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OPINION REVIEW

Very early onset perinatal constipation: Can it be cow's milk protein allergy?

Rajalakshmy Arakoni, Hebat Kamal, Sam Xianjun Cheng

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Abstract

Delayed passage of meconium or constipation during the perinatal period is traditionally regarded as a signal to initiate further work up to evaluate for serious diagnoses such as Hirschsprung's disease (HD), meconium ileus due to Cystic Fibrosis, *etc.* The diagnosis of HD particularly warrants invasive testing to confirm the diagnosis, such as anorectal manometry or rectal suction biopsy. What if there was another etiology of perinatal constipation, that is far lesser known? Cow's milk protein allergy (CMPA) is often diagnosed in infants within the first few weeks of life, however, there are studies that show that the CMPA allergen can be passed from mother to an infant in-utero, therefore allowing symptoms to show as early as day one of life. The presentation is more atypical, with perinatal constipation rather than with bloody stools, diarrhea, and vomiting. The diagnosis and management would be avoidance of cow's milk protein within the diet, with results and symptom improvement in patients immediately. Therefore, we discuss whether an alternative pathway to address perinatal constipation should be further discussed and implemented to potentially avoid invasive techniques in patients. This entails first ruling out CMPA with safe, noninvasive techniques with diet modification, and if unsuccessful, then moving forward with further diagnostic modalities.

Key Words: Delayed passage of meconium; Perinatal constipation; Cow's milk protein allergy; In-utero; Alternate pathway; Hirschsprung's disease

Core Tip: Cow's milk protein allergy (CMPA) is a far lesser known cause of perinatal constipation compared to more frequently considered diagnoses such as Hirschsprung's, Cystic fibrosis related meconium ileus, *etc.* The presentation during the perinatal period is considered atypical caused by a non-immunoglobulin E (IgE) mechanism as opposed to the typical presentation caused by an IgE-mediated mechanism. The likelihood of CMPA is significant in the perinatal period, therefore should be considered more often. Here we discuss an alternative pathway for the workup of perinatal constipation focusing on CMPA as an etiology. The use of this pathway can avoid invasive tests among patients.

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INTRODUCTION

Delayed passage of meconium or constipation is defined as failure to pass stool within the first 48 h after birth[1]. With a positive history of delayed meconium passage, providers traditionally regard this as a signal towards serious diagnoses such as Hirschsprung's disease (HD), meconium ileus (MI) due to cystic fibrosis (CF), or intestinal obstruction, and initiate work up such as barium enema, anorectal manometry, and or rectal suction biopsy per current recommendations [2]. We rarely consider cow's milk protein allergy (CMPA), even though it has been described to occur in the perinatal period[3,4] and causes disease that mimics HD[5-8]. Since there's still no available diagnostic laboratory tests, the diagnosis of CMPA has to be clinical. We recently reported 3 cases of infants who had delayed passage of meconium with subsequent early-onset perinatal constipation that did not respond to conventional therapies and required rectal stimulation to defecate. The symptoms resolved when the milk protein component was withheld and recurred when milk proteins were reintroduced for the patients. The symptoms subsequently resolved again when switched to an extensively hydrolyzed or amino acid-based formula[9]. Thus, it had not only avoided more aggressive and invasive workup, but had also significantly improved the care of this very vulnerable young patient population. Unfortunately, per our recent survey, this early-onset atypical perinatal form of CMPA was rarely recognized by a large majority of care providers including gastroenterologists, contrasting the typical form of CMPA that occurs later in life. This is not surprising; it indicates that more presentations of this condition to gastroenterology communities are necessary. In fact, even for its typical, more known counterpart of CMPA, it has taken approximately 20 years of time before it became widely recognized by providers. CMPA was rarely diagnosed before 1950, but since 1970 the condition has been further documented[10]. This paper will discuss 4 burning questions regarding the atypical CMPA, typical CMPA, and HD.

WHAT IS THE PREVALENCE OF HD *VS.* CMPA IN INFANTS WITH CONSTIPATION?

A number of conditions can cause delayed passage of meconium or early-onset perinatal constipation. This includes HD, intestinal obstruction, MI, meconium plug syndrome, functional ileus, small left colon, drug effect, hypothyroidism, and megacystis-microcolon-intestinal hypoperistalsis syndrome[2]. However, these conditions might be much less common than we think when thinking about perinatal constipation. For example, a study conducted in 2008 looked at causes of meconium plug syndrome (radiological form of perinatal constipation) in the largest cohort of a patient population to that date, which actually indicated that only about 13% were due to HD, and none were caused by CF[11]. Table 1 below summarizes the incidences in pubmed of HD, meconium plug syndrome, MI, anorectal malformation, and CMPA in infants, whereas Table 2 lists the frequencies of HD relative to CMPA. As shown, the incidences of these congenital diagnoses are extremely low, less than 0.1%[2] for all four diagnoses individually, in contrast to CMPA, which is estimated to occur in 0.5%-17%[10,12]. Thus, it is possible that the majority of the cases of perinatal constipation are not caused by HD or other congenital etiologies, but by acquired CMPA. In accordance with this, we recently observed 25 neonates/young infants referred to our clinic for intractable perinatal constipation and found 23 responded to cow's milk protein (CMP) avoidance, suggesting that this very early onset constipation is largely related to CMPA, specifically the atypical CMPA.

WHAT IS ATYPICAL CMPA AND HOW DOES IT DIFFER FROM TYPICAL CMPA?

CMPA is an abnormal immunological response to CMP. According to time of onset, CMPA can be categorized into two forms: Atypical form that occurs early in life before, during, or shortly after birth, and typical form that commonly happens later in life, typically weeks or months after birth. Table 3 compares their differences in clinical presentation, way of allergen transmission, type of allergy reaction, and intestinal tissue and brief mechanism involved as well as their diagnosis, management, and relative awareness. The development of CMPA requires exposure followed by an immunological response to the milk allergen, which can take up to months to occur. Therefore, CMPA has been thought to most

Table 1 Incidences of various diagnoses in infants with very early-onset constipation

Diagnosis	Incidence	Ref.
Hirschsprung's disease	0.025%	Loening-Baucke and Kimura[2]
Meconium plug syndrome	0.1%-0.2	Loening-Baucke and Kimura[2]
Meconium ileus	0.04	Loening-Baucke and Kimura[2]
Anorectal malformation	0.025%-0.05%	Loening-Baucke and Kimura[2]
CMPA	0.5%-17%	Høst[10], Rona *et al*[12]

CMPA: Cow's milk protein allergy.

Table 2 Relative frequencies of Hirschsprung's disease *vs* cow's milk protein allergy in infants with very early-onset constipation

Ref.	HD (%)	CMPA (%)
Burge and Drewett[18]	38.1	
Kubota *et al*[19]	34.6	65.4
Van Leeuwen *et al*[20]	25.0	
Keckler *et al*[11]	13.0	
Cheng *et al* (unpublished observation)	8.0	92.0

CMPA: Cow's milk protein allergy; HD: Hirschsprung's disease.

Table 3 Comparison of atypical *vs* typical cow's milk protein allergy

	Atypical CMPA	Typical CMPA
Time of onset	Perinatal period[3]	Early infancy[21,22]
Way of transmission of allergen	Vertically *via* placenta and amniotic fluid[3]	Vertically *via* breast feeding Horizontally by oral ingestion[21,22]
Typical presentation	Intractable constipation[5,6]	Vomiting and diarrhea, bloody stools[21]
Type of allergy reaction	Non IgE mediated[5]	IgE mediated[5]
Tissues involved	Enteric neurons and smooth muscle[5-8]	Intestinal mucosa[5]
Mechanism involved	Immune mediated neuromuscular dysfunction leading to persistent spasm or failure to relax of the anorectum (HD like changes)[5-8]	Allergic enterocolitis[5]
Diagnosis	CMP avoidance and challenge. Both blood and skin allergy testings are of no diagnostic value[9]	CMP avoidance and challenge with or without blood and skin allergy testing[21]
Treatment	CMP avoidance[9]	CMP avoidance[9]
Awareness to providers	Rarely aware/reported	Well known/reported

CMP: cow's milk protein; CMPA: cow's milk protein allergy; HD: Hirschsprung's disease; IgE: Immunoglobulin E.

commonly occur within weeks or months after their first postnatal feed. However, cow's milk allergens are not only transported postnatally *via* the oral route; they can also pass through the placenta and amniotic fluid to sensitize fetuses and cause allergy[3-4]. This indicates that the process of developing CMPA can occur prenatally. To support this, there are studies that show α-lactalbumin, -lactoglobulin, and α-casein were found in full-term neonates, which are indications of responses of cord blood lymphocytes to cow's milk allergen[3,13]. Figure 1 demonstrates the passage of CMP from maternal ingestion through the placenta, affecting the infant in-utero, eventually leading to perinatal constipation. The typical, late-onset form of CMPA is now well known to care providers, however the early-onset form is often overlooked, particularly when this type of CMPA presents with atypical symptoms. Most pediatricians including pediatric gastroenterologists are not aware of the early-onset CMPA. As a result, many atypical cases of neonate infants CMPA were

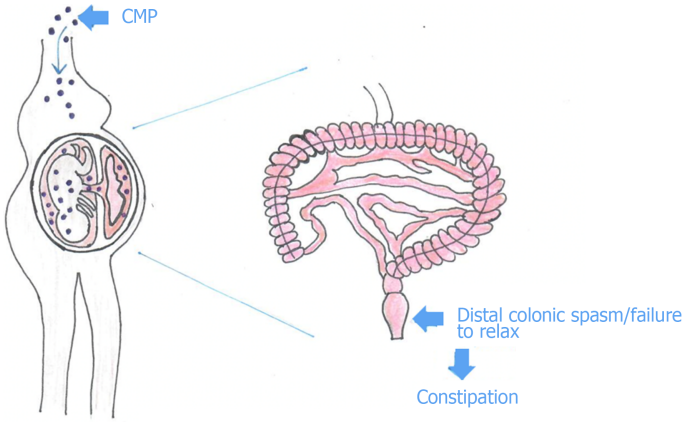

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Figure 1 How cow's milk protein allergen from maternal diet passes into the fetus causing distal colon spasm or failure to relax leading to Hirschsprung's disease-like constipation. As mentioned in the text, the suspected mechanism involves neuromodulation of the enteric nervous system, with increased anal pressure at rest. CMP: Cow's milk protein.

missed, leaving them undiagnosed or misdiagnosed. This resulted in delay of initiating the appropriate treatment for this very treatable condition or led to many unnecessary workup procedures.

WHAT IS THE MECHANISM BY WHICH CMPA CAUSES PERINATAL CONSTIPATION?

The exact mechanism by which CMPA causes perinatal constipation has remained incompletely understood. CMP can cause constipation in at least two ways: Nonspecifically through CMP constipating effect and specifically through CMPA. CMPA, rather than CMP constipation, is considered a more probable etiology for perinatal constipation, because for the CMP constipation to occur, patients will require a prolonged consumption of the CMP and this is unlikely to occur in this neonatal patient population, during the perinatal period. Then, how does CMPA lead to perinatal constipation? CMPA occurs by two primary mechanisms: an immunoglobulin E (IgE)-mediated immediate hypersensitivity reaction and a non-IgE-mediated delayed hypersensitivity reaction[9]. As shown in Table 3, the former is primarily seen in the typical CMPA, while the latter is speculated to cause the atypical CMPA. The IgE-mediated reaction is described as IgE antibodies that are secreted in response to the allergen and bind to the surface of mast cells and basophils, causing subsequent release of histamine and inflammatory mediators, leading to eosinophilia, allergic colitis and proctitis, and bloody/mucousy stools[5]. The non-IgE mediated reaction is not as well-known as compared to the IgE-mediated reaction, but hypotheses include milk antigens binding to immune complexes of immunoglobulin A or immunoglobulin G or directly binding and stimulating T cells, resulting in activation of an inflammatory cascade[14] that involves neuromodulation of the enteric nervous system[15], leading to alteration of the function of smooth muscle and intestinal motility that is functionally similar to that of HD[5], with increased anal pressure at rest[16] causing difficulties in stooling. As illustrated in one of our cases[9], early-onset constipation had normal appearing colorectal histology, with no evidence of lamina propria/muscularis mucosa eosinophilia nor increased mast cell infiltration, therefore the etiology of the perinatal constipation is likely not the IgE-mediated but more consistent with the non-IgE mediated mechanism. Also, all of our observed cases mentioned in the previous case series[9] and subsequent new cases did not respond to stool softeners but required rectal stimulation/rectal insertion for bowel movement, suggesting that the mechanism of constipation is likely not simply related to the hardness of the stool but the dysmotility of the muscle of the distal colon, either due to distal colon spasm or failure to relax (Figure 1).

WHAT IS THE BEST MANAGEMENT ALGORITHM FOR CARE PROVIDERS TO DIFFERENTIATE ATYPICAL CMPA FROM HD IN PATIENTS WITH PERINATAL CONSTIPATION?

How to effectively diagnose the infants with acquired condition from the infants with congenital disease as in HD and other anatomical obstructions without extensive testing remains clinically challenging.

Current guidelines recommend a moderate level of suspicion for HD, although practice varies regarding the evaluation of these infants. At a minimum, they should be closely observed and evaluated promptly for HD if they develop symptoms of constipation even though a great majority of those patients are not due to HD[2]. We designate this management algorithm the "top-down" approach (Figure 2A).

Our practice has adopted a new management guideline in managing these patients[9] given that CMPA rather than HD causes most of these presentations. The new guideline has recommended performing a 2-week trial of CMP avoidance as the initial diagnostic procedure before performing a contrast enema, anorectal manometry, and or suction biopsy. We designate this new management algorithm the "bottom-up" approach (Figure 2B).

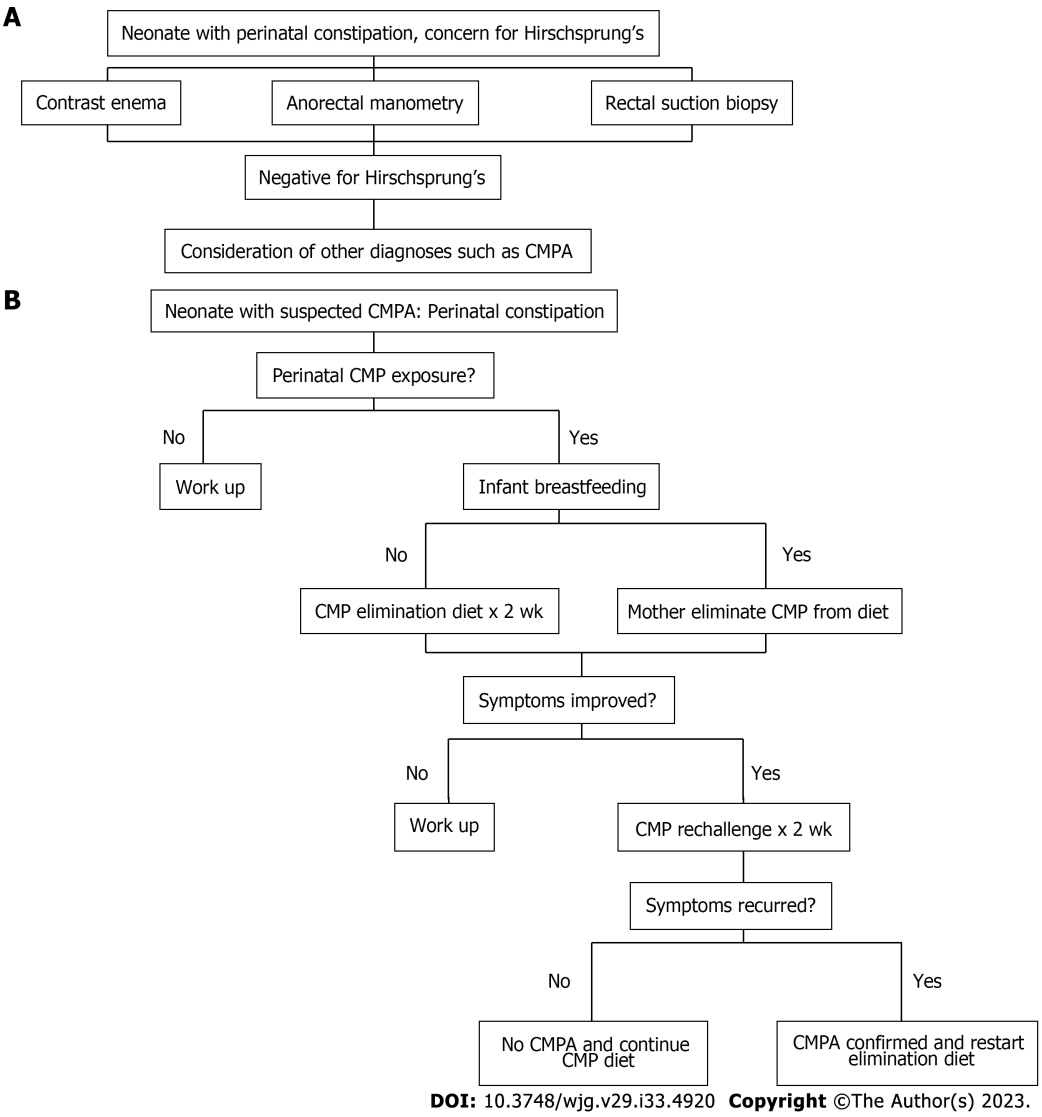

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Figure 2 Two algorithms for clinical management of patients with intractable early onset perinatal constipation. A: Top-down approach and current guideline for management of perinatal constipation. B: Bottom-up approach; new guideline to consider which suspects cow's milk protein allergy as a diagnosis. CMP: Cow's milk protein; CMPA: Cow's milk protein allergy.

The top-down approach relies on highly sophisticated investigations (*i.e.*, contrast enema, anorectal manometry or suction biopsy) to first rule out HD, which are rare and expensive; whereas the bottom-up protocol uses relatively simple food elimination diet to first rule in CMPA before rare entities are considered, which is common and inexpensive. With this new approach, we have promptly identified and successfully treated CMPA in 23 of 25 infants with intractable early-onset constipation without the need to go to any of the invasive testings (Cheng *et al*, unpublished observation). Thus, if this is validated by other clinical centers or practices, the new recommendation would largely help avert many unnecessary investigations, which are not only costly but are also invasive and expose patients to various potential complications.

CONCLUSION

Further understanding of the mechanism of CMPA makes it clear that infants can present with the manifestations immediately after birth. Therefore, shouldn't we consider CMPA in our patients who present with perinatal constipation early on? There is no doubt that the serious diagnoses such as HD and CF related MI cannot be missed, however, if there is a way to eliminate invasive tests, we should, as providers, incorporate it into our medical practice. There are many unanswered questions that have yet to be further studied. For example, in our studies, all the mothers of patients with perinatal constipation consumed dairy products during pregnancy, however, this does not mean all mothers who consume dairy products during pregnancy will necessarily give birth to infants who will develop CMPA or symptoms of perinatal constipation. What factor(s) determines who develops and who does not develop sensitization and allergy remains unclear. Also unclear is the factor(s) that determines the type of CMPA, typical *vs* atypical. Similarly, although

we know CMP allergens can pass the placenta and amniotic fluid to sensitize fetuses and cause allergy, we still do not know how long this sensitization process lasts and how long the allergens remain effective. The fact that our patients' symptoms resolved following the 2-week elimination period of the allergen, however, seems to suggest that this could approximate the timeline of allergen effectiveness. Also, the overall prognosis of typical CMPA is good, with a total recovery of 56% at 1 year, 77% at 2 years, 87% at 3 years, 92% at 5 and 10 years and 97% at 15 years of age[17]. Whether this applies to atypical CMPA in the perinatal population requires further investigation. Regardless of what the type of CMPA is, our recommendation for treatment of CMPA is the same, that is, once a diagnosis of CMPA is confirmed, a milk-free diet will be continued until a new milk challenge has shown development of tolerance. All infants with CMPA will be rechallenged at 12 mo of age and, in the event of continued clinical sensitivity to CMP, controlled rechallenges will be performed every 6 mo up to 3 years of age; and thereafter every 12 mo until tolerance develops.

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REVIEW

Non-coding RNAs: The potential biomarker or therapeutic target in hepatic ischemia-reperfusion injury

Jia-Li Shao, Li-Juan Wang, Ji Xiao, Jin-Feng Yang

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Abstract

Hepatic ischemia-reperfusion injury (HIRI) is the major complication of liver surgery and liver transplantation, that may increase the postoperative morbidity, mortality, tumor progression, and metastasis. The underlying mechanisms have been extensively investigated in recent years. Among these, oxidative stress, inflammatory responses, immunoreactions, and cell death are the most studied. Non-coding RNAs (ncRNAs) are defined as the RNAs that do not encode proteins, but can regulate gene expressions. In recent years, ncRNAs have emerged as research hotspots for various diseases. During the progression of HIRI, ncRNAs are differentially expressed, while these dysregulations of ncRNAs, in turn, have been verified to be related to the above pathological processes involved in HIRI. ncRNAs mainly contain microRNAs, long ncRNAs, and circular RNAs, some of which have been reported as biomarkers for early diagnosis or assessment of liver damage severity, and as therapeutic targets to attenuate HIRI. Here, we briefly summarize the common pathophysiology of HIRI, describe the current knowledge of ncRNAs involved in HIRI in animal and human studies, and discuss the potential of ncRNA-targeted therapeutic strategies. Given the scarcity of clinical trials, there is still a long way to go from pre-clinical to clinical application, and further studies are needed to uncover their potential as therapeutic targets.

Key Words: Hepatic ischemia-reperfusion injury; Non-coding RNAs; MicroRNAs; Long non-coding RNAs; Circular RNAs

Core Tip: This review focuses on the recent progress in understanding non-coding RNAs (ncRNAs) in hepatic ischemia-reperfusion injury (HIRI). HIRI can alter ncRNAs expressions, which in turn modulates the pathophysiological processes that contribute to the development of HIRI. Differentially expressed ncRNAs from different sources (the liver tissues, serums and cells) are involved in oxidative stress, inflammatory responses, cell death and so on. ncRNAs are regarded as biomarkers for the diagnosis and assessment of liver damage severity, or as therapeutic targets for HIRI; however, their clinical transformation will still take a long time.

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INTRODUCTION

Hepatic ischemia-reperfusion injury (HIRI) is a common clinical issue that occurs during major liver resection, liver transplantation, and liver trauma[1-3]. HIRI usually causes liver injury, early transplantation failure, liver failure and even multiple-organ failure. Although existing studies have shown that several signaling pathways, such as oxidative stress, inflammatory response, and cell death, participate in the pathological process of HIRI[4-6], current treatments and pharmacological approaches cannot completely address this problem. Therefore, much more new molecules need to be explored for the diagnosis and treatment of HIRI.

Non-coding RNAs (ncRNAs) are a cluster of functional RNAs that cannot encode protein[7,8]. In recent years, ncRNAs have become a hot area of research and have been reported to play a significant role in various diseases, including cancers, nervous system diseases, and ischemia/reperfusion injuries[9-11]. ncRNAs mainly consist of microRNAs (miRNAs), long ncRNAs (lncRNAs), and circular RNAs (circRNAs), which have been shown to modulate genes expression, and participate in many critical biological processes at different levels (*e.g.* immune responses, oxidative stress reactions, apoptosis, autophagy, and energy metabolism)[12-14]. The role of ncRNAs in HIRI is explored in a few studies, and has attracted the attention of many researchers.

ncRNAs are regarded as potential biomarkers or therapeutic targets for assessing or attenuating HIRI development. As the present studies are mainly in the preclinical stage, and clinical investigations are lacking, there are still large research prospects for the diagnosis, prevention, and treatment of HIRI. This review briefly illustrates the well-studied molecular mechanisms of HIRI and summarizes the relevant ncRNAs and their roles in the pathological process of HIRI to provide a reference for further research.

HIRI AND THE UNDERLYING MECHANISMS

HIRI is usually classified into two types, warm IRI *in situ* and cold IRI *in vitro*, and the cells involved are different. Warm IRI is characterized by hepatocellular injury, which is mainly caused by Kupffer cells (KCs) induced oxidative stress and neutrophils recruitment[15]. Cold IRI is associated with sinusoidal endothelial cells (SECs) damage[11]. Although the initial cells are different, they do share common subsequent reactions: activation of cell death programs such as apoptosis, necrosis, pyroptosis, and autophagy, thus contributing to the development of inflammation[1,16-18].

The mechanisms involved in HIRI pathogenesis are multifactorial and complex. Numerous studies have demonstrated several molecular mechanisms that contribute to the development of HIRI[19], such as anaerobic metabolism, immune response[20], microcirculatory dysfunction[21], and gene transcription[6,21].

Oxidative stress

The most widely studied mechanism is oxidative stress, which is defined as an imbalance between the oxidant and antioxidant systems, resulting in tissue damages[22,23]. For instance, CeO2, NO, and chlorogenic acid have a liver-protective effect by reducing oxidative stress during HIRI[20,24,25]. In contrast, Han *et al*[26] found that hyperglycemia aggravates HIRI by inducing reactive oxygen species (ROS) mediated oxidative stress. ROS are the most critical reactive molecules involved in HIRI. Enormous amounts of ROS are usually generated by mitochondria and KCs during reperfusion, which can result in apoptosis, autophagy, inflammation, protein and DNA damage, and worsened hepatocyte injury[5,22]. Prussian blue scavenger is a potential therapeutic agent for treating HIRI with ROS-scavenging and anti-inflammatory properties[27]. OX40 expression in neutrophils can increase ROS production, which in turn activates neutrophils and, aggravates HIRI[28]. Dugbartey *et al*[29] designed a reversible redox probe REPOM to monitor ROS during HIRI for early diagnosis and timely intervention. Furthermore, an increasing number of studies on gene therapy for oxidative stress have attracted the attention of researchers in recent years. Therefore, strategies that are aimed at inhibiting oxidative stress or scavenging ROS may alleviate HIRI, and novel antioxidant regulatory molecules are required.

Inflammatory response and immune response

The inflammatory response is another major mechanism underlying HIRI. During HIRI development, KCs and SECs are initially activated[30,31], and generate a range of inflammatory mediators including cytokines, such as PAF, TNFα, and interleukins (*e.g.*, IL-1, IL-6, IL-12, and IL-23), which could lead to the inflammatory response involved in HIRI development[32-34]. In addition, several cytokines (*e.g.*, PAF, leukotriene B4, IL-8, and IL-17) induce neutrophil accumulation, which plays a role in the process of HIRI[35]. Furthermore, activated neutrophils can form neutrophil extracellular traps (NETs) *via* TLR-dependent pathways, that initiate inflammatory responses during HIRI[36]. This local inflammatory state can cause a systemic inflammatory response, leading to systemic inflammatory response syndrome and even multiple-organ failures.

Both innate and adaptive immune responses play important roles in HIRI[37,38]. KCs can increase the production of damage-associated molecular patterns (DAMPs), such as ATP, histones, high mobility group box 1 (HMGB1), S100, and heat shock proteins, which are released into the circulation and induce cytokine/chemokine storms to attract neutrophils and other immune cells[39]. In contrast, DAMPs can bind to Toll-like receptors (TLRs) and drive immune responses. Targeting the DAMP pathways alleviates HIRI. For example, HMGB1/NLRP3 inflammasome inhibition attenuated HIRI [40]. In addition, the complement system serves as an important contributor to the process of HIRI[41-43]. Therefore, targeting inflammation-oriented therapies may alleviate HIRI.

Cell death

Cell death is a stable pathological indicator of I/R injury, including apoptosis, necrosis, autophagy, pyroptosis, and ferroptosis. Among them, apoptosis, necrosis, and autophagy are the most common types of cell death during HIRI and may share the same stimuli and signaling pathways[44]. For example, hydrogen sulfide (H_2S) effectively alleviates HIRI by attenuating hepatocyte apoptosis *via* inhibition of the endoplasmic reticulum (ER) stress response[22]. Eucommia ulmoides polysaccharide administration notably reduced the area of liver necrosis in a rat HIRI model[45]. Cafestol preconditioning can inhibit apoptosis and autophagy in hepatocytes, thus attenuating HIRI, by suppressing the extracelluar signal-regulated/eroxisome proliferator-activated receptor gamma (ERK/PPARγ) pathway[46]. Moreover, some studies suggest that mitochondrial autophagy is a key pathological mechanism underlying age-dependent hypersensitivity to HIRI[16]. In our previous studies, we found that octreotide pretreatment mitigated HIRI and attenuated kidney injury caused by HIRI by inhibiting hepatocellular apoptosis and enhancing autophagy[47-49]. These three processes usually coexist and participate in the development of HIRI.

The role of pyroptosis in HIRI remains unclear. Pyroptosis is a form of regulated cell death driven by perturbations in extracellular or intracellular homeostasis related to innate immunity. It is usually associated with IL-1β and IL-18 secretion and hence mediates robust pro-inflammatory effects[50]. Adipose-derived mesenchymal stem cells reduce pyroptosis in HIRI by inhibiting the NF-κB pathway and activating the Wnt/β-catenin pathway[51,52]. In the hepatic tissue of HIRI mice, lncRNA KCNQ1OT1 increased, which modulated miR-142a-3p/HMGB1, thereby promoting pyroptosis[15]. Under HIRI condition, IKZF1 increased, but sirtuin 1 (SIRT1) decreased in both human and mouse livers. Further investigation indicated that IKZF1 augmented pyroptosis through negatively regulating SIRT1 expression. Hence, pyroptosis plays an important role in the development of HIRI, and targeting pyroptosis is a promising approach for attenuating HIRI. However, the role of pyroptosis in HIRI requires further investigations.

In addition to the common types of cell death, ferroptosis has recently been reported to participate in HIRI. In 2012, Dixon *et al*[53] first proposed the definition of ferroptosis, which is characterized by the iron-dependent accumulation of lethal lipid ROS. Then ferroptosis was proved contributed to HIRI pathogenesis. Deferoxamine, (an iron chelator) attenuated HIRI and lipid peroxidation, whereas iron overload was a novel risk factor for HIRI[53]. Bioinformatics analysis was conducted to predict the genes related to ferroptosis in HIRI. Five genes (ATF3, IL6, IL1B, CDKN1A, and PTGS2) and several miRNAs (miR-128-3p and miR-24-3p) were identified[54]. Subsequently, Maresin conjugate in tissue regeneration 1 ameliorated ferroptosis-induced HIRI by promoting nuclear factor erythroid-derived 2-like 2 expression [55]. However, μ opioid receptor alleviated ferroptosis in HIRI *via* the HIF-1α/ axis[56]. These results indicate that ferroptosis plays a critical role in HIRI.

Other mechanisms

Many other regulatory mechanisms, in addition to those mentioned in previous sections, are involved in the progress of HIRI. For instance, mitochondrial dysfunction (such as mitophagy and impairment of mitochondrial permeability) plays a vital role in HIRI[16]. Acidic microenvironment has been reported to be a key factor affecting HIRI through the regulation of PPAR-γ[26]. Besides, lipid metabolites[57,58], calcium overload[56], adenosine triphosphate depletion[55], gap junctions[6], dysfunctional microcirculation[5,53], and endoplasmic reticulum stress[26,29] can affect the development of HIRI.

miRNAs

miRNAs are a class of endogenous single-chain, small ncRNAs with (21-25 nucleotides in length). The first known miRNAs (lin-4 and let-7) were identified in C. elegans in 1993[59]. Subsequently, more miRNAs have been found in plants, viruses, and animals. miRNAs produced from hairpin-like precursor transcripts are regulators of posttranscriptional and transcriptional gene expression[7,60], and are involved in various biological processes, such as development, apoptosis, metabolism, and proliferation[61]. miRNAs play important roles in human diseases, such as cancer, cardiovascular diseases, and genetic diseases[62,63]. In HIRI, numerous miRNAs have been thus far identified as

biomarkers or therapeutic targets in the past decades (shown in Table 1).

In 2009, Xu *et al*[64] reported for the first time that under the criteria of fold change > 2 and *P* value < 0.5, 78 miRNAs (40 down-regulated/38 up-regulated) were identified in the liver upon I/R injury. Among them, four miRNAs (miR-23a, miR-326, miR-346_MM1, and miR-370) were significantly downregulated by ischemic preconditioning (IPC) compared to non-preconditioned controls, implying a potential role of these miRNAs in the protective mechanism of IPC against hepatic injury[64]. Subsequently, more miRNAs were identified and their specific regulatory mechanisms were reported. The rise or fall of miRNAs determines their modulatory effects on HIRI development, and the different target genes of miRNAs allow them to be involved in the different mechanisms underlying HIRI, such as inflammation, apoptosis, and oxidative stress. The relevant information is presented in Tables 1 and 2.

miR-34

During HIRI, miR-34 is upregulated and mediates several important signaling pathways involved in various biological processes, such as inflammatory responses and apoptosis. Carbon monoxide (CO) inhalation or p-coumaric acid reduces miR-34a expression in the liver tissue, thus increasing SIRT1, which in turn mitigates HIRI by alleviating inflammatory responses, hepatocellular apoptosis and autophagy[64,65]. Similarly, H_2S and crocin exerted hepatoprotective effects in a rat model of HIRI by regulating the miR-34a/Nrf-2 pathway[66,67]. H_2S significantly modulated miR-34a expression in hepatocytes, whereas crocin regulated its expression in serum. Both zinc sulfate and gallic acid decreased the miR-34a serum level of miR-34a as an anti-miR to ameliorate HIRI[68-70]. Zheng *et al*[71] revealed that high miR-34a-5p expression may reduce liver injury during hepatectomy in adults. Further, agomir-miR-34a-5p could attenuate HIRI in rats, and *in vitro* experiments indicated that miR-34a-5p/HNF4α might be the underlying mechanism.

miR-122

miR-122 is a hepato-specific miRNA that accounts for nearly 70% of the total miRNAs pool in the liver tissue and exerts modulatory effects in liver diseases[71-74]. Recent studies have implicated its role in HIRI. van Caster *et al*[75] reported that miR-122 is a potential biomarker of warm HIRI of rats. In clinical research, serum miR-122 Levels were significantly elevated in patients with acute liver failure (ALF) than in healthy individuals. Further investigation revealed higher miR-122 levels in the serum and liver tissue of ALF survivors compared with those in the non-recovered patients[75]. Furthermore, miR-122 downregulation is involved in the hepatoprotective effects of crocin, gallic acid, and zinc sulfate [67-69]. However, John *et al*[76] found that hepatocyte-specific miR122 deletion in mice exacerbated liver injury during HIRI and that nanoparticle-mediated miR122 overexpression attenuated liver injury. These controversial modulatory effects require further investigations.

miR-370

In 2009, miR-370 was detected in a mouse model of HIRI by using miRNA microarrays. Subsequent analysis revealed that miR-370 was upregulated and positively correlated with the severity of ischemic injury[63,77]. Li *et al*[78] reported that miR-370 was significantly upregulated in a mouse model of HIRI, and that miR-370 inhibition efficiently attenuated liver damage *via* TbRII. Further investigation revealed that downregulation of miR-370 reduced the levels of proinflammatory cytokines, but had no effect on the apoptosis and proliferation of hepatocytes during HIRI. Moreover, the NF-κB gene was suggested as a potential target of miR-370[78]. Mesenchymal stem cells (MSCs) display immunomodulatory functions and have been proven to alleviate HIRI[79-81]. Zare *et al*[82] revealed that bone marrow-derived mesenchymal stem cells (BM-MSCs) downregulated miR-370 and inhibited inflammatory responses and apoptosis, thus attenuating liver damage during HIRI.

miR-494

In addition, miR-494 warrants further investigation. In a mouse model of HIRI, miRNA microarrays have indicated a upregulation of miR-494[63]. Besides, miR-494 expression was reported to significantly increase during hypoxia for 4 h in L02 cells, and its overexpression protected against hypoxia-induced apoptosis[82]. Another study suggested in a rat model of HIRI, miR-494 was elevated, and reducing its expression with propofol had a protective effect during HIRI[83, 84]. However, we observed an opposite trend in miR-494 expression of in other studies. Su *et al*[85] showed that miR-494 was downregulated in a rat model of HIRI and H_2O_2-induced apoptosis in hepatic AML12 cells. Furthermore, overexpression of miR-494 attenuated HIRI by modulating the PTEN/ PI3K/AKT signaling pathway[85]. We speculated that the opposite trend of miR-494 during HIRI might be caused by the duration of ischemia and reperfusion, cell lines, and different models. Based on previous studies, the researchers chosen different durations of ischemia (75 min, 30 min, and 60 min), different durations of reperfusion (2 h, reperfusion moment, 6 h), and different cell models (hypoxia-induced apoptosis in L02 cells and H_2O_2-induced apoptosis in AML-12 cells) for their studies. Further studies are required to confirm this hypothesis.

In addition to these above miRNAs, many other miRNAs have been identified, including miR-17, miR-155, miR-223, miR-494, miR-27-5p, miR-191, miR-450-5p, and miR-218-5p. Functional tests revealed they might be involved in HIRI development by modulating various biological processes such as cell death, inflammatory immune responses, and oxidative stress by targeting different downstream genes. Tables 1 and 2 present detailed information on the upregulated miRNAs.

The downregulated miRNAs

Several miRNAs were downregulated and exhibited modulatory effects on HIRI. miR-146b, miR-124, miR-20b-5p, miR-133a-5p, miR-449b-5p, miR-9-3p, and miR-124-3p were significantly downregulated in a rat HIRI model[85]. In terms of a

Table 1 MicroRNAs and their function in hepatic ischemia-reperfusion injury

miRNAs	Change	Targets	Effect on HIRI	Models	Ref.
miR-34a	Up	Nrf-2, SIRT1	Overexpression aggravates, downregulation alleviates	Rats, mice and RAW264.7	[66,68-71]
miR-34a-5p	Up	NF-κB /JNK/ P38	Overexpression alleviates	Human, rats, 7702 cells, AML12 cells	[72]
miR-122	Up	Nrf2, PHD1	Overexpression aggravates, overexpression alleviates	Rats, mice and human	[69-71, 78]
miR-370	Up	TbRII, NF-κB, Blc2/BAX	Downregulation alleviates	Mice and AML12	[79,80, 83]
miR-17	Up	Stat3	Overexpression aggravates	Mice and AML12	[96]
miRNA-155	Up	CD80, CD86, MHC-II	Knock out alleviates	Mice, Kupffer cells, AML12 cells, primary hepatocytes	[97]
MiR-223	Up		Biomarker	Mice and human	[98]
miR-494	Up	HIF-1α/HO-1, PI3K/Akt pathway	Overexpression alleviates	Mice, L02 cells, rats	[65,84, 85]
miR-27a-5p	Up	Bach1	Overexpression alleviates, downregulation aggravates	Mice and AML12 cells	[99]
miRNA-191	Up	ZONAB/Cyclin D1	Overexpression aggravates, knock out alleviates	Mice and LO2 cells	[100]
miR-450b-5p	Up	CRYAB/NF-κB, Akt1/mTOR	Downregulation alleviates	Mice and RAW 264.7 cells	[101]
miR-218-5p	Up	GAB2/PI3K/AKT	downregulation alleviates, overexpression aggravates	mice	[102]
miR-494	Down	PTEN/PI3K/AKT	Overexpression alleviates	Rats and AML12 cells	[86]
miR-146b	Down	TRAF6, NF-κB	Downregulation aggravates	Rats	[103]
miR-330-3p	Down	PGAM5	Overexpression alleviates, downregulation aggravates	Mice and AML12 cells	[104]
miR-1246	Down	IL-6-gp130-STAT3	Downregulation aggravates	Mice and hUCB-MSCs	[105]
miR-142	Down	HMGB1/TLR4/NF-κB	Overexpression alleviates, downregulation aggravates	Mice and NCTC 1469 cells	[93]
miR-96	Down	FOXO4	Overexpression alleviates, downregulation aggravates	Mice and primary hepatocytes	[92]
miR-494	Down	PTEN/PI3K/AKT	Overexpression alleviates	L02 cells; rats, AML12 cells	[84,86]
miR-30b	Down	Atg12-Atg5	Downregulation aggravates	Mice and AML12	[106]
miR-146a	Down	IRAK1, TRAF6	Overexpression alleviates	Mice and RAW264.7	[107]
miR-124	Down	Rab38, AKT pathway	Overexpression alleviates	Rats and L02 cells	[108]
miR-20b-5p	Down	SIRT1	Overexpression alleviates	Rats	[67]
miRNA-182-5p	No detect	TLR4	Overexpression alleviates	Mice and RAW264.7	[109]
miR-192-5p	Up/Down	Zeb2	Downregulation alleviates	Mice and Hepa1-6 cells, human	[90]
125b-5p	Down	Myd88, c-Fos and A20	No functional tests	Mice	[87]
miR-501-3p	Down	Myd88, c-Fos and A20	No functional tests	Mice	[87]
miR-133a-5p	Down	MAPK6	Overexpression alleviates, downregulation aggravates	Rats and QSG-7701	[95]
miR-214	Down	TRAF1/ASK1/JNK	Overexpression alleviates	Mice and AML12 cells	[110]
miR-449b-5p	Down	HMGB1, NF-κB	Overexpression alleviates	Rats and L02 cells	[111]
miR-142-3p	Down	MARCKS	Overexpression alleviates, downregulation aggravates	Mice and AML12, HepG2 cells	[112]
miR-24-3p	Down	STING	Overexpression alleviates	Mice	[113]
miR-9-5p	Down	CXCR4	Overexpression alleviates	Liver sinusoidal endothelial cells	[114]

miR-141-3p	Down	Keap1/Nrf2	Overexpression alleviates, downregulation aggravates	Human, mice and LO2 cells	[89]
miR-194	Down	PHLDA1	Overexpression alleviates	Mice and RAW 264.7 cells	[115]
miR-9-3p	Down	FNDC3VB	Overexpression alleviates, downregulation aggravates	Rats	[94]
miR-29a-3p	No change	Ireb2	Overexpression alleviates, downregulation aggravates	Rats and BMMSC	[116]
miR-124-3p	Down	TRAF3/CREB, Steap3	Overexpression alleviates, downregulation aggravates	Mice and Normal BNL Rats, CL.2 hepatocytes, BMMSC	[117, 118]
miR-140-5p	Down	CAPN1	Overexpression alleviates, downregulation aggravates	Mice and AML12 cells	[119]

miRNAs: MicroRNAs; HIRI: Hepatic ischemia-reperfusion injury.

Table 2 MicroRNAs and the involved mechanisms in hepatic ischemia-reperfusion injury

Mechanisms	miRNAs	Ref.
Apoptosis	miR-1, miR-17, miR-133, miR-205, miR-34a, miR-124, miR-146a, miR-494, miR-192-5p, miR-133a-5p, miRNA-155, miR-146b, miR-27a-5p, miR-214, miRNA-191, miR-370, miR-449b-5p, miRNA-142-3p, miRNA-24-3p, miR-9-5p, miR-96, miRNA-141-3p, miR-9-3p, miR-218-5p, miR-124-3p, miR-34a-5p, miR-142, miR-140-5p	[68,72,83,84,86,89-95, 97,99,100,102,107,108, 110-113,114,117,119]
Inflammatory responses	miRNA-182-5p, miR-370, miR-34a, miR-146a, 125b-5p and miR-501-3p, miRNA-155, miR-146b, miR-148a, miR-1246, MiRNA-142-3p, miRNA-24-3p, miR-9-5p, miR-194, miR-9-3p, miR-124-3p, miR-218-5p, miR-450b-5p, miR-142, miR-140-5p	[68,79,80,87,93,94,97, 101-103,105,107,109, 112-115,117,119,120]
Oxidative stress	miR-34a, miR-122, miR-494, miR-9-3p, miR-218-5p, miR-142	[69,70,71,86,93,94,102]
Autophagy	miR-17, miR-30b, miR-330-3p	[96,104,106]
Ferroptosis	miR-29a-3p, miR-124-3p	[116,118]

miRNAs: MicroRNAs.

mouse HIRI model, the downregulated miRNAs, including miR-330-3p, miR-1246, miR-142, miR-30b, miR-146a, miR-96, 125b-5p , miR-501-3p, miR-214, miR-142-3p, miR-24-3p, miR-141-3p, miR-194, miR-124-3p, miR-140-5p, miR-153-3p, miR-210-5p, miR-107-3p, miR-103-3p, miR-205-5p, miR-296-5p, miR-183-3p, and miR-698-5p were detected[86,87]. Most of these miRNAs were confirmed and their functions were verified using cell models. Detailed information on the downregulated miRNAs is presented in Tables 1 and 2.

Only two miRNAs (miR-141-3p and miR-192-5p) have been identified in humans. Li *et al*[88] collected serum samples from 27 Liver transplantation patients at different time points (pre-operatively, 4 h after reperfusion, and on postoperative days 1, 2, and 3) and measured the expression of miR-141-3p, ALT, and AST. They found that 4 h after perfusion, miR-141-3p was lower than pre-operation and then gradually increased over time, which manifested a negative correlation with ALT/AST levels[87]. Roy and his colleagues detected miR-192-5p expression in the liver tissues and sera of patients with acute liver injury. The results shown that miR-192-5p decreased in liver samples, but elevated in serum levels from patients with acute hepatic injury; further investigation revealed that miR-192-5p concentrations in serum were positively correlated with AST, ALT, and miR-122 Levels, which might represent a hepatocyte-specific serum biomarker[88].

Of note, several miRNAs have been indicated to participate in the hepatoprotective effect of several pharmacological agents during HIRI, including inhaled anesthetics and propofol. For instance, sevoflurane preconditioning ameliorates liver injury by the inhibitory effects of several miRNAs (*e.g.*, miR-133 and miR-205) on the Akt–GSK–cyclin D1 pathway [89]. Others have reported that sevoflurane preconditioning promotes the expression of miR-96 and inhibits FOXO4, thus alleviating HIRI[90]. In contrast, sevoflurane postconditioning exhibited the same hepatoprotective effect by counteracting miR-142 downregulation induced by I/R[91]. Moreover, isoflurane upregulates miR-9-3p to protect rats from HIRI by inhibiting FNDC3VB[92]. In addition, propofol exhibited protective effects against HIRI in rats by increasing the expression of miR-133a-5p and decreasing that of MAPK6[93].

Collectively, HIRI alters the expression of miRNAs. In turn, differentially expressed miRNAs play vital roles in HIRI development. Currently, a lot of miRNAs have been identified and their specific modulatory roles have been verified. However, it is important to note that human trails are lacking. Future research should focus on clinical transformation, which remains a significant challenge.

LNCRNAS

lncRNAs are a subset of noncoding RNAs with over 200 nucleotides (200 nt) and are localized to both the nucleus and cytoplasm[8,119]. Typically, lncRNAs are transcribed by Pol II, and have 5′-end 7-methyl guanosine (m^7G) caps and 3′-end polyadenylated [poly(A)] tails. lncRNAs were considered transcription junks without protein-coding capacity until their modulatory effects on gene expression were established. lncRNAs modulate chromatin structure and function, transcription, post-transcription, and sponge miRNAs by interacting with DNA, RNA and proteins, and in turn participate in diverse cellular processes such as cell differentiation, cell apoptosis, stem cell pluripotency, and stress response[12,120,121]. Recent studies show that lncRNAs can affect various diseases (*e.g.*, nervous disorders, immune systems, and cancers)[122,123]. The role of lncRNAs in the pathophysiology of I/R has been explored in multiple oxygen-dependent organs, such as the heart, brain, and kidney[124-129]. In terms of HIRI, it is still at an early stage. Here, we summarize the lncRNAs that have been reported, and detailed information is shown in Table 3.

In 2013, Chen *et al*[130] first revealed that in mouse livers after I/R treatment, 71 upregulated lncRNAs (fold change ≥ 1.5, and P value < 0.5) and 27 downregulated lncRNAs (fold change ≤ 0.7, and P value < 0.5) were identified. Four up-regulated lncRNAs (AK139328, AK087277, AK054386 and AK028007) and six down-regulated lncRNAs (AK143693, NR-028310, NR-015462, NR-036616, ENSMUST00000151138 and AK143294) were validated using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Further investigation suggested that silencing of AK139328 could ameliorate HIRI by activating the Akt/ NF-κB signaling pathway[128]. The same research team detected the lncRNA profile in mouse plasma after HIRI and found that under the same criteria, 64 up-regulated lncRNAs and 244 down-regulated lncRNAs were detected. The authors then conducted a comparative analysis of dysregulated lncRNA profiles between plasma and liver and revealed that all dysregulated lncRNAs in plasma remained either unchanged or absent in mice livers after HIRI, as did dysregulated lncRNAs in the livers, which strongly indicated that the source of these dysregulated lncRNAs may not be restricted to liver cells during HIRI[129]. Another study suggested that blood cells secrete large amounts of lncRNAs during heart failure[130,131].

With the development of novel technologies, an increasing number of differentially expressed lncRNAs have been identified, and their roles have been explored in HIRI models. Current studies indicate that lncRNAs participate in various biological processes involved in HIRI development. A few studies revealed that some lncRNAs, including TUG1 [132], NEAT1[133], MALAT1[134] and Hnf4αos[135], could modulate the processes of apoptosis and inflammatory response. Other lncRNAs, such as MEG3[136], Gm4419[137], CCAT1[138] and AK054386[139] were verified to regulate apoptosis, whereas AK139328 was only found to regulate the inflammatory response[130]. In addition to regulating these common biological processes, several lncRNAs participate in uncommon processes. HOTAIR expression in the liver was upregulated in a mouse model of HIRI, and further investigation indicated that HOTAIR regulates hepatocyte autophagy by targeting miR-20b-5p/ATG7[140]. Similarly, HIRI downregulates the expression of KCNQ1OT1 in mice livers, which promotes proliferation and inhibits pyroptosis by serving as a competing endogenous RNA to modulate the miR-142a-3p/HMGB1 axis[5]. Moreover, AK054386 upregulation may lead to sustained ERS and increased cell apoptosis and death in mice HIRI models[139]. Detailed information is shown in Table 4.

Li *et al*[141] successfully constructed HIRI-related lncRNA-miRNA-mRNA networks such as LOC1201029870-miRNA-331-3p/miRNA-128-5p-CDH3/UPK3B and LOC120094223-miRNA-92b-5p-KRT7, which may play an important role in HIRI. However, these specific modulatory mechanisms stay uncovered, and further investigation is needed.

In summary, these studies support the use of lncRNAs as highly attractive targets for diagnosing and treating HIRI. An increasing number of lncRNAs are known to be involved in HIRI. Overexpression and knockdown of lncRNAs attenuated or aggravated the extent of HIRI *in vivo* and *in vitro*, respectively, indicating the significance of lncRNAs in HIRI. A comprehensive understanding of lncRNAs in HIRI not only provides a new dimension to the molecular mechanisms, but also paves the way for future treatments. Indeed, future studies need more functional experiments *in vivo* and *in vitro* to reveal the specific roles of lncRNAs and to further explore its secretory and transport mechanisms in HIRI.

CIRCULAR RNAS

Circular RNA is a special subclass of ncRNAs characterized by a covalent bond joining the 3′ and 5′ ends generated by the back-splicing of exons[142-144]. Once produced, most circRNAs are exported from the nucleus to the cytoplasm[145]. Compared to their cognate linear RNAs, circRNAs are more stable and are not easily degraded by RNase L, RNase P, or RNase MRP. In addition to regulating transcription, splicing, and chromatin interactions, circRNAs act as decoys for miRNAs and proteins, interact with proteins, and function as templates for translation, and as sources of pseudogene generation[13,144]. circRNAs are involved in various biological processes, including immunity, neuronal function, cell proliferation, and transformation[146-149]. Existing evidence indicates the potential of circRNAs in treating diverse diseases.

Advances in RNA sequencing technologies have allowed the detection and exploration of many circRNAs in various pathological conditions. Currently, circRNAs have been implicated in the process of I/R injury, particularly myocardial I/R injury. For instance, circ_SMG6 deteriorates myocardial I/R injury by activating the miR-138-5p/EGR1/TLR4/TRIF signaling, whereas circ_CNEACR and circ_ACR alleviate myocardial I/R injury by suppressing autophagy[150-152]. Moreover, circ-FoxO3 attenuates blood-brain barrier damage by inhibiting mTORC1 activity during cerebral I/R[153]. circ-AKT3 aggravates renal I/R injury by regulating the miR-144-5p /Wnt/β-catenin pathway[154]. However, only few studies have investigated the role of circRNAs in HIRI.

Table 3 Long non-coding RNAs and their function in hepatic ischemia-reperfusion injury

lncRNAs	Change	Targets	Effect on HIRI	Models	Ref.
TUG1	Up	Brg1, miR-194/SIRT1	Alleviate	Mice, AML12 cells, WRL-68 cells	[133,144]
SNHG1	Up	miR-186-5p/YY1	Alleviate	AML12 cells	[145]
NEAT1	Up	-	Aggravate	HL7702 cells	[134]
Gm4419	Up	miR-455/SOX6 axis	Aggravate	Rats and BRL-3A	[138]
AK139328	Up	Akt signaling pathway and NF-κB	Aggravate	Mice and primary mouse hepatocyte	[130]
AK054386	Up	miR-199	Aggravate	Mice and BNL-CL2 cells	[140]
HOTAIR	Up	miR-20b-5p/ATG7	Aggravate	Mice and primary mouse hepatocyte	[141]
MALAT1	Up	HMGB1-TLR4	Aggravate	HL7702 cells	[135]
Hnf4αos	Up	Hnf4α/miR-23a	Aggravate	Human, mice, primary mouse hepatocyte and L02 cells	[136]
KCNQ1OT1	Up	miR-142a-3p/HMGB1	Aggravate	Mice and primary mouse hepatocyte	[15]
CCAT1	Down	caspase-3, cyclin D1	Aggravate	HL7702 cells	[139]
MEG3	Down	miR-34a/Nrf2	Aggravate	Mice and HL7702 cells	[137]

lncRNAs: Long non-coding RNAs; HIRI: Hepatic ischemia-reperfusion injury.

Table 4 Long non-coding RNAs and the involved mechanisms in hepatic ischemia-reperfusion injury

Mechanisms	lncRNAs	Ref.
Apoptosis	MEG3, TUG1, NEAT1, Gm4419, CCAT1, AK054386, MALAT1, Hnf4αos	[133-140]
Inflammatory responses	TUG1, NEAT1, AK139328, MALAT1, Hnf4αos	[130,133-136]
Oxidative stress	TUG1	[133,144]
Autophagy	HOTAIR	[141]
Pyroptosis	KCNQ1OT1	[142]
Endoplasmic reticulum stress	AK054386	[140]

lncRNAs: Long non-coding RNAs.

To date, only two studies have reported the potential role of circRNAs in HIRI. Zhang *et al*[87] conducted a circRNA microarray in mice for the first time in 2019 and found that, compared to the sham group, 706 circRNAs were differentially expressed in the I/R group, including 213 upregulated and 493 downregulated circRNAs. Compared to the ischemic postconditioning (IPO) group, 641 up-regulated and 252 down-regulated circRNAs were identified in the I/R group (fold change ≥ 2.0 and P value < 0.05). Among these, circRNA_005186 was upregulated in the I/R group, whereas IPO treatment downregulated its expression. A subsequent *in vitro* experiment showed that circRNA_005186 functioned as a miRNA sponge for miR-124-3p, thereby enhancing Epha2 expression. In other words, the circRNA_005186-miR-124-3p-Epha2 pathway might be a possible protective mechanism of IPO against HIRI[87]. The other five circRNAs (circRNA_011137, circRNA_013703, circRNA_29140, circRNA_36837, and circRNA_43819) were validated by RT-qPCR, and may be the focus of future research. In addition to IPO, IPC can attenuate HIRI. Tian *et al*[155] reported the circRNA profiles of mice with ischemic livers, with and without IPC. The data revealed that there were 77 circRNAs and 686 mRNAs in the IRI group, 50 circRNAs and 95 mRNAs in the IPC group (fold change ≥ 1.5, and P value < 0.05), respectively, when compared with those in the sham group. Next, they compared the circRNA alterations in the three groups and selected circRNA_017753 for further study. The prediction of the circRNA–miRNA–mRNA pathway implied a potential role of the circRNA_017753–miR-218-5p/miR-7002-3p/miR-7008-3p–Jade1 pathway in the mechanisms of IPC protection in HIRI. However, further investigations are required[155].

Although circRNA research in the field of HIRI is still in its infancy, the present data indicate great prospects for research. With the increasing attention of the scientific community, a thorough understanding of circRNA mechanisms will provide new insights and therapeutic targets for treating HIRI.

ADDITIONAL NCRNAS

Other ncRNAs such as PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs) and tRNA-derived small RNAs (tsRNAs), have attracted widespread attention in recent years. For instance, some studies have revealed that piRNAs were abnormally expressed and might play a regulatory role in liver cancer, non-alcoholic fatty liver disease and liver injury[156-158]. Moreover, snoRNAs and tsRNAs are involved in several liver diseases as biomarkers and therapeutic targets[159-162]. However, only few studies have investigated their roles in HIRI. As our knowledge of these ncRNAs expands, their potential role in HIRI will be confirmed.

CONCLUSION

Although ncRNAs have not been fully identified in the development of HIRI, current data indicate that ncRNAs are important regulators of various biological processes involved in the pathology of HIRI, and can serve as biomarkers for the diagnosis and assessment of therapeutic targets for treating HIRI. However, we noticed that most of the data were collected from animal studies, and the majority of ncRNAs described in this review were isolated from total liver tissue. So, establishing large clinical trials with diverse sample sources is necessary. Meanwhile, exploring the role of lncRNAs and circRNAs in HIRI is still in the start-up phase, and more attention needs to be paid in the future. In summary, our expanding knowledge of the capabilities of ncRNAs in HIRI will pave the way for novel diagnostic indicators and therapeutic inventions for HIRI.

FOOTNOTES

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REVIEW

Prevention and management of hepatitis B virus reactivation in patients with hematological malignancies in the targeted therapy era

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Abstract

Hepatitis due to hepatitis B virus (HBV) reactivation can be serious and potentially fatal, but is preventable. HBV reactivation is most commonly reported in patients receiving chemotherapy, especially rituximab-containing therapy for hematological malignancies and those receiving stem cell transplantation. Patients with inactive and even resolved HBV infection still have persistence of HBV genomes in the liver. The expression of these silent genomes is controlled by the immune system. Suppression or ablation of immune cells, most importantly B cells, may lead to reactivation of seemingly resolved HBV infection. Thus, all patients with hematological malignancies receiving anticancer therapy should be screened for active or resolved HBV infection by blood tests for hepatitis B surface antigen (HBsAg) and antibody to hepatitis B core antigen. Patients found to be positive for HBsAg should be given prophylactic antiviral therapy. For patients with resolved HBV infection, there are two approaches. The first is pre-emptive therapy guided by serial HBV DNA monitoring, and treatment with antiviral therapy as soon as HBV DNA becomes detectable. The second approach is prophy-lactic antiviral therapy, particularly for patients receiving high-risk therapy, especially anti-CD20 monoclonal antibody or hematopoietic stem cell transplantation. Entecavir and tenofovir are the preferred antiviral choices. Many new effective therapies for hematological malignancies have been introduced in the past decade, for example, chimeric antigen receptor (CAR)-T cell therapy, novel monoclonal antibodies, bispecific antibody drug conjugates, and small molecule inhibitors, which may be associated with HBV reactivation. Although there is limited evidence to guide the optimal preventive measures, we recommend antivi-ral prophylaxis in HBsAg-positive patients receiving novel treatments, including Bruton's tyrosine kinase inhibitors, B-cell lymphoma 2 inhibitors, and CAR-T cell

therapy. Further studies are needed to determine the risk of HBV reactivation with these agents and the best prophylactic strategy.

Key Words: Hepatitis B; Hematologic neoplasms; Chimeric antigen receptor-T cell therapy; Monoclonal antibodies; Bruton's tyrosine kinase inhibitors; Antiviral agents

Core Tip: Patients with chronic or past resolved hepatitis B virus (HBV) infection are at risk of reactivation of the virus when they receive chemotherapy or immunosuppressive therapy. Therefore, before treatment, patients should be screened for HBV markers, specifically hepatitis B surface antigen (HBsAg) and antibody to hepatitis B core antigen. Prophylactic antiviral therapy is important for HBsAg-positive patients, and is a reasonable option for patients with resolved HBV infection who are scheduled to receive high-risk therapy such as anti-CD20 monoclonal antibodies, anti-CD79 monoclonal antibodies, bispecific antibodies, chimeric antigen receptor-T cell therapy, or hematopoietic stem cell transplantation. For other patients with resolved HBV infection, pre-emptive antiviral therapy guided by serial monitoring of HBV DNA is a reasonable option.

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INTRODUCTION

Patients with chronic or resolved hepatitis B virus (HBV) infection are at risk of viral reactivation during chemotherapy or immunosuppressive therapy, commonly in patients receiving anti-cancer therapy for hematological malignancies or hematopoietic stem cell transplantation (HSCT). The earliest reports of HBV reactivation were in patients with lymphoma [1], and the highest risk of HBV reactivation is in patients receiving potent anti-CD20 monoclonal antibodies such as rituximab or obinutuzumab, which result in profound B-cell depletion.

There have been major advances in the development of new targeted therapy in the treatment of hematological malignancies in the past two decades. Bruton's tyrosine kinase (BTK) inhibitors are increasingly used in chronic lymphocytic leukemia (CLL) and lymphoma. Because these agents block B-cell antigen receptor signaling and thus reduce malignant B-cell proliferation, BTK inhibitors may potentially reactivate HBV. Other examples include bispecific antibodies in the treatment of non-Hodgkin lymphoma (NHL), CD79b-targeted antibody-drug conjugate, *i.e.*, polatuzumab vedotin, for diffuse large B cell lymphoma (DLBCL), and anti-CD38 monoclonal antibodies used in multiple myeloma (MM) patients.

Chimeric antigen receptor (CAR)-T cell therapy is a promising intervention which can be applied to lymphoid malignancies and plasma cell diseases including acute lymphoblastic leukemia (ALL), NHL, and MM. There is prolonged B-cell aplasia after CAR-T cell therapy which may potentially cause fatal HBV reactivation[2]. Hence, an understanding of the risk of HBV reactivation during treatment with novel therapies is important to prevent a fatal outcome.

This article will review the current published data on the clinical course and risk factors for HBV reactivation when using these novel therapies in patients with hematological malignancies. The recommended choice and duration of antiviral prophylaxis together with monitoring after stopping antiviral prophylaxis will also be discussed.

DEFINITIONS OF HBV REACTIVATION AND CLINICAL MANIFESTATIONS

Antibody to hepatitis B core (anti-HBc) is a good marker of current and past HBV infection as it persists even after hepatitis B surface antigen (HBsAg) is no longer detectable, while anti-HBs can be present due to successful hepatitis B vaccination or previous infection.

HBV reactivation is defined as exacerbation of chronic hepatitis B (CHB) or reactivation of past resolved hepatitis B infection. In general, reactivation is characterized by an increase from baseline in the HBV DNA level in patients with CHB, but it can also be defined as reverse HBsAg seroconversion, or the appearance of HBV DNA in serum when there is absence of HBsAg. The definition of HBV reactivation varies among different international guidelines and the information is summarized in Supplementary Table 1[3-7]. Hepatitis flare is defined as a 3-fold or more rise in alanine aminotransferase (ALT) level compared with baseline and ALT level more than 100 U/L[7].

When a patient has been infected with HBV, the virus enters the hepatocytes where the viral genome is released and transported into the nucleus. Once inside the nucleus, the viral genome is then converted into plasmid-like covalently closed circular DNA (cccDNA), which can persist in the hepatocytes in a latent and stable state[8].

HBV reactivation may occur at any time during or after chemotherapy. There are five stages in the course of HBV reactivation[9]. The first stage includes an asymptomatic elevation in markers of viral replication, with detectable HBV DNA levels in patients who are HBsAg-positive or -negative, or the reappearance of HBsAg in previously HBsAg-negative patients. In the second phase, serum HBV DNA levels continue to raise, and serum ALT and aspartate aminotransferase (AST) concentrations start increasing within a few weeks or days. This stage is also regarded as hepatic flare or HBV reactivation-related hepatitis. Most patients remain asymptomatic but a small number may experience constitutional symptoms, jaundice, and right upper-quadrant pain. Patients may then enter a spontaneous or on-treatment improvement, *i.e.*, the third stage, in which the ALT and AST levels improve spontaneously or after administration of antiviral therapy. A small proportion of patients experience the fourth stage if the hepatic injury cannot be resolved, characterized by a decrease in hepatic synthetic function, deranged clotting profile, and a rise of serum bilirubin levels. Fortunately, the majority of patients will go into the fifth stage with resolution of HBV reactivation after cessation of immunosuppressive therapy and the initiation of antiviral therapy. However, some may remain in stage 4, warranting liver transplantation in some severe cases.

In patients receiving immunosuppressive therapy, the loss of immune control may result in viral replication inside the hepatocytes without any increase in ALT levels. Nevertheless, upon immune reconstitution, sometimes during immunosuppressant tapering or withdrawal, the immune system will target the hepatocytes to clear the virus, resulting in liver damage[10].

RISK FACTORS FOR HEPATITIS B REACTIVATION

Host factors

Male sex and older age were identified to be risk factors for HBV reactivation[11-13]. A study in 626 HBsAg-positive patients who were undergoing chemotherapy for a variety of malignancies showed that there was almost a 3-fold increase of the incidence of HBV reactivation in men but the exact mechanism was not clear[13]. Chen *et al*[14] analyzed the risk of HBV reactivation among 1962 patients with hematological malignancy in Taiwan. The presence of hepatocellular carcinoma (HCC) and absence of antiviral prophylaxis were independent risk factors for HBV reactivation in HBV carriers. Among patients who were HBsAg negative at diagnosis, liver cirrhosis, diabetes mellitus, allogeneic stem cell transplantation, and low anti-HBs titers were independent risk factors for HBV reactivation[14]. Lymphoma is also associated with a higher risk of HBV reactivation[15]. Both the underlying disease and the anti-cancer therapy may contribute to HBV reactivation, indicating that the immunocompromised state is an important risk factor.

Virologic factors

The identified virologic risk factors for HBV reactivation include the presence of intrahepatic cccDNA and detectable HBV DNA levels[16-18]. Signs of increased viral replication, such as HBsAg or hepatitis B e antigen (HBeAg) positivity and detectable baseline HBV DNA, before treatment, are predictive of the patient meeting the criteria for HBV reactivation during treatment with cytotoxic chemotherapy or autologous stem cell transplantation[19-22].

HBV genotype is also related to treatment response and disease severity and progression[7,9]. For example, it was found that HBV genotype B is associated with HBeAg seroconversion at an earlier age, less active hepatic necroinflammation, more prolonged remission after HBeAg seroconversion, a slower rate of cirrhotic progression, and a reduced rate of HCC development compared with genotype C[7].

Salpini *et al*[23] identified mutations in HBsAg as being risk factors for reactivation. Using population-based and ultradeep sequencing, they analyzed the genetic diversity of HBsAg in 29 patients and found that 75.9% of HBV-reactivated patients carried mutations localized in immune-active HBsAg regions compared with only 3.1% of control patients ($P < 0.001$)[23]. The majority of these mutations resided in the B-cell epitopes of the HBs antigenic loop. Some of the mutations are known to hamper HBsAg recognition by humoral response, which may explain the frequent reactivation of HBV in patients receiving immunosuppressive therapy targeting B cells.

Types of anticancer therapies

Chemotherapy: Anthracycline chemotherapy (*e.g.*, doxorubicin, daunorubicin, and idarubicin) is a common form of treatment for hematological cancers such as lymphoma and acute myeloid leukemia (AML). The risk of HBV reactivation is significant in patients receiving doxorubicin as part of the chemotherapeutic regimen[6].

Chen *et al*[24] found that there was an increase in p21 expression during treatment with doxorubicin. The increase in p21 expression promotes the expression of CCAAT/enhancer-binding protein α (C/EBPα), which helps to activate HBV replication by enhancing the binding of C/EBPα to the HBV promoter. Kostyusheva *et al*[25] studied the effects of DNA-damaging compounds such as doxorubicin and hydrogen peroxide on the replication or reactivation of HBV and found that both doxorubicin and hydrogen peroxide dose-dependently activated HBV replication[25]. If doxorubicin is planned, anti-HBV prophylaxis is recommended for patients who are receiving doxorubicin if they have either CHB or a past resolved HBV infection.

Steroids: Steroids are commonly combined with chemotherapy or immunomodulatory drugs in the treatment of many hematological malignancies such as lymphoid malignancies and MM. Steroids can increase the HBV replication through two mechanisms. First, they can prevent T and B cell proliferation by suppressing cell-mediated immunity through the inhibition of interleukins[26]. Second, they exert a direct suppressive effect on T cell-mediated immunity through the stimulation of the glucocorticoid-responsive element present in the HBV genome[27].

Cheng *et al*[28] randomized 50 lymphoma patients who were HBsAg-positive and receiving the same chemotherapeutic regimen with or without the addition of corticosteroids, and compared the rate of HBV reactivation. The cumulative incidence of HBV reactivation was significantly higher in the corticosteroid group at 9 mo (73% *vs* 38%, respectively, $P = 0.03$). In a separate prospective cohort study with 6 years of follow-up, HBV reactivation occurred at 4 to 32 mo (median 10 mo) after the administration of steroids[29]. Most patients had malignancies or rheumatologic diseases.

The risk of HBV reactivation is further increased in patients who receive high-dose steroids (> 20 mg/d of prednisolone) and/or a long duration of therapy (> 4 wk)[6]. In a recent prospective study of 1303 patients with rheumatic diseases and past resolved HBV infection, it was found that patients taking steroids at a time-weighted average dose of higher than 20 mg/d prednisone-equivalents are at high risk for HBV reactivation or even hepatitis flare[30]. Prophylactic anti-HBV therapy should be considered for these high-risk patients.

Tyrosine kinase inhibitors: Currently, treatment with tyrosine kinase inhibitors (TKIs), *e.g.*, imatinib, nilotinib, dasatinib, and ponatinib, is a standard therapy for chronic myeloid leukemia (CML). The exact mechanism for HBV reactivation with TKIs is not known but it may be related to immune restoration. There are some published data on the risk of HBV reactivation in patients receiving TKIs. One hundred and forty-two adult Taiwanese CML patients were enrolled in a study to assess the rate of HBV reactivation during TKI therapy, including imatinib ($n = 43$, 30.3%), dasatinib ($n = 48$, 33.8%), nilotinib ($n = 37$, 26.1%), ponatinib ($n = 1$, 0.7%), and two or more TKIs ($n = 13$, 9.2%)[31]. Nineteen patients were HBV carriers and the rate of HBV reactivation was 26.3%; HBV reactivation was detected between 3 and 51 mo after the use of TKIs. Three patients experienced HBV-related hepatitis with an increase in ALT of more than 100 U/L[29]. One of the patients with HBV reactivation had received antiviral prophylaxis with entecavir; he was then given tenofovir after HBV reactivation.

A Korean study involved 69 patients with CHB being assessed for HBV reactivation[30]. Forty-six patients did not receive antiviral prophylaxis and the rate of HBV reactivation was 26% in this group of patients[32]. HBV reactivation was detected in seven patients who received imatinib, two patients receiving dasatinib, one nilotinib recipient, and one patient treated with radotinib therapy.

We would recommend prophylactic antiviral therapy to HBV carriers, and monitor HBV DNA and liver enzymes every 1 to 3 mo in patients with past resolved HBV infection, during TKI treatment. If the HBV DNA level rises, pre-emptive treatment with antiviral agents should be given. TKIs may also be used in combination with chemotherapy in the treatment of Philadelphia-positive ALL[33,34]. The combination with chemotherapy will likely lead to a deeper immunosuppressive effect, so prophylactic antiviral therapy is recommended in any patient with either CHB or past resolved HBV infection.

Anti-CD20 monoclonal antibodies: Treatment for a number of different hematological malignancies, including CLL and B-cell lymphoma, often includes B cell-depleting agents such as anti-CD20 monoclonal antibodies. Rituximab, obinutuzumab, and ofatumumab target the CD20 B-lymphocyte antigen and lead to marked depletion of the B cells involved in priming specific cytotoxic T cells[35]. Rituximab also worsens the impairment of antigen-presenting B cells which is seen in patients with CHB, resulting in inadequate induction of $CD4^+$ T cell activation and proliferation, and a T cell hyporesponsive state[36]. There is more than a 5-fold increase in the risk of HBV reactivation associated with the use of rituximab[37].

Both HBsAg-positive patients and those with resolved HBV infection are susceptible to HBV reactivation when they receive rituximab[38-41]. The incidence varies from 8.3% to 25% in patients with resolved HBV infection receiving rituximab-based chemotherapy[42-46]. Rituximab is a significant risk factor for HBV reactivation.

Obinutuzumab is a second-generation anti-CD20 monoclonal antibody. It has an engineered fragment crystallizable portion and a modified elbow hinge region[47]. Obinutuzumab has shown better efficacy than rituximab in several types of lymphoid diseases, by inducing direct cell death and enhancing antibody-dependent cellular cytotoxicity[48,49]. It can potentially cause more profound suppression of CD20 than rituximab[48,49]. It is used in patients with CLL and follicular lymphoma with promising results[48,49].

Kusumoto *et al*[50] performed a prospective study in 326 B-cell lymphoma patients with past resolved HBV infection who received obinutuzumab- ($n = 155$) or rituximab-containing immunochemotherapy ($n = 171$) in the phase 3 GALLIUM [48] and GOYA[51] studies. Of the 326 patients with resolved HBV infection, 119 (36.5%) received nucleos(t)ide analog treatment (NAT). Among these 119 patients, 94 received prophylactic NAT and 25 received pre-emptive NAT. The rate of HBV reactivation was 10.8% without antiviral prophylaxis, whereas only two of the 94 patients who received prophylactic NAT (2.1%) had HBV reactivation[48]. It was shown that the baseline detectable HBV DNA was strongly associated with an increased risk of reactivation while prophylactic NAT significantly decreased the risk on multivariate Cox analysis[50].

The reactivation rate in patients receiving obinutuzumab- and rituximab-based chemotherapy was 13.2% and 6.1%, respectively[50]. Although no significant difference in the risk of HBV reactivation between these two different immunochemotherapy regimens was demonstrated in the multivariate analysis, it might be due to confounding factors including imbalance of baseline risk factors. Anti-HBV prophylaxis is recommended in patients with either CHB or past resolved infection receiving anti-CD20 monoclonal antibody.

Monoclonal antibodies other than anti-CD20

Polatuzumab vedotin: Polatuzumab vedotin is an antibody-drug conjugate targeting CD79b, which is universally expressed on the surface of malignant B cells. CD79b is a signaling component of the B-cell receptor which is located on the surface of normal B cells as well as most of the mature B-cell tumors and 95% of DLBCL[52]. Polatuzumab vedotin was found to be useful in combination with bendamustine and rituximab (pola-BR) for patients with relapsed or refrac-

tory DLBCL[53]. It can also be used in a modified regimen of polatuzumab vedotin, rituximab-cyclophosphamide, doxorubicin, and prednisolone (pola-R-CHP) with success in the frontline treatment of DLBCL[54]. The risk of disease progression, relapse, and death was all reduced among those who received pola-R-CHP than among those who received standard rituximab-cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) therapy in previously untreated DLBCL patients with an intermediate or high risk.

To date, there are no published data or reported cases of HBV reactivation in patients receiving polatuzumab vedotin. In view of the profound B-cell suppression that occurs when these regimens are used to treat lymphoma, we would recommend antiviral prophylaxis for patients receiving polatuzumab vedotin if they have either CHB or past resolved HBV infection.

Inotuzumab ozogamicin: Inotuzumab ozogamicin is an antibody conjugate in which a humanized monoclonal antibody against CD22 is conjugated to the cytotoxic antibiotic calicheamicin[55]. After binding to CD22 on the leukemic cell surface, the CD22-conjugate complex is rapidly internalized, releasing the calicheamicin. Once released, the cytotoxic portion of the conjugate binds to the minor groove of DNA in these leukemic cells, and induces double-strand cleavage and subsequent apoptosis.

More than 90% of patients with B-cell ALL express CD22 and this cell-surface glycoprotein is not shed into the extracellular matrix, making it a logical target for B-cell cancer therapy[56]. It has been used with success in the treatment of ALL and it can deplete B cells. There have been no reports to date of HBV reactivation in patients with CHB or past resolved infection receiving inotuzumab ozogamicin. However, in view of the profound B-cell depletion, we would recommend antiviral prophylaxis during inotuzumab ozogamicin in patients with either CHB or past resolved infection.

Blinatumomab: Blinatumomab is a bispecific T-cell engager, with two binding sites: One for CD3-positive cytotoxic T cells and the other for CD19-positive B cells. By drawing the two types of immune cells together, blinatumomab facilitates the recognition and destruction of CD19-positive ALL blasts by the patient's own endogenous T cells[57]. It has been used with success in the treatment of relapsed/refractory ALL or as consolidation therapy[58,59].

We did not identify any published cases of HBV reactivation in patients with CHB or past resolved infection receiving blinatumomab. HBV prophylaxis is recommended for both chronic and resolved HBV infection in patients receiving blinatumomab, in view of profound B-cell depletion seen with this agent.

Daratumumab and isatuximab: Daratumumab is a human immunoglobulin G1 monoclonal antibody which targets CD38-expressing cells. Several of the combination regimens containing daratumumab have shown promising results in the treatment of newly diagnosed and refractory/relapsed MM[60-63]. It also has an emerging role in the treatment of amy-loid light-chain amyloidosis[64,65].

The principal mechanism of daratumumab in MM is to induce death of CD38-expressing myeloma cells *via* antibody-dependent and complement-dependent cytotoxicity, as well as *via* antibody-dependent cellular phagocytosis[66]. Daratumumab also targets normal plasma cells expressing CD38, leading to a reduction in the humoral immunity against reactivation of HBV[67].

There was a report of HBV reactivation occurring on day 15 of the third course of a daratumumab-containing regimen in a MM patient with resolved HBV infection[68]. Lee *et al*[69] also conducted a retrospective study of 93 patients with resolved HBV infection who had been treated with daratumumab and found that the risk of HBV reactivation was 6.5% at a median follow-up period of 8.7 mo. One patient later died of hepatic failure despite treatment with tenofovir. These reports highlight the risk of reactivation of resolved HBV infection after daratumumab treatment in MM patients.

Isatuximab, another anti-CD38 monoclonal antibody, has demonstrated benefits in the treatment of patients with relapsed/refractory and high-risk MM[70-73]. We would recommend anti-HBV prophylaxis in both HBV carriers or those with resolved HBV infection during treatment with either daratumumab or isatuximab in view of the similar mecha-nisms of action of both drugs.

Novel therapies for hematological malignancies

CAR-T cell therapy: CAR-T cell therapy is a promising immunotherapy with curative intent for several types of hematological malignancies including NHL[74-77], ALL[78], and MM[79,80]. CAR-T cell therapy involves removal of the patient's own T cells, reprogramming these cells with a CAR construct, and then returning them to the patient's bloodstream, where these programmed T cells attack the cancer cells[81]. The activated CAR-T cells identify targets on cancer cells, specifically leading to the destruction of these cancer cells. Because it is easier to target an adequate tumor antigen in hematological malignancies (such as CD19 in lymphoid malignancies) than it is in solid cancers, CAR-T cell therapy has been first applied to hematological malignancies.

The issue of HBV reactivation in patients receiving CAR-T cell therapy remains unexplored, and the data on HBV reactivation in these patients are limited. CAR-T cells may predispose HBV immune patients to reactivation due to its cytotoxicity against B cells. The proper prevention strategy and duration of antiviral prophylaxis in patients receiving CAR-T cell therapy are still unclear and should be further investigated. There has been a fatal case of HBV reactivation after CAR-T cell therapy[2]. HBV reactivation can be a significant complication in CAR-T cell treatment and clinicians should be cautious about this complication particularly in areas where HBV is still prevalent.

Patients can also have late HBV reactivation occurring more than 1 year after CAR-T cell therapy. CAR-T cells can persist in the blood for a long time, resulting in prolonged B-cell aplasia and a persistent reduction in immunoglobulin production, thus contributing to late reactivation[82].

Table 1 summarizes the published data on the HBV reactivation in patients receiving CAR-T cell therapy[83-89]. The rate of HBV reactivation ranged from 0% to 20% for CHB patients[83-89]. We recommend that clinicians administer anti-HBV prophylaxis during the CAR-T cell therapy and for at least 1 year afterwards in patients who had CHB or past resol-

Table 1 Studies of hepatitis B virus reactivation in patients receiving chimeric antigen receptor-T cell therapy

Ref.	Indication for CAR-T	*N*	CHB, *n*	Past resolved HBV infection, *n*	Antiviral prophylaxis, % patients	Definition of HBV reactivation	Rate of HBV reactivation	HBV-related death
Prospective studies								
Liu *et al*[87], 2020	B-cell lymphoma	17	6	11	100% for CHB, and 45.5% for past infection (entecavir)	Elevation of HBV DNA levels to > 1000 IU/mL and/or HBsAg reverse seroconversion in HBsAg-negative patients	0	0
Yang *et al* [89], 2020	DLBCL	15	15	0	100% (lamivudine, entecavir, tenofovir, or adefovir dipivoxil)	Positive follow-up HBV-DNA test if the baseline HBV-DNA is undetectable/negative or > 10-fold increase from baseline	20%	0
Li *et al* [86], 2021	ALL, B-cell lymphoma	30	0	30	No prophylaxis	Elevation of HBV DNA ≥ 100 IU/mL for two consecutive measurements	6.6%	0
Wang *et al* [88], 2020	ALL, B-cell lymphoma, PCM	70	12	29	100% for CHB (entecavir, tenofovir disoproxil, or lamivudine). Nil for patients with past HBV infection	> 1 log increase in HBV DNA, HBV DNA-positive when previously negative, HBV DNA > 2000 IU/mL if no baseline level was available, or reverse sero-conversion from HBsAg-negative to positive	16.7% with chronic infection and 34.4 % with past infection	0
Retrospective studies								
Cao *et al*[83], 2020	ALL, NHL	89	19	37	100% for chronic infection, and 5.4% for past infection	100-fold increase in HBV DNA when compared with baseline or HBV DNA ≥ 10^3 IU/mL in a patient with a previously undetectable level or reverse seroconversion from HBsAg negative to HBsAg positive	5.3% for CHB	0
Han *et al*[85], 2020	Multiple myeloma	9	1	8	100% for CHB, 25% for past infection (lamivudine/entecavir)	HBsAg seroconversion or increase in HBV DNA levels by at least 10-fold or 1 × 10^9 copies/mL	12.5% for past infection	0
Cui *et al*[84], 2021	DLBCL, B-ALL	20	5	15	100% for CHB (entecavir or tenofovir), 13.3% for past HBV infection (entecavir)	For CHB: (1) ≥ 2 log increase in HBV DNA compared to the baseline level; (2) HBV DNA ≥ 3 log IU/mL in a patient with previously undetectable level; and (3) HBV DNA ≥ 4 log IU/mL if the baseline level is not available. For resolved HBV infection: HBV DNA is detectable; reverse HBsAg seroconversion	6.2% for past infection	0

AASLD: American Association for the Study of Liver Diseases; ALL: Acute lymphoblastic leukemia; CAR: Chimeric antigen receptor; CHB: Chronic hepatitis B; CLL: Chronic lymphocytic leukemia; DLBCL: Diffuse large B-cell lymphoma; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; NHL: Non-Hodgkin lymphoma; PCM: Plasma cell myeloma.

ved HBV infection[82].

Bispecific antibodies: The development of bispecific antibodies has been an important advance in the treatment of relapsed or refractory B-cell lymphomas[90-92], including DLBCL[93-95] and follicular lymphoma[96]. Bispecific antibodies target both T cells and CD19 or CD20 on malignant B cells and is a promising immunotherapy in the treatment of NHL. These dual binding sites draw malignant B cells close to endogenous T cells, thereby directly activating T-cell cytotoxicity. Examples of the bispecific antibodies include glofitamab, mosunetuzumab, epcoritamab, and odronextamab. Some of these bispecific antibodies have a fixed duration of therapy and some, such as epcoritamab, are administered until disease progression.

Since there are a lack of prospective or retrospective studies on the risk of HBV reactivation in patients receiving bispecific antibodies, the real incidence of HBV reactivation is unclear. However, bispecific antibodies will profoundly suppress B-cell activity. These drugs are highly potent and the effect on B-cell depletion is expected to be significant. Therefore, we recommend antiviral prophylaxis against HBV in patients with either CHB and past resolved HBV infection.

BTK inhibitors: BTK inhibitors such as ibrutinib, acalabrutinib, and zanubrutinib have shown success in the treatment of many lymphoid malignancies including CLL[97-102], mantle cell lymphoma[103,104], marginal zone B-cell lymphoma [105], and Waldenström's macroglobulinemia[106]. Ibrutinib blocks B-cell antigen receptor signaling, thus reducing malignant proliferation of B cells and inducing cell death[107].

The effect of ibrutinib or other BTK inhibitors on HBV reactivation has not been extensively studied, and there are no guidelines on the prophylaxis and management of HBV reactivation during treatment with ibrutinib. Table 2 summarizes the current data on the risk of HBV reactivation and their outcomes in patients receiving BTK inhibitors[108-111]. Most existing data are from retrospective studies. The rate of HBV reactivation ranged from 1.9% to 8.3% for past resolved infec -tion. BTK inhibitors will induce profound B-cell suppression. Hence, we recommend anti-HBV prophylactic treatment with nucleotide analogues for lymphoma patients with positive HBsAg or resolved HBV infection with detectable HBV DNA who are receiving BTK inhibitors. For those with resolved HBV infection and negative HBV DNA, we recommend monitoring HBV DNA levels and liver function every 1 to 3 mo, and giving pre-emptive antivirals when the HBV DNA level rises.

B cell lymphoma-2 inhibitors: The B cell lymphoma (BCL)-2 inhibitor venetoclax is commonly used in the treatment of CLL[112,113] and in combination with azacitidine or low-dose cytarabine in AML patients who are not fit for intensive chemotherapy[114,115]. Venetoclax is a potent inhibitor of the antiapoptotic BCL-2 protein. AML stem cells express BCL-2 and depend on BCL-2 for survival. Venetoclax has synergistic effects when used in combination with azacitidine.

There is a lack of large retrospective or prospective studies on the incidence of HBV reactivation in patients receiving venetoclax, so the risk is unclear. Because of its mechanism of action, venetoclax will profoundly suppress B-cell activity. Thus, the same prophylactic or pre-emptive antiviral management approach used for patients receiving BTK inhibitors should be applied in patients receiving venetoclax.

Proteasome inhibitors: The proteasome inhibitors have become the backbone treatment for MM. They include bortezomib, carfilzomib, and ixazomib. Bortezomib is the most commonly used proteasome inhibitor. Bortezomib can target cellular pathways essential for the proliferation of malignant plasma cells. However, it may also negatively impact the functions of healthy B cells and plasma cells, which are important in the immune control of HBV. It can also dysregulate cell-mediated immunity and may increase HBV reactivation by affecting the number and functions of $CD8^+$ T cells and $CD56^+$ natural killer cells[116].

Ataca Atilla *et al*[117] conducted a retrospective study in 178 MM patients who had received lenalidomide and/or bortezomib. They found that the rate of HBV reactivation was 3% after bortezomib and 8% after bortezomib and lenalidomide[117]. Lee *et al*[118] reported HBV reactivation in 5.2% of 230 MM patients with past resolved HBV infection after a median follow-up of 2.4 years. One hundred and thirty-three patients (58%) had received bortezomib-based therapy. In this study, the cumulative rate of HBV reactivation was 5% at 2 years and 8% at 5 years[118].

Mya *et al*[116] reported an HBV reactivation incidence of 5.5% in 273 relapsed or refractory MM patients who had received bortezomib and dexamethasone therapy. Li *et al*[119] conducted a retrospective study of HBV reactivation in patients receiving regimens containing bortezomib. Twenty-seven of the 139 patients were HBsAg positive and 22 of them were given antiviral prophylaxis with lamivudine or entecavir. HBV reactivation occurred in six HBsAg-positive and two HBsAg-negative/anti-HBc-positive cases from a total of 139 patients[119]. Antiviral prophylaxis is recommended for both CHB patients and those with past resolved HBV infection who are receiving proteasome inhibitors.

Immune checkpoint inhibitors: Immune checkpoint inhibitors (ICI) are effective in the treatment of solid tumors, and have also shown efficacy in the treatment of lymphoma[120-124]. ICIs can block the localization and traffic of activated lymphocytes, thus inhibiting the inflammatory response associated with immune-mediated diseases[125]. They may also reduce the local immune control of HBV replication in the liver, predisposing patients to HBV reactivation.

Table 3 summarizes the data on HBV reactivation in patients receiving ICIs[126-129]. The rate of HBV reactivation among HBsAg-positive cancer patients is 0.5% to 5.3% during ICI therapy. Prophylactic antiviral treatment is recommended for HBsAg-positive patients to prevent HBV reactivation. For those with resolved HBV infection and undetectable HBV DNA levels, we recommend monitoring liver function and HBV DNA levels every 1 to 3 mo, and administering pre-emptive treatment with antiviral agents if an increase in HBV DNA levels is detected.

PREVENTION OF HEPATITIS B REACTIVATION

Existing guidelines on the drug classes and the corresponding risk of HBV reactivation are summarized in Table 4, while Table 5 summarizes the international guidelines on the management of patients with HBV infection receiving chemotherapy. The recommendations for management of HBV-infected cancer patients receiving novel agents for hematological malignancies are shown in Table 6.

Screening for hepatitis B

In order to prevent HBV reactivation among patients with hematological malignancies, it is essential to identify those with HBV infection before starting chemotherapy or immunotherapy. This starts with screening for the presence of HBsAg and anti-HBc in blood. The commercial immunoassays usually capture HbsAg, having specificity for epitopes present on the antigenic α determinant. The enzyme-linked immunosorbent assay method used in HBsAg detection has a sensitivity and specificity of both about 80%, compared with more than 90% using the immunochromatographic test [130]. Complete loss of anti-HBc with chronic and high viremic HBV infection after allogeneic stem cell transplantation has been reported[131]. However, there might be some rare scenarios where HBsAg or anti-HBc is falsely negative. For example, mutations within the α determinant may affect the conformation of the surface epitope such that it is unrecognizable to the test, or mutations in other parts of the viral genome may affect HbsAg secretion or expression, resulting in diagnostic escape[132]. Moreover, there has been a case report describing complete loss of anti-HBc after allogeneic stem

Table 2 Studies of hepatitis B virus reactivation in patients receiving Bruton's tyrosine kinase inhibitors (all studies are retrospective)

Ref.	Disease type	Therapy	*N*	CHB, *n*	Past resolved HBV infection, *n*	Antiviral prophyl-axis, % patients	Definition of HBV reactivation	Rate of HBV reactivation, % patients	HBV-related death
Hammond *et al*[108], 2018	CLL, MCL, LPL	Ibrutinib	21	0	21	4.8%	HBV DNA > 100 IU/mL on 2 consecutive measurements ± reappearance of HBsAg	9.5%	0
Innocenti *et al*[109], 2019	CLL	Ibrutinib	34	0	12	42% for past infection (lamivudine)	Increase in serum ALT and HBV DNA in HBsAg-positive patients or elevation of HBV DNA ± HBsAg recurrence in anti-HBc-positive patients	8.3%	0
Innocenti *et al*[110], 2022	CLL	Ibrutinib	108	0	108	67.6% (lamivudine)	HBsAg seroconversion and/or an increase of serum HBV DNA by ≥ 1 log above the LLD of the assay	1.9%	0
Ni *et al*[111], 2022	DLBCL	Ibrutinib or zanu-brutinib	55	4	26	100% for CHB and 34.6% for past infection (entecavir)	> 1 log increase in HBV DNA, HBV DNA-positive when previously negative, HBV DNA > 2000 IU/mL if no baseline level was available, or reverse seroconversion from HBsAg-negative to -positive	7.69% for past infection	0

CHB: Chronic hepatitis B; CLL: Chronic lymphocytic leukaemia; DLBCL: Diffuse large B-cell lymphoma; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; LLD: Lower limit of detection; LPL: Lymphoplasmacytic lymphoma; MCL: Mantle cell lymphoma; ALT: Alanine aminotransferase; anti-HBc: Antibody to hepatitis B core.

cell transplantation in a patient with resolved HBV infection who previously had positive anti-HBs and anti-HBc prior to the stem cell transplant[131]. Isolated anti-HBs without anti-HBc may be present in pretreated patients without previous hepatitis B vaccination[133,134]. Thus, a more sensitive combined screening strategy is advisable, including serological testing for HBsAg, anti-HBc, and anti-HBs and a sensitive test for HBV DNA.

HBsAg-positive patients without hepatitis at baseline

A preventive strategy is more effective than a pre-emptive strategy in HBsAg-positive patients[135,136]. We recommend giving NAT for prophylaxis in all HBsAg-positive candidates prior to immunosuppressive therapy irrespective of their HBV DNA status because the risk of HBV reactivation is high in this group of patients. This approach is highly effective, with a number needed to treat to prevent one episode of HBV reactivation of three[135].

It is not always possible to prevent the development of hepatitis or hepatitis flares if antiviral therapy is started after the onset of HBV reactivation[42], since it will take some weeks or even months for the antiviral therapy to reduce viral loads, and the inflammation and necrosis of the liver will be ongoing during this period[137].

HBsAg-negative and anti-HBc-positive patients

The risk of HBV reactivation in this group varies considerably, depending on the level of viremia and the immunosuppressive regimens administered. In general, if HBV DNA is detectable, the patient would be given anti-HBV prophylaxis and treated similarly to HBsAg-positive patients. If HBV DNA is undetectable, then the risk of reactivation associated with the immunosuppressive regimen will be assessed. High-risk groups such as those receiving anti-CD20 monoclonal antibodies should receive antiviral prophylaxis with NAT. Pre-emptive treatment is recommended for moderate- and low-risk groups, with HBV DNA monitoring every 1-3 mo.

Huang *et al*[43] compared pre-emptive with prophylactic entecavir therapy during R-CHOP chemotherapy in patients with lymphoma and resolved hepatitis B. Prophylactic entecavir treatment significantly reduced the risk of HBV reactivation compared with pre-emptive antiviral therapy (17.9% *vs* 2.4%, $P = 0.027$)[43]. Therefore, the prophylactic strategy is a better option in patients receiving high-risk immunosuppressive regimens (Table 5).

HBsAg-negative/anti-HBc-negative/anti-HBs-negative patients

HBV vaccination can be considered in patients who are HBsAg-negative, anti-HBc-negative, and anti-HBs-negative[3]. Anti-HBs potentially provide a protective effect against HBV reactivation[45,138-141]. The results of a meta-analysis showed that, among patients not receiving antiviral prophylaxis, the reactivation risk was 14% in the 388 patients who had anti-HBc only *vs* 5.0% in 1284 patients with concomitant anti-HBs. The pooled odds ratio of HBV reactivation was 0.21 (95% confidence interval: 0.14-0.32) in those with anti-HBs compared with anti-HBc only[141].

Table 3 Studies of hepatitis B virus reactivation in patients receiving immune checkpoint inhibitors (all studies were retrospective)

Ref.	Disease type	Therapy	*N*	CHB, *n*	Past resolved HBV infection, *n*	Antiviral prophylaxis, % patients	Definition of HBV reactivation	Rate of HBV reactivation	HBV-related death
Zhang *et al*[129], 2019	Solid tumors, lymphoma (7%)	PD-1/PD-L1 inhibitors (pembrolizumab, nivolumab, toripalimab, camrelizumab, sintilimab, atezolizumab)	114	114	0	74.6% received prophylaxis (entecavir, tenofovir, lamivudine, telbivudine, adefovir)	AASLD 2018 guidelines	6 (5.3%)	0
Wong *et al*[127], 2021	Solid tumors	PD-1 inhibitors (nivolumab, pembrolizumab, spartal-izumab), PD-L1 inhibitors (atezolizumab, avelumab, durvalumab), CTLA-4 inhibitors (ipilimumab, tremelimumab)	990	397	225	100% for CHB, and 11.3% for past HBV infection (entecavir, TAF, TDF, lamivudine, telbivudine, ADV)	AASLD 2018 guidelines	2/397 (0.5%); none in the resolved HBV group	0
Yoo *et al* [128], 2022	Solid tumors, lymphoma (1.8%)	PD-1 inhibitors (nivolumab, pembrolizumab), PD-L1 inhibitors (atezolizumab, avelumab), CTLA-4 inhibitors (ipilimumab, tremelimumab)	3465	511	564	90.8% for CHB, 1.1% for HBsAg negative patients (entecavir, tenofovir, lamivudine, telbivudine, adefovir, clevudine)	AASLD 2018 guidelines	1% for chronic HBV infection, 0% for past HBV infection	0
Lasagna *et al* [126], 2023	Solid tumors	Pembrolizumab, nivolumab, atezolizumab	150	0	150	Nil	AASLD 2018 guidelines	0%	0

AASLD: American Association for the Study of Liver Diseases; ADV: Adefovir dipivoxil; CHB: Chronic hepatitis B; CTLA4: Cytotoxic T-lymphocyte-associated protein 4; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; PD-1: Programmed cell death protein 1; PD-L1: Programmed cell death ligand 1; TAF: Tenofovir alafenamide; TDF: Tenofovir disoproxil fumarate.

Table 4 Drug classes and corresponding risk of hepatitis B virus reactivation[6]

Drug class	Drug or dose	Risk of HBV reactivation	
		For HBsAg-positive patients	For HBsAg-negative/anti-HBc-positive patients
Anti-CD20 monoclonal antibodies	Rituximab, obinutuzumab, ofatumumab	High (30%-60%)	High (> 10%)
Anthracycline chemotherapy	Doxorubicin, daunorubicin, epirubicin	High (15%-30%)	High (> 10%)
Steroids	Moderate/high dose ≥ 4 wk	High (> 10%)	Moderate (1%-10%)
	Low dose ≥ 4 wk	Moderate (1%-10%)	Low (< 1%)
	Low dose ≤ 1 wk	Low (< 1%)	Low (< 1%)
Tyrosine kinase inhibitors	Imatinib, nilotinib, dasatinib	High to moderate	Low (< 1%)
Immune checkpoint inhibitors	Nivolumab, pembrolizumab	High (> 10%)	Uncertain
Proteasome inhibitor	Bortezomib	Moderate (1%-10%)	Moderate (1%-10%)

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; anti-HBc: Antibody to hepatitis B core.

HBsAg-negative/anti-HBc-negative/anti-HBs-positive patients

It is rare to have HBV reactivation in patients with isolated anti-HBs, but there have been occasional reports of HBV reactivation in patients with only anti-HBs seropositivity[133,134]. In one report, a patient with follicular lymphoma, who had not been vaccinated for hepatitis B, was positive for anti-HBs but negative for anti-HBc prior to starting chemotherapy. He subsequently developed high HBV DNA levels (1.8×10^8 copies/mL), and was found to have an HBV escape mutant, which was difficult to detect using the standard HBsAg assays[133]. HBV escape mutants harbor mutations in the essential antigenic area of HBsAg, and are capable of growing in the presence of anti-HBs. In these circumstances,

Table 5 International guidelines on prevention of hepatitis B in patients with a history of hepatitis B virus infection who are candidates for chemotherapy

Guideline	HBV screening	Screening tests	HBsAg-positive patients	HBsAg-negative, anti-HBc-positive patients	Choice of antiviral agent	Duration of antiviral therapy	Monitoring after prophylaxis	Ref.
American Gastroenterological Association 2015 guideline	High risk of HBV reactivation (> 10%) and moderate risk of HBV reactivation (1%-10%). Routine screening not recommended for low risk of HBV reactivation (< 1%)	HBsAg, anti-HBc, HBV DNA if serology positive	Prophylactic antiviral therapy	Antiviral prophylaxis over monitoring for patients if the chemotherapy is associated with high or moderate risk of HBV reactivation	Drug with high barrier to resistance is favored over LMV	6 mo after discontinuation of therapy and at least 12 mo for B-cell depleting agents	Not defined	[6]
European Association for the Study of the Liver 2017	All candidates for CT or IST	HBsAg, anti-HBc, and anti-HBs	Anti-HBV prophylaxis	Anti-HBV prophylaxis if they are at high risk of HBV reactivation. Pre-emptive therapy for moderate (10%) or low (1%) risk of HBV reactivation, and monitor HBsAg and/or HBV DNA every 1-3 mo during and after IST	ETV or TDF or TAF	At least 12 mo (18 mo for high-risk therapy) after the last course of therapy	LFT and HBV DNA every 3 to 6 mo during prophylaxis and for ≥ 12 mo after NA withdrawal	[3]
American Association for the Study of Liver Diseases 2018	All patients for CT and IST	HBsAg and anti-HBc	Anti-HBV prophylaxis	On-demand therapy except for patients receiving anti-CD20 antibody therapy or SCT (monitor ALT, HBV DNA, HBsAg every 1-3 mo)	ETV or TDF or TAF	At least 6 mo after discontinuation of IST. At least 12 mo for B cell-depleting agents	For up to 12 mo after cessation of anti-HBV therapy	[7]
American Society of Clinical Oncology 2020 update	All candidates for CT or IST	HBsAg, anti-HBc, and anti-HBs	Anti-HBV prophylaxis	High risk, *e.g.*, anti-CD20 antibody therapy or stem cell transplantation: Prophylaxis. Others: On-demend therapy (monitor HBsAg and HBV DNA every 3 mo)	ETV, TDF, TAF	At least 12 mo after cessation of IST	High risk: Monthly for the first 3 mo after NA withdrawal and then every 3 mo (duration not specified). Resolved HBV and not high risk: Not necessary	[4]
The Asian Pacific Association for the Study of the Liver 2021	All patients planned to receive IST	HBsAg, anti-HBs and anti-HBc, quanti-tative HBV DNA for HBsAg-positive patients	Anti-HBV prophylaxis in high and moderate-risk groups, and low-risk group with advanced liver fibrosis or cirrhosis. Pre-emptive treatment in low-risk group without advanced liver fibrosis or cirrhosis	Anti-HBV prophylaxis in high-risk group and moderate-risk group with advanced liver fibrosis or cirrhosis. Pre-emptive treatment in low-risk group without advanced liver fibrosis or cirrhosis	ETV, TDF or TAF	6 mo after the completion of IST for HBsAg-positive patients, without advanced liver fibrosis or cirrhosis and with low level of HBV DNA	HBV DNA every 3 mo	[5]

High risk of hepatitis B virus reactivation (> 10%), moderate risk (1%-10%), low risk (< 1%). ALT: Alanine aminotransferase; CT: Chemotherapy; ETV: Entecavir; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; IST: Immunosuppresive therapy; LFT: Liver function test; LMV: Lamivudine; NA: Nucleotide analog; SCT: Stem cell transplant; TDF: Tenofovir; TAF: Tenofovir alafenamide fumarate; anti-HBc: Antibody to hepatitis B core; TAF: Tenofovir alafenamide; TDF: Tenofovir disoproxil fumarate.

Table 6 Recommendations for management strategy of hepatitis B virus-infected cancer patients receiving novel agents for hematological malignancies

Therapy	Chronic HBV infection	Past resolved HBV infection
CAR-T (*e.g.*, axicabtagene ciloleucel, tisagenlecleucel, and lisocabtagene maraleucel)	Antiviral prophylaxis	Antiviral prophylaxis
Bispecific antibodies (*e.g.*, glofitamab, mosunetuzumab)	Antiviral prophylaxis	Antiviral prophylaxis
BTK inhibitors (*e.g.*, ibrutinib, acalabrutinib, zanubrutinib)	Antiviral prophylaxis	Antiviral prophylaxis or monitoring and pre-emptive therapy[1]
BCL-2 inhibitors (venetoclax)	Antiviral prophylaxis	Antiviral prophylaxis or monitoring and pre-emptive therapy[1]
Anti-CD19 monoclonal antibody (blinatumumab)	Antiviral prophylaxis	Antiviral prophylaxis
Anti-CD22 monoclonal antibody (inotuzumab)	Antiviral prophylaxis	Antiviral prophylaxis
Anti-CD79 monoclonal antibody (polatuzumab)	Antiviral prophylaxis	Antiviral prophylaxis
Anti-CD38 monoclonal antibody (daratumumab)	Antiviral prophylaxis	Antiviral prophylaxis

[1]Pre-emptive therapy is monitoring of serum hepatitis B virus DNA every 1-3 mo during and after immunosuppression, and starting antiviral therapy with entecavir or tenofovir in the case of detectable hepatitis B virus DNA levels.
HBV: Hepatitis B virus; CAR: Chimeric antigen receptor; BTK: Bruton tyrosine kinase; BCL: B-cell lymphoma.

anti-HBc may appear very late.

In a separate report, a patient with DLBCL (also without a record of hepatitis B vaccination) had a pre-chemotherapy HBV profile that was positive for anti-HBs (127 IU/mL) but negative for HBsAg and anti-HBc. She developed HBV reactivation after completing rituximab-based chemotherapy. Antiviral treatment with entecavir was started after HBV reactivation was detected. Despite that, she had clinical deterioration with development of hepatic encephalopathy and died of liver failure finally[134]. Figure 1 shows a suggested algorithm for HBV testing and management of patients with hematological malignancies receiving anticancer therapy.

CHOICE OF ANTIVIRAL THERAPY

For the treatment of chronic HBV infection, entecavir and tenofovir are the preferred antiviral agents because they have high genetic barriers to resistance compared with lamivudine. Huang *et al*[142] performed a prospective randomized study in 121 HBsAg-positive patients with untreated DLBCL. Sixty patients received lamivudine prophylaxis and 61 received entecavir prophylaxis. Various endpoints occurred at a significantly lower rate in the entecavir than the lamivudine group, including HBV reactivation (6.6% *vs* 30%, $P = 0.001$), HBV-related hepatitis (0% *vs* 13.3%, $P = 0.003$), and chemotherapy disruption (1.6% *vs* 18.3%, $P = 0.002$)[142].

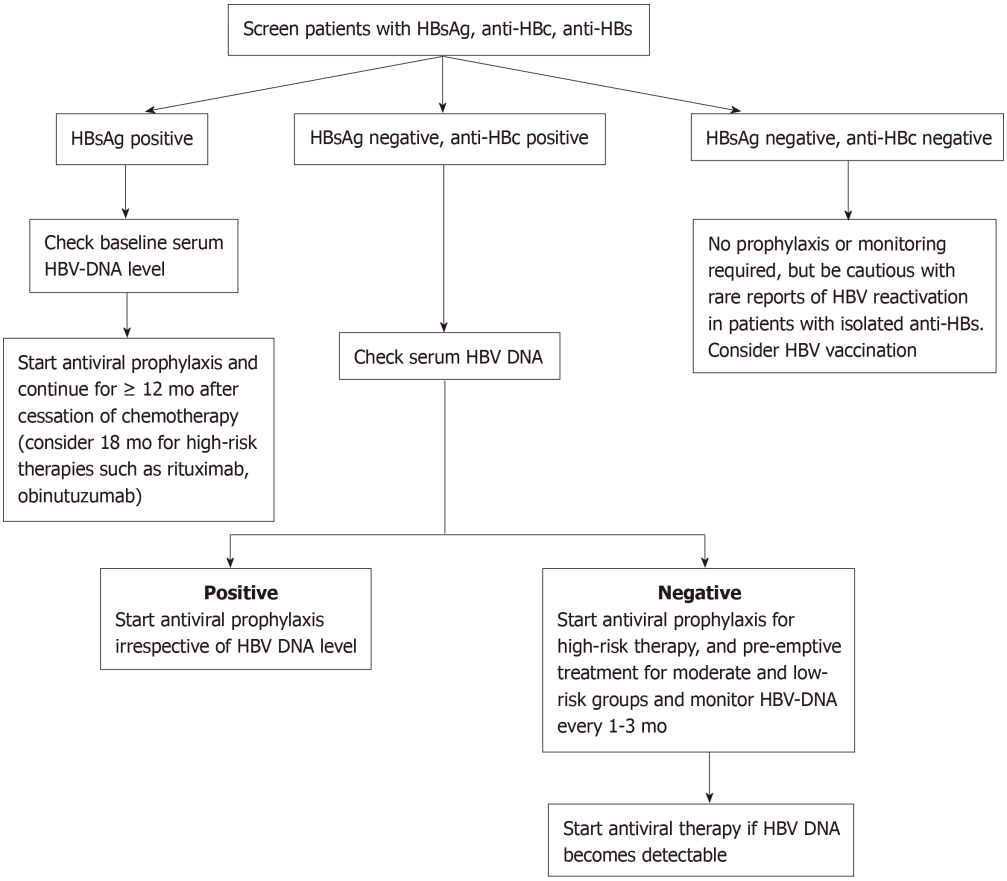
A meta-analysis of 770 patients with lymphoma showed that, in patients with CHB, the risk of HBV reactivation was significantly higher in those receiving prophylactic lamivudine compared with entecavir ($P < 0.001$)[143]. The superior prophylactic efficacy of entecavir is supported by studies in allogenic HSCT recipients and solid tumor patients, which showed a lower rate of HBV reactivation with entecavir compared with lamivudine[20,144]. Meta-analyses have also shown that tenofovir and entecavir are the most effective antiviral agents for the prevention of HBV reactivation[145,146]. Entecavir treatment of HBV patients with lamivudine-resistant viral strains is usually unsuccessful due to the rapid selection of additional mutants[147], highlighting the importance in choosing an effective initial anti-viral therapy.

Duration of antiviral therapy

Most guidelines recommend continuing antiviral therapy for 1 year after the cessation of anti-cancer therapy, and some guidelines recommend extending antiviral treatment for up to 18 mo after the last dose of cancer therapy (Table 6)[3,4,148]. Delayed HBV reactivation has been reported in patients who received anti-CD20 antibody therapy such as rituximab since rituximab will delay the immune recovery[149,150]. HBV DNA levels should be checked before stopping antiviral treatment.

Monitoring after stopping antiviral prophylaxis

HBV reactivation can develop after cessation of NAT[149,151,152], so monitoring for HBV reactivation is recommended after stopping anti-HBV prophylaxis (Table 6). In general, liver function tests and HBV DNA are monitored every 3 mo for a minimum of 12 mo after discontinuation of antiviral agents[3,4,7]. Monitoring for more than 12 mo is recommended for patients who received anti-CD20 monoclonal antibody therapy.



DOI: 10.3748/wjg.v29.i33.4942

Figure 1 Recommended algorithm for hepatitis B virus testing and treatment in patients with hematological malignancies receiving anti-cancer therapy. HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HBc: Hepatitis B core.

Management of HBV reactivation

Prophylaxis is better than treatment because fatal outcomes may still occur in patients with HBV reactivation even with antiviral treatment[153]. Foont and Schiff[154] performed a systematic review on the use of lamivudine for the prophylaxis of HBV reactivation in patients on chemotherapy. In the ten trials with 173 patients included in the analysis, two patients taking lamivudine prophylaxis developed fatal HBV reactivation[154]. If a patient is not on antiviral prophylaxis, treatment with an antiviral agent such as entecavir or tenofovir should be initiated. Hepatitis B flare-ups are generally uncommon in patients receiving anti-HBV prophylaxis with potent antiviral agents, but drug resistance can develop to prophylactic lamivudine. In this instance, salvage therapy such as entecavir or tenofovir may be beneficial to them. Some patients achieve biochemical and virological recovery after combination treatment with entecavir + adefovir or lamivudine + adefovir[155].

The purpose of the treatment is prevention of severe hepatitis and also hepatic failure, which are potentially fatal. It is important to closely monitor the patient's liver enzymes, clotting profile, and bilirubin levels. Patients can still progress to hepatic failure despite therapy with nucleoside analogs[42], especially when there is already a marked increase in liver enzymes or jaundice. Liver transplantation is an option for patients with liver failure and there have been reported cases of successful transplantation in patients with chemotherapy-induced HBV reactivation[156-160]. Benten *et al*[161] found a low recurrence of pre-existing extrahepatic malignancies after liver transplantation.

CONCLUSION

Many novel therapies have emerged for the treatment of hematological malignancies in the past two decades and the results are promising. The issue of prevention of HBV reactivation is an important part of the management. Hepatitis due to HBV reactivation is a potentially fatal complication of cancer chemotherapy in patients with hematological malignancies. HBV reactivation can be prevented through blood test screening and, in patients with moderate or high risk of HBV reactivation, prophylactic antiviral therapy. We recommend screening all hematology patients for HBsAg and anti-HBc prior to receipt of anticancer therapy, and risk stratification based on the types of therapies planned and the serologic status of the patients. Prophylactic antiviral therapy is important for HBsAg-positive patients. Two options are available for HBsAg-negative/anti-HBc-positive patients. One is routine prophylactic antiviral therapy. The other is serial HBV DNA monitoring, and pre-emptive antiviral drug administration as soon as HBV DNA is detected. While there is

still limited evidence on the risk of HBV reactivation with newer therapies, we recommend antiviral prophylaxis in patients with resolved HBV who are scheduled to receive high-risk therapies like anti-CD20 monoclonal antibodies, anti-CD79 monoclonal antibodies, bispecific antibodies, BTK inhibitors, BCL-2 inhibitors, CAR-T cell therapy, or HSCT.

Entecavir and tenofovir are the preferred choices for prophylactic therapy. Preventative antiviral therapy should be continued for at least 12 mo after the cessation of chemotherapy; longer durations are recommended for patients who received rituximab or those who had high levels of serum HBV DNA before starting chemotherapy. Checking the HBV DNA before the cessation of antiviral therapy is recommended. We would also recommend monitoring liver function and HBV DNA levels for at least 12 mo after the cessation of antiviral prophylaxis.

FOOTNOTES

Author contributions: Mak JWY, Law AWH, Law KWT, Ho R, Cheung CKM, and Law MF were involved in the analysis of data/references; Mak JWY revised critically the manuscript; Law AWH, Law KWT, Cheung CKM, and Law MF contributed to the acquisition of data/references; Mak JWY, Cheung CKM, and Law MF contributed to the interpretation of data/references; Cheung CKM and Law MF drafted the manuscript; and all authors approved the manuscript.

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REVIEW

Direct oral anticoagulants for the treatment of splanchnic vein thrombosis: A state of art

Giovanni Monaco, Luca Bucherini, Bernardo Stefanini, Fabio Piscaglia, Francesco Giuseppe Foschi, Luca Ielasi

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Abstract

Splanchnic vein thrombosis (SVT) is a manifestation of venous thromboembolism in an unusual site. Portal, mesenteric, and splenic veins are the most common vessels involved in SVT which occurs mainly in patients with liver cirrhosis, although non-cirrhotic patients could be affected as well. Thrombosis of hepatic veins, also known as Budd-Chiari syndrome, is another manifestation of SVT. Prompt diagnosis and intervention are mandatory in order to increase the recalization rate and reduce the risk of thrombus progression and hypertensive complications. Traditional anticoagulation with heparin and vitamin-K antagonists is the treatment of choice in these cases. However, recent studies have shown promising results on the efficacy and safety of direct oral anticoagulants (DOACs) in this setting. Available results are mainly based on retrospective studies with small sample size, but first clinical trials have been published in the last years. This manuscript aims to provide an updated overview of the current evidence regarding the role of DOACs for SVT in both cirrhotic and non-cirrhotic patients.

Key Words: Splanchnic vein thrombosis; Portal vein thrombosis; Budd-Chiari syndrome; Direct oral anticoagulants

Core Tip: The term splanchnic vein thrombosis (SVT) includes portal vein thrombosis and Budd-Chiari syndrome. Both conditions could occur in patients with and without an underlying liver disease. The cornerstone of treatment is anticoagulation. Direct oral anticoagulants (DOACs) are a novel class of drugs that have strongly affirmed their role in the management of patients with atrial fibrillation and venous thromboembolism. In the last few years, several studies have been published showing promising results in efficacy and safety of DOACs in patients with SVT.

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INTRODUCTION

Splanchnic vein thrombosis (SVT) is a rare but potentially life-threatening condition that occurs when blood clots form in the veins that drain the digestive system from the lower esophagus to the upper two-thirds of the rectum. Among different SVT, we can distinguish two main conditions: Budd-Chiari syndrome (BCS) and portal vein thrombosis (PVT).

BCS is caused by the thrombotic obstruction of hepatic venous outflow, localized anywhere from the hepatic veins to the entry of the inferior vena cava into the right atrium. BCS could also be caused by extra-vascular compression (secondary BCS), but this non-thrombotic form of the disease will not be discussed further.

There is no standardized definition of PVT. Generally, it refers to the thrombosis of the main portal trunk or its lobar branches with or without extension to the splenic or mesenteric veins.

SVT can develop both in patients with and without underlying liver disease[1].

In the first case, SVT represents a rare condition with a prevalence of less than 0.2% in the general population and it is commonly associated with strong risk factors for thrombosis[2].

In the second case, liver cirrhosis represents the mainstay of the pathogenesis of SVT and the co-presence of thrombophilic risk factors is uncommon. Cirrhotic patients generally present a PVT with an incidence that ranges from 11% to 24% at 5 years; prevalence increases according to liver disease severity (10% in compensated cirrhosis, 17% in decompensated cirrhosis, and 26% in liver transplant candidates)[3-5].

In patients with SVT, the development of portal hypertension is common; the increase of portal venous pressure could be caused by either pre-hepatic (in PVT) or post-hepatic (in BCS) venous flow obstruction.

As a thrombotic condition, anticoagulation is generally required for these patients as first line treatment. Over the last few years, interventional endovascular approaches (*e.g.* transjugular intrahepatic portosystemic shunt placement, angioplasty, suction thrombectomy, catheter-directed thrombolysis) have shown interesting results mainly in the management of acute symptomatic PVT with an inadequate response to medical treatment[6-8]. They could be used in isolation or in conjunction with systemic anticoagulation. Description of these procedures and their indications go beyond the aim of this paper, so it will not be discussed further.

Traditional anticoagulants commonly used for SVT are heparins and vitamin-K antagonists (VKA).

Low-molecular-weight heparin (LMWH) is generally preferred to unfractionated heparin (UFH) due to its lower incidence of heparin-induced thrombocytopenia, unless there are contraindications to LMWH such as severe renal failure. LMWH also has the advantage that it has a short half-life and no need of monitoring, but daily subcutaneous administration may reduce patients' compliance.

VKA are usually used for long-term anticoagulation. They have the advantage of oral administration and reversibility with vitamin K supplementation, but they require international normalized ratio (INR) monitoring and a personalized dose schedule.

Beside traditional anticoagulants, in recent years direct oral anticoagulants (DOACs) have become the first choice of treatment in several conditions, such as stroke prophylaxis in atrial fibrillation[9] and treatment of deep vein thrombosis and pulmonary embolism[10].

DOACs have the advantage of oral administration, fixed dosing schedule, predictable anticoagulant effect, and they do not require frequent monitoring.

DOACs exert their activity by directly inhibiting factor X-activated (such as rivaroxaban, apixaban and edoxaban) or factor II-activated (such as dabigatran). Their metabolism is generally both renal and hepatic, with different percentage among single drugs. Rivaroxaban, apixaban and edoxaban are metabolized by cytochromes without forming active metabolites; dabigatran is a prodrug not metabolized by cytochromes and it is the DOAC with the higher amount of renal excretion (approximatively 80%)[11].

Despite the aforementioned considerations, at present the use of DOACs for SVT remains poorly investigated. If chosen as anticoagulation therapy, they have to be prescribed off-label as they are currently not licensed for this indication in many countries.

Nevertheless, on the thrust of the advantages demonstrated in other conditions, interest on the use of DOACs in this setting is recently emerging, and data obtained by several recent reports are encouraging[12,13].

In this review, we analyzed all the studies available in the literature concerning patients with cirrhotic and non-cirrhotic PVT and BCS treated with DOACs; case reports were systematically excluded.

NON-CIRRHOTIC PVT

Causes of SVT in patients without underlying liver disease could be classified as systemic acquired risk factors for thrombosis, inherited thrombophilia and local factors. More than one risk factor is found in 10%-23% of patients[14,15].

Systemic acquired thrombophilic factors represent the cause of up to 50% of SVT[16]. The main related conditions are myeloproliferative neoplasms (mostly those related to JAK2-V617F mutation)[17,18], hormonal factors (oral contraceptive or pregnancy)[19,20], antiphospholipid antibody syndrome[21], and other systemic inflammations/infections (*e.g.* connective tissue disease, sarcoidosis, cytomegalovirus infection[22], severe acute respiratory syndrome coronavirus 2 infection[23,24], sepsis).

Inherited thrombophilic disorders could be detected in about 20% of cases[16]. The most common clotting factor alteration is factor V Leiden mutation (8% of cases), followed by G20210A prothrombin mutation and antithrombin deficiency (5% of cases each); protein S and protein C deficiency are less frequent (less than 2% and 1%, respectively)[25-27].

Local factors are involved in about 20% of cases[16]. These are represented mainly by abdominal surgery and infectious or inflammatory diseases involving abdominal organs, such as pancreatitis[28], diverticulitis, inflammatory bowel disease, abdominal vasculitis and abdominal cancers[17].

Notably, in 15%-40% of cases of SVT without cirrhosis no causative factors are identified. The treatment of the underlying disease is crucial in the management of patients, so an accurate work-up should be performed at SVT diagnosis[16].

Although not all guidelines agree on this definition, it is widely accepted that PVT can be divided in acute or chronic, based on the onset of the disease within 6 mo or beyond, respectively. The latter also includes the transformation in portal cavernoma, that is the replacement of the native portal vein with multiple tortuous collateral venous vessels that develop in response to chronic venous outflow obstruction.

In case of acute non-cirrhotic PVT, the main goal is to achieve portal recanalization and to prevent extension of the clot and sequelae such as intestinal infarction and the development of portal hypertension. Spontaneous resolution of acute PVT is rare, and early anticoagulation treatment is associated with higher rates of recanalization[29]. Therefore, full dose anticoagulation treatment should be started at diagnosis[15,29-33]. Moreover, a study showed that the risk of developing recurrent thrombotic events among subjects with non-abdominal thromboembolism and non-cirrhotic PVT is comparable [34].

Treatment should be continued for at least 3-6 mo for all patients. Similar to guideline recommendations for deep vein thrombosis occurring in typical sites, indefinite anticoagulation is recommended in all cases of persistent identified risk factors, such as acquired or congenital thrombophilia, but should also be considered in case the evidence of a persistent underlying prothrombotic factor is lacking[30,35].

As mentioned above, PVT may evolve into portal cavernoma if left untreated. In the presence of chronic PVT or portal cavernoma, even though the benefit of anticoagulation is less clear, it is recommended to treat patients as in the case of acute PVT[36-38]. However, since bleeding is the most common complication of chronic PVT[39], in patients with high risk esophageal varices anticoagulation treatment should be postponed until an adequate prophylaxis for portal hypertensive bleeding has been initiated[35].

Regarding the choice of anticoagulants, initial treatment with LMWH and subsequent switch to VKA is supported by extensive evidence and still represents the established therapy for most patients. The treatment is administered with the same therapeutic regimens and dose adjustments as for typical site venous thromboembolism.

Several studies have been recently published regarding the use of DOACs in this setting showing their efficacy and safety; at present, no randomized controlled trial has been published yet (Table 1).

Janczak *et al*[40] were the first to investigate the use of DOACs for thrombosis in atypical sites. They conducted a prospective study enrolling patients that were treated with anticoagulants for thromboembolism occurring both in typical and atypical sites. Considering the subgroup with PVT, 16 patients were treated with DOACs (rivaroxaban and apixaban), and 13 patients were treated with LMWH. The results did not reveal any statistically significant difference between DOACs and LMWH both in terms of efficacy and safety[40].

Scheiner *et al*[41] performed a retrospective study with 51 cirrhotic patients with concomitant non-malignant PVT. No anticoagulation therapy was started in 39 patients, whereas 12 patients received warfarin. Additionally, they also enrolled 10 patients treated with DOACs after traditional anticoagulation. In particular, 4 patients received edoxaban 30 or 60 mg once daily (OD), 3 apixaban 5 mg twice daily (BID), 2 rivaroxaban 10 mg OD, 1 dabigatran 100 mg BID. The mean follow-up time was 9.2 mo. In the DOAC group 70% of patients were non-cirrhotic. Regression of thrombus was observed in 20% of patients, and stability in 80%; no thrombus progression has been reported. Since cavernous transformation of the chronic PVT was already present in all patients treated with DOACs (therefore achieving recanalization could be difficult), the authors could not extrapolate data to compare the success rates of conservative or traditional therapy to DOACs. Only one bleeding episode was described in a patient in therapy with DOAC, so authors concluded that there was no statistically significant difference in bleeding events between DOAC and VKA groups[41].

Naymagon *et al*[42] published several retrospective studies comparing traditional anticoagulants *vs* DOACs for treatment of SVT in non-cirrhotic patients. In a study that compared VKA/LMWH and DOACs for non-cirrhotic PVT, recanalization rates (defined as complete radiological resolution) were higher in DOAC group compared to VKA, but similar to the group treated with enoxaparin. Nevertheless, a lower rate of bleeding was observed in patients treated with DOACs[42].

Another retrospective study from the same authors evaluated a cohort of 58 patients with inflammatory bowel disease associated-PVT who were treated either with DOACs or traditional anticoagulants. Complete radiological response rate in the DOAC group was two-fold higher than in the warfarin group; moreover, the DOAC group needed a shorter course

Table 1 Characteristics of studies on non-cirrhotic patients with portal vein thrombosis treated with direct oral anticoagulants

Study	Population	Outcomes	Adverse events	Ref.
Prospective	Non-cirrhotic, atypical sites (including PVT); Riva and Api for PVT (n = 16) *vs* enoxa for PVT (n = 13)	Riva and Apixaban are effective and safe in patients with venous thrombosis of atypical locations	No major difference in bleeding rate	Janczak *et al* [40], 2018
Retrospective	Non-malignant PVT, both cirrhotic and non-cirrhotic; Edo (n = 4), Api (n = 3), Riva (n = 2), Dabi (n = 1) *vs* traditional AC (n = 12), no AC (n = 39)	Favourable outcomes with DOACs with regression/resolution of thrombus in 20% of patients and stability or nonprogression in 80%	One bleeding episode in DOACs	Scheiner *et al* [41], 2018
Retrospective	Non-cirrhotic PVT; Riva (n = 65), Api (n = 20), Dabi (n = 8) *vs* Warf (n = 108), Enoxa (n = 70), Fondap (n = 2)	Resolution rate: Dabi (75%), Api (65%), Riva (65%), Enoxa (57%), Warf (31%); Recanalization rates are higher in DOACs compared to Warf but similar to Enoxa	Less major bleeding incidence in DOACs	Naymagon *et al*[42], 2020
Retrospective	IBD-associated PVT; DOACs (n = 23) *vs* Warf (n = 22), Enoxa (n = 13)	Resolution rate: DOACs (96%), Warf (55%); DOACs group needed a shorter course of anticoagulation (median 3.9 *vs* 8.5)	N/A	Naymagon *et al*[43] 2021
Retrospective	Intraabdominal surgery < 3 mo prior to PVT diagnosis; DOACs (n = 35) *vs* Warf (n = 31), Enoxa (n = 29), no AC (n = 12)	Complete resolution rate: DOACs (77%), Enoxa (69%), Warf (45%), no AC (17%)	N/A	Naymagon *et al*[44], 2021
Retrospective	PVT with/without cirrhosis; DOACs (n = 13; 8 non-cirrhotic) *vs* Warf (n = 20; 15 non cirrotic)	Treatment failure: DOACs (n = 0); Warf (n = 4)	Major bleedings: DOACs: n=0; VKA: n=1	Ilcewicz *et al* [45], 2021
Prospective	SVT without cirrhosis; Riva 15 BID for 3 wk + Riva 20 mg OD for 3 mo (n = 100)	Recanalization > 80% at 3 mo (47% complete)	2 major bleeding; 2 SVT recurrence	Ageno *et al* [46], 2022

AC: Anticoagulation; Api: Apixaban; BID: Twice daily; Dabi: Dabigatran; DOACs: Direct oral anticoagulants; Edo: Edoxaban; Enoxa: Enoxaparin; Fondap: Fondaparinux; IBD: Inflammatory bowel disease; OD: Once daily; PVT: Portal vein thrombosis; Riva: Rivaroxaban; SVT: Splanchnic vein thrombosis; VKA: Vitamin K antagonists; Warf: Warfarin.

of anticoagulation to achieve recanalization[43].

Similar results in terms of vein recanalization have been shown in patients who developed PVT within three months after abdominal surgery. The first group was treated with DOACs, the second with conventional anticoagulants or no anticoagulation. Recanalization rate was higher with DOAC than with VKA (77% *vs* 45%), but similar to LMWH. Of note, in the group receiving no anticoagulation treatment, only 17% of patients recanalized spontaneously[44].

Ilcewicz *et al*[45] analyzed retrospectively a cohort of 33 patients with PVT, including 10 patients with cirrhosis. Patients were treated with either warfarin or DOACs; 4 treatment failure and one major bleeding were recorded in the warfarin group but none was recorded in the DOAC group[45].

Recently, Ageno *et al*[46] conducted the first interventional study evaluating the safety and efficacy of DOACs in non-cirrhotic PVT. The study was a single-arm prospective multicentric study enrolling patients presenting with a first episode of non-cirrhotic, symptomatic, objectively diagnosed SVT who were treated with rivaroxaban 15 mg BID for 3 wk followed by 3 mo of rivaroxaban 20 mg OD. Major bleeding was the primary endpoint of the study; secondary endpoints included death, recurrent SVT, and complete vein recanalization within 3 mo. During the 6-months follow-up period, non-life-threatening major bleeding events occurred in 2 patients; recurrence of thrombosis was observed in 2 patients, and 1 death unrelated to thrombosis was recorded. The recanalization at 3 mo was achieved in more than 80% of patients, with a complete recanalization rate of 47%[46].

From what has emerged from the aforementioned studies, the use of DOACs in non-cirrhotic PVT seems to be promising; results suggest that DOACs are superior to traditional anticoagulants in terms of recanalization rate[42-44,46] although they have a similar safety profile to VKA[40].

However, it is important to emphasize that these results are affected by several limitations: Firstly, at present no randomized controlled trial has been published; secondly, the results are based on small patients cohorts, the therapeutic regimens of DOACs vary widely between studies and the duration of follow-up was also extremely heterogeneous.

CIRRHOTIC PVT

Liver cirrhosis is an irreversible end-stage liver disease characterized by the progressive deposition of fibrotic tissue and a diffuse conversion of the normal liver architecture into structurally abnormal nodules, eventually leading to impaired liver function.

The increased liver stiffness causes a reduced portal blood flow and an increase of portal pressure, (*i.e.*, portal hypertension); the blood stasis together with the pro-thrombotic status typical of cirrhotic patients lead to a higher cumulative risk of splanchnic thrombosis, mainly PVT[47,48].

A recent meta-analysis on cirrhotic PVT not treated with anticoagulation showed an improvement in 30% of cases and a progression of thrombus in approximately 25% of cases[49].

According to the Baveno VII consensus, anticoagulation is recommended in cirrhotic patients with recent (< 6 mo) and > 50% occlusive thrombosis of the main portal vein trunk, in those with symptomatic PVT or in potential candidates for liver transplantation. In the last group of patients, the aim of anticoagulation is the prevention of recurrence of thrombosis or the progression of thrombus in order to with the aim facilitate the portal anastomosis during the surgical procedure.

Anticoagulation should also be considered in patients with < 50% occlusive thrombosis of the main portal vein trunk with progression during follow-up or with extension to the superior mesenteric vein.

Once anticoagulation is started, it should be maintained until portal vein recanalization and for a minimum of 6 mo; longer anticoagulation therapy should always be considered in patients awaiting liver transplantation, even after complete portal vein recanalization[35].

Early initiation of anticoagulation seems to be related to a higher recanalization rate[50,51].

Different classifications, indications and duration of treatment, and anticoagulation of choice according to the main clinical practice guidelines[30,35,38,52] are resumed in Table 2; a deep analysis of the differences among guidelines is not the aim of this paper, so it will not be discussed further.

The assessment of the bleeding risk in cirrhotic patients is mandatory but it is always challenging. Profound alteration in coagulation pathways, related to a reduced synthesis of prothrombotic and antithrombotic clotting factors, as well as thrombocytopenia, related to hypersplenism and decreased hepatic thrombopoietin synthesis, define a hemostatic imbalance and, consequently, the management of anticoagulation therapy in cirrhotic patient could be very difficult in clinical practice[53-55].

However, anticoagulation therapy in cirrhotic patients seems to be quite safe, as demonstrated in a meta-analysis of Loffredo *et al*[56] reporting no difference in major and minor bleeding rates between patients with or without anticoagulation therapy for PVT. Moreover, a recent competing-risk meta-analysis showed that anticoagulation in patients with cirrhosis and PVT reduces all-cause mortality independently of portal recanalization[57].

The presence of hepatocellular carcinoma does not contraindicate anticoagulation for non-malignant PVT; safety and efficacy of anticoagulation seem to be similar to patients without hepatocellular carcinoma[58,59].

The choice of the best anticoagulation is still debated, and guidelines do not give strong recommendations on this topic. LMWH is the best-known treatment option, largely used and with the most solid data in the literature; for these reasons consensus panels suggest at least to start anticoagulation with this drug class[35]. Fondaparinux may be another option, although there are no significant data in the literature, especially on safety[60,61]. VKA are potentially usable[62], but physicians have to be aware that INR accuracy for treatment monitoring is significantly lower in patients with liver dysfunction[63].

Over the last few years, the clinical experience in using DOACs in patients with liver cirrhosis has been growing[64].

Despite cirrhotic patients have been excluded from phase III trials of DOACs for atrial fibrillation[65-68] and venous thromboembolism[69-72], several studies on their use in this cohort of patients have been published, demonstrating DOACs safety in patients with compensated liver disease (Child-Pugh A)[73-77]. DOACs should be used with caution in Child-Pugh B patients[78,79] and they are contraindicated in Child-Pugh C patients[80,81].

Moreover, further pharmacokinetics considerations should be considered in DOACs prescription in patients with underlying liver disease, such as altered plasma protein binding, cytochrome P450-mediated metabolism and biliary excretion[53].

Another issue is the possible hepatotoxicity of DOACs. All four available DOACs can induce hepatotoxicity with an idiosyncratic mechanism; rivaroxaban seems to have a minimally higher risk of liver injury compared to other three molecules[82]. However, recent studies have definitively shown that liver injury is a very rare adverse event and, more importantly, this rate is significantly lower than with warfarin[83-85].

Recently, several studies have been published investigating the efficacy and safety of DOAC in patients with liver cirrhosis and PVT (Table 3); In 2019, Hanafy *et al*[86] published a randomized controlled trial on rivaroxaban 10 mg BID *vs* warfarin, but it has been recently retracted for methodological issues, therefore it will not be considered in our review.

First data were obtained by Hum *et al*[87] in a single-centre retrospective cohort study of cirrhotic patients treated with anticoagulants for any indications. In the small subgroup of patients with PVT (7 patients), 4 received DOACs (rivaroxaban or apixaban) and 3 received LMWH or VKA. Of particular note, the total number of bleeding events was similar in both groups even if results are given for the entire population of study[87].

As already mentioned above, Scheiner *et al*[41] investigated a cohort of both cirrhotic and non-cirrhotic patients presenting with non-neoplastic PVT. Out of the 10 patients receiving DOACs, only 30% presented concomitant liver disease[41]. For more details about this study, refer to the previous paragraph on non-cirrhotic PVT.

De Gottardi *et al*[88] retrospectively analyzed data from 17 European centers on cirrhotic and non-cirrhotic patients all treated with DOACs (either rivaroxaban, apixaban, or dabigatran at different doses) for any indication, mainly PVT. Patients were either initially prescribed with DOACs or switched to DOACs after traditional anticoagulants. The main reasons for switching were the development of recurrent thrombosis, clinically relevant side effects, and INR instability or unreliability for monitoring cirrhotic patients. Among the entire population of 94 patients, there were 22 and 38 patients with cirrhotic and non-cirrhotic PVT, respectively. The median follow-up time was 9.6 mo. In the group of non-cirrhotic patients, bleeding event rate was 15.5% *vs* 13.9% in the cirrhotic group, suggesting that the safety of DOACs is comparable between two groups. Despite the majority of the patients presented a PVT, the results presented by the authors are referred to the entire population and actual conclusions on PVT patients alone cannot be extrapolated.

Table 2 Comparison of main clinical practice guidelines for the management of portal vein thrombosis in non-cirrhotic patients

	EASL 2016[30]	AASLD 2020[38]	ACG 2020[52]	Baveno VII 2022[35]
Classification	Acute; Chronic	Recent: < 6 mo; Chronic: > 6 mo	Acute; Chronic	Recent: < 6 mo; Chronic: > 6 mo
Treatment	Acute: AC; Chronic: Not specified	Recent PVT: AC; Chronic complete PVT or cavernous transformation: No benefit from AC	Acute PVT: AC; Chronic: thrombophilia, progression of thrombus into mesenteric veins, current or previous evidence of bowel ischemia	Recent PVT: At diagnosis; Chronic PVT: After prophylaxis for portal hypertensive bleeding in high-risk varices
Choice of anticoagulation	LMWH, VKA	LMWH, VKA, DOACs	UFH, LMWH for initiation; LMWH or VKA for maintenance (DOACs absorption limited in the presence of intestinal oedema)	LMWH, VKA, DOACs
Duration of treatment	At least 6 mo in presence of transient risk factor; long term for persistent risk factor or in case of chronic PVT with history of intestinal ischemia or recurrent thrombosis	AC for 3 mo	At least 6 mo for acute without thrombophilia; long term with thrombophilia	Recent PVT: At least 6 mo; Chronic: Long term for patient with permanent prothrombotic state
Notes				EVL can be performed safely without withdrawing VKA

AASLD: American Association for the Study of Liver Diseases; AC: Anticoagulation; ACG: American College of Gastroenterology; DOACs: Direct oral anticoagulants; EASL: European Association for the Study of the Liver; EVL: Endoscopic variceal ligation; LMWH: Low molecular weight heparin; PVT: Portal vein thrombosis; UFH: Unfractioned heparin; VKA: Vitamin K antagonists.

Another study examining DOACs safety in cirrhosis, but this time in comparison with conventional anticoagulants, was conducted by Intagliata *et al*[89]. After collecting data from a research database, a cohort of 39 cirrhotic patients treated with anticoagulants for various indications was identified. Since no patients with decompensated liver disease (Child-Pugh C) were treated with DOACs, only patients with Child-Pugh A or B cirrhosis were included. In the group treated with DOACs (apixaban or rivaroxaban, either in therapeutic or prophylactic doses) 20 patients were included, and the most common indication for treatment was PVT (60%). In contrast, most patients treated with VKA or LMWH presented non-splanchnic venous thromboembolism (63%). No statistically significant difference in bleeding rates was observed between the two groups.

Also Davis *et al*[90] investigated the safety of cirrhotic patients treated with DOACs or VKA for any indication. Since only 3 patients received DOAC for PVT, this study was not included in our review.

Nagaoki *et al*[91] conducted a retrospective cohort study to evaluate the efficacy of edoxaban as maintenance therapy in 50 cirrhotic patients with PVT. Child–Pugh classification was grade A in 29 patients, B in 16, and C in 5. All patients were initially treated with danaparoid sodium for two weeks and then switched to either warfarin or edoxaban 60 or 30 mg OD, depending on renal function (creatinine clearance < 30 mL/min), body weight (< 60 kg) and concomitant treatment with a strong P-glycoprotein inhibitor. Among study population, 17 patients had concomitant hepatocellular carcinoma, but all were diagnosed with non-neoplastic PVT. All patients were screened with endoscopy before the initiation of anticoagulation. In case of high risk esophageal and/or gastric varices, endoscopic prophylactic treatment was systematically performed. Median time from PVT to treatment was similar between edoxaban and VKA group (4.2 *vs* 4.3 mo, respectively). Complete recanalization, assessed by computed tomography (CT) scan at 6 mo, was observed in 14 of 20 patients (70%) in the edoxaban group and in 6 of 30 patients (20%) in the warfarin group. However, given the potential risk of bleeding, a target INR of 1.5–2.0 was chosen for patients undergoing warfarin treatment. This underdosing in VKA therapy, may explain the low efficacy rate in this cohort. Additionally, safety was considered comparable between edoxaban and warfarin groups with 3 and 2 gastrointestinal bleedings, respectively[91].

In a prospective cohort study performed by Ai *et al*[92] 80 patients with cirrhosis and chronic PVT were examined. Patients with history of recent bleeding (< 3 mo), high risk esophageal varices, systemic malignancies, severe renal impairment (creatinine clearance < 30 mL/min), concomitant antiplatelet therapy and low platelet count (< 50 × 10^9/L) were excluded. Of the 40 patients treated with DOACs, 26 Child-Pugh A patients were treated with rivaroxaban 20 mg OD and 14 Child-Pugh grade B or C patients with dabigatran 150 mg BID. The other 40 patients received no anticoagulation. Recanalization rates and improvements in portal vein flow velocity were analyzed at 3 and 6 mo. The recanalization rate was higher in the DOAC group than in the control group, especially after 6 mo of treatment (12.8% at 3 mo *vs* 28.2% at 6 mo), whereas the bleeding rate was similar between the 2 groups. Of note, authors considered PVT as chronic if lasting more than one month, commensurate to definition of chronic deep vein thrombosis. Overall recanalization rates were low compared to previous studies; authors suggested that the delayed initiation of anticoagulation therapy might be associated with a worse outcome[92].

Finally, Lv *et al*[93] designed a prospective observational study investigating the role of both anticoagulation and transjugular intrahepatic porto-systemic shunt (TIPS) in 396 cirrhotic patients with non-malignant PVT either acute or chronic, confirmed with CT scan. Patients with intra or extrahepatic malignancy at baseline, presence of previous TIPS, isolated mesenteric or splenic vein thrombosis, and liver transplantation recipients were excluded. Forty-eight patients

Table 3 Characteristics of studies on cirrhotic patients with portal vein thrombosis treated with direct oral anticoagulants

Study	Population	Aim of study	Doses and duration	Outcomes	Adverse events	Ref.
Retrospective	Cirrhotic, CP A/B/C; any indication (incl. PVT); subgroup with PVT: Riva or Api (n = 4) *vs* Enoxa or VKA (n = 3)	Efficacy and safety of DOACs *vs* traditional AC in cirrhosis	Riva 15 mg OD +/- 20 mg OD load; Api 5 mg BID +/- 10 mg BID load 10.6 mo (mean)	Recurrent thrombosis: DOACs (n = 1); Trad AC (n = 1)	Total bleeding events were similar in the two groups (with lesser major bleeding in the DOACs group)	Hum *et al* [87], 2017
Retrospective	Cirrhotic, CP A/BAny indication (incl. PVT); subgroup with PVT: Riva or Api (n = 12) *vs* LMWH or Warf (n = 6)	Compare the bleeding rates in cirrhotic patients	Riva 20 mg OD; Api 5 mg BID 10.6 mo (mean)	No statistical difference between therapeutic and prophylactic dosing between groups	Similar rates of major and minor bleeding in the two groups	Intagliata *et al* [89], 2016
Retrospective	Both cirrhotic and non, CP A/B; any indication (incl. PVT); subgroup with cirrhosis and PVT: Riva, Api or Dabi (n = 22)	Indication for starting or switching to DOACs and report short-term efficacy and safety	Cirrhotic: Different doses 9.6 mo (mean)	Cirrhotic: recurrent PVT (n = 1, 4.5%)	Cirrhotic group any indication: Major bleeding (n = 1), minor bleeding (n = 4)	De Gottardi *et al*[88], 2017
Retrospective	Both cirrhotic and non, CP A/B/C; non-malignant PVT; Edo (n = 4), Api (n = 3), Riva (n = 2), Dabi (n = 1) *vs* traditional AC (n = 12), no AC (n = 39)	Efficacy and safety of AC in non-malignant PVT	Edo 30/60 mg OD, Api 5 mg BID, Riva 10 mg OD, Dabi 110 mg BID 9.2 mo (median)	Favourable outcomes with DOACs: Regression/resolution 20%; stability/non-progression 80%	Portal hypertensive gastropathy bleeding	Scheiner *et al* [41], 2018
Retrospective	Cirrhotic, CP A/B; non-malignant PVT; Edo (n = 20) *vs* Warf (n = 30) (following 2 wk Danaparoid)	Compare the efficacy and safety of Edo and Warf for treatment of chronic PVT in cirrhotic patients	Edo 60 mg OD, (if CrCl > 50; n = 4) or Edo 30 mg OD (if CrCl < 50; n = 16) 6 mo (max)	Edo group had more complete resolution and less PVT progression than Warf group	Major GI bleeding: Edo (n = 3; 7%); Warf (n = 2; 15%)	Nagaoki *et al* [91], 2018
Prospective	Cirrhotic, CP A; chronic PTV; Riva (n = 26), Dabi (n = 14) *vs* no AC (n = 40)	Compare the efficacy and safety of DOACs and no AC in chronic PVT in cirrhotic patients	Riva 20 mg OD; Dabi 150 mg BID; 6 mo (max)	Recanalization rate with DOACs 28.2% (statistically higher) and improvement of liver function	No statistically significant difference between the DOACs and the control group in bleeding events	Ai *et al*[92], 2020
Prospective	Cirrhotic, CP A/B/C; non-malignant PVT; TIPS + AC (n = 197, 18 Riva) *vs* AC only (n = 63, 4 Riva) *vs* TIPS only (n = 88) *vs* nothing (n = 48)	Compare the management using a wait-and-see strategy, AC, and TIPS to treat PVT in cirrhosis	Riva 10 mg OD; 21.0 mo (median)	Recanalization: 0% with Riva only (all with PVT and SMV thrombosis), 100% with Riva + TIPS	Major bleeding events: AC only (n = 14); TIPS+AC (n = 30)	Lv *et al*[93], 2021

AC: Anticoagulation; Api: Apixaban; VKA: Vitamin K antagonists; BID: Twice daily; CP: Child-Pugh score; Dabi: Dabigatran; DOACs: Direct oral anticoagulants; Edo: Edoxaban; GI: Gastrointestinal; Enoxa: Enoxaparin; LMWH: Low molecular weight heparin; OD: Once daily; PVT: Portal vein thrombosis; Riva: Rivaroxaban; SMV: Superior mesenteric vein; TIPS: Transjugular intrahepatic portosystemic shunt; Warf: Warfarin.

received no treatment, 63 patients were treated with anticoagulants only, 88 patients received TIPS only, and 197 started anticoagulation after TIPS insertion. When patients received anticoagulation, they were treated with either VKA, LMWH, or rivaroxaban 10 mg OD, and anticoagulation treatment was extended for 12 mo after complete recanalization was achieved. A combined strategy with TIPS and subsequent anticoagulation showed the highest complete recanalization rate (188/197 patients); long-term anticoagulation with LMWH or rivaroxaban resulted in minor incidence of re-thrombosis and longer survival compared with VKA[93].

Overall, the proposed studies show that DOACs are at least non-inferior to conventional anticoagulants in cirrhotic non-malignant PVT, both in terms of efficacy and safety, but several limitations pose some issues regarding the results obtained.

First, most studies were conducted retrospectively with a limited number of patients and very heterogeneous cohorts.

Second, PVT classification, definition of bleeding events, drug dosage, and treatment duration vary widely among studies, making it difficult to compare results and to identify a standardized treatment algorithm.

Nonetheless, DOACs may represent a viable alternative to conventional anticoagulants in cirrhotic PVT, but further evidence and RCTs are needed.

BCS

Causes of primary BCS are essentially the same of non-cirrhotic PVT[16]. Compared with PVT, there is a greater prevalence of association with myeloproliferative neoplasm (30%-57% of cases)[17,94]. Some acquired thrombophilic conditions, such as paroxysmal nocturnal hemoglobinuria and Behçet's disease have also a higher causative link in BCS compared with PVT (12% *vs* < 1%, respectively)[95-97]. To the contrary, BCS caused by local factors is rare, with the only exception of hepatic hydatid cysts in countries where *Echinococcus granulosus* is endemic[98].

As for PVT, more than one risk factor could be found in 26%-46% of patients and no causative factors are identified in 10%-29% of patients[16,99].

Prompt identification and treatment of an underlying disease is mandatory for the management of BCS patients since both are positively related with outcome[96,100]. Anticoagulation is the cornerstone of BCS treatment and it should be initiated at diagnosis; long-term anticoagulation is generally recommended even in the absence of an identified prothrombotic disorder[35]. LMWH is currently the drug of choice, based on several previous studies reporting a higher rate of heparin-induced thrombocytopenia in BCS patients treated with UFH[101,102]. When a stability of the disease is achieved, a switch to VKA is usually the preferred choice in clinical practice.

The role of DOACs in BCS patients has been poorly investigated compared to PVT patients.

First data came from the aforementioned retrospective study of De Gottardi *et al*[88] about the use of DOACs in both cirrhotic and non-cirrhotic patients with SVT. In the study population (94 patients) there were 9 patients with BCS treated with DOACs (dabigatran, rivaroxaban or apixaban), but as results are presented for the entire population, it is not possible to extrapolate conclusions about efficacy and safety in this cohort of patients[88].

A recent multicentric Austrian study aimed to analyze the outcