cheng SX finalized the manuscript; and all authors approved the final version of the article.

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LETTER TO THE EDITOR

Use of curcumin and its nanopreparations in the treatment of inflammatory bowel disease

Zi-Wen Meng, Bing Chang, Li-Xuan Sang

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Abstract

Inflammatory bowel disease (IBD) is a nonspecific inflammatory disease of the intestine that includes Crohn's disease and ulcerative colitis. Because IBD is difficult to heal and easily relapses, it could worsen patient quality of life and increase economic burdens. Curcumin (CUR) is a bioactive component derived from the rhizome of turmeric (Curcuma longa). Many basic and clinical studies have shown that CUR can efficiently treat IBD by decreasing the activity of proinflammatory cytokines by communicating with transcription factors and signaling molecules. However, due to the limitations of being almost insoluble in aqueous solutions and having low oral bioavailability, it is important to select appropriate pharmaceutical preparations.

Key Words: Curcumin; Inflammatory bowel disease; Bioavailability; Nanotherapeutics;

Core Tip: Curcumin (CUR) can efficiently decrease the activity of proinflammatory cytokines by communicating with transcription factors and signaling molecules. It is a new area of research that may be promising in the future to treat patients with inflammatory bowel disease, especially in patients with ulcerative colitis. How to improve the bioavailability of CUR *in vivo* was also discussed.

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TO THE EDITOR

With great interest, we have read the article by Zheng *et al*[1], who found that curcumin (CUR) regulated mTh/mTfh cell homeostasis by inhibiting the c-Jun amino-terminal kinases (JAK) 1/STAT3/SOCS signaling pathway, thus alleviating dextran sulfate sodium (DSS) induced pathological injury in the colon. Various studies have shown that CUR can also efficiently decrease the activity of proinflammatory cytokines by communicating with other transcription factors and signaling molecules. For example, CUR inhibits the activation of transcription factors, multiple protein kinases, and antiapoptotic proteins and modulates various inflammatory cytokines by suppressing the inflammatory transcription factor nuclear factor-κB[2]. Khan *et al*[3] reported the inhibitory effects of CUR on JNKs, extracellular-signal-regulated kinases, and stress-activated protein kinases. These inhibitory effects involve decreasing the expression and release of proinflammatory mediators, such as tumor necrosis factor (TNF)-α and adhesion molecules. Current research indicates that CUR has high medicinal value, including anti-inflammatory, antioxidant, antitumor, antiapoptotic, antifibrotic, immunoregulatory and other effects, and can be used to treat a variety of diseases[4].

Although CUR has few adverse effects and is highly safe for use, it still has several disadvantages. CUR is hardly soluble in water solution due to its lipophilic properties and low bioavailability after oral administration[5]. Therefore, it is particularly important to choose a combination of CUR and other treatments or a modified CUR formula to treat ulcerative colitis (UC). First, Xu *et al*'s evaluation of the in vivo therapeutic effects on DSS-induced UC in mice revealed that dexamethasone (DEX)-loaded hydroxyethyl starch-CUR nanoparticles could enhance the efficacy of free DEX and significantly alleviate the lesions caused by UC[6]. Second, A nanocarrier of CUR coated with tannic acid and genipin crosslinked human serum albumin was prepared into CUR nanoparticles by Luo *et al*[7]. The synthetic nanoparticles prolonged the colonic adhesion of CUR and improved its absorption in Caco-2 cells. In addition, a study demonstrated that oral administration of turmeric-derived nanoparticles containing a specific preparation could ameliorate colitis in mice and accelerate colitis resolution by regulating the expression of proinflammatory cytokines, including TNF-α, interleukin (IL)-6, and IL-1β, and the antioxidant gene *HO-1*[8].

Notably, CUR nanomaterials have been tested not only in preclinical animal models but also in human clinical trials for the treatment of various diseases[9]. Further clinical studies on the possible benefits and associated risks of CUR nano preparations in patients with IBD are also warranted in the future[10].

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LETTER TO THE EDITOR

Cholecystokinin and cholecystokinin-A receptor: An attractive treatment strategy for biliary dyskinesia?

Jun Chang, Yan Liu, Ting-Can Jiang, Lan Zhao, Jian-Wei Liu

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Abstract

Biliary dyskinesia is a relatively common gastrointestinal disease that is increasing in incidence as living standards improve. However, its underlying pathogenesis remains unclear, hindering the development of therapeutic drugs. Recently, "Expression and functional study of cholecystokinin-A receptors on the interstitial Cajal-like cells of the guinea pig common bile duct" demonstrated that cholecystokinin (CCK) regulates the contractile function of the common bile duct through interaction with the CCK-A receptor in interstitial Cajal-like cells, contributing to improving the academic understanding of biliary tract dynamics and providing emerging directions for the pathogenesis and clinical management of biliary dyskinesia. This letter provides a brief overview of the role of CCK and CCK-A receptors in biliary dyskinesia from the perspective of animal experiments and clinical studies, and discusses prospects and challenges for the clinical application of CCK and CCK-A receptors as potential therapeutic targets.

Key Words: Cholecystokinin; Cholecystokinin-A receptor; Biliary dyskinesia; Interstitial Cajal-like cell; Therapeutic target

Core Tip: Biliary dyskinesia has an estimated 10% morbidity rate and its cause is unknown, hindering the development of appropriate treatments. Traditional surgical treatments have side effects and there is thus an urgent need to identify safe and effective therapeutic targets. This letter agrees with the findings of "Expression and functional study of cholecystokinin-A receptors on the interstitial Cajal-like cells of the guinea pig common bile duct" and provides a brief overview of the prospects and challenges of cholecystokinin (CCK) and CCK-A receptors as potential targets in biliary dyskinesia from the perspective of animal experiments and clinical studies.

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TO THE EDITOR

We were interested to read an original article "Expression and functional study of cholecystokinin-A receptors on the interstitial Cajal-like cells of the guinea pig common bile duct" by Xu *et al*[1]. We agree with the authors' findings that cholecystokinin (CCK)-A receptors are highly expressed by common bile duct (CBD) interstitial Cajal-like cells (ICLC) and that CCK interacts with ICLC CCK-A receptors to regulate CBD smooth muscle contraction in a dose-dependent manner. We are grateful to the authors for their commitment to the study of CCK and CCK-A receptors in biliary dyskinesia, as this will assist in the elucidation of the key cells and receptors involved in biliary dyskinesia and thus provide promising directions for the development of clinical treatments for the disorder.

Gallbladder motility is regulated by hormonal interactions. CCK is a peptide hormone found in neurons and the gastrointestinal tract that regulates digestive, cardiovascular, and neurological functions by binding to CCK receptors on target cells. In the digestive system, CCK regulates cholecystic contraction, pancreatic enzyme secretion, and gastrointestinal peristalsis. CCK binds to CCK receptors to induce gallbladder contraction and promote cholecystic emptying and bile release[2] and also mediates rhythmic contraction of the gallbladder and diastole of the sphincter of Oddi, resulting in the release of bile from the gallbladder into the duodenum to participate in food digestion. An animal study found that increased levels of CCK enhanced cholecystic contractile function, while on the contrary, reduced CCK levels led to cholecystic contractile dysfunction and ultimately led to gallstone formation[3]. Notably, Xu *et al*[1] found that in guinea pigs, CCK interacted with ICLC CCK-A receptors to regulate CBD smooth muscle contractility in a dose-dependent manner[1], suggesting that CCK and CCK-A receptors play a key role in regulating CBD smooth muscle contraction. The CCK-A receptor is a major mediator of gallbladder smooth muscle contraction and is highly expressed by guinea pig CBD ICLCs[1]. Reduced expression of the CCK-A receptor in the mouse gallbladder is an important cause of cholelithiasis[4]. These animal studies suggest that both CCK and CCK-A receptors may be attractive targets for combating biliary dyskinesia.

However, there have been few studies on the safety and efficacy of targeting CCK and CCK-A receptors in humans. A clinical study explored whether a CCK-A agonist (GI181771X) was beneficial in reducing body weight in obese patients. GI181771X was found to have no significant effect on body weight and waist circumference, nor on hepatobiliary, pancreatic, and other cardiometabolic markers, but had mild side effects in the gastrointestinal tract[5]. In contrast, another clinical study analyzed the role of CCK-A receptors in patients with functional dyspepsia and found that a CCK-A antagonist (dexloxiglumide) reduced gastric volume and dyspepsia during duodenal lipid infusion, and also reduced gastric compliance during gastric distension[6], which implies that CCK-A receptors play a significant role in gastric distension and duodenal lipid-induced symptoms of dyspepsia. Similarly, clinical studies used a CCK-A antagonist (loxiglumide) to assess the role of CCK-A receptors in postprandial satiety and nausea and their influence on duodenal lipids, and found that loxiglumide reduced both postprandial satiety and nausea[7], indicating the involvement of CCK-A receptors in inducing these symptoms. Despite these findings, research on the effectiveness and safety of targeting CCK-A receptors in the treatment of organic digestive diseases is still in the preliminary stage, and more in-depth exploration is required to provide a scientific basis for the prevention and treatment of these diseases and biliary dyskinesia in particular.

As an important hormone that affects the contraction of gallbladder tissue, CCK plays a unique role in the maintenance of physiological homeostasis in the body. However, current animal and clinical studies have not fully elucidated its biological effects, and its safety and effectiveness warrant further investigation. It has been reported that while CCK promotes gastric motility in guinea pigs, it has the opposite effect in both humans and dogs[8], indicating that the effect of CCK on gastric motility is species-dependent. Further investigation into species differences in the effects of CCK on biliary motility is required. In addition, the biological mechanisms underlying the interaction between CCK and the CCK-A receptors, which mediate the cholecystic contractile function, require further study. Once the safety and effectiveness of targeting the CCK-CCK-A receptor interaction have been clarified in animal studies, it will be necessary to conduct large-scale clinical trials to promote the clinical transformation of basic research results and better serve patients.

In conclusion, while biliary dyskinesia is traditionally treated with cholecystectomy, this can cause side effects such as diarrhea, dyspepsia, and duodenal gastrointestinal reflux, as well as damage to the patient's immune system. Thus, in recent years, treatment involving gallbladder conservation has tended to be used for biliary dyskinesia-related disorders,

which makes the search for potential targets for the prevention and treatment of biliary dyskinesia particularly important. The study of biliary tract dynamics represents a research hotspot in extra-biliary science. Evidence from in-depth basic and clinical research on biliary tract dynamics is expected to clarify the key cells and receptors together with their functions and regulatory mechanisms, allowing the identification of therapeutic targets for biliary dyskinesia and the design of drugs against these targets, which will, in turn, provide a theoretical basis for the standardized treatment of biliary dyskinesia.

FOOTNOTES

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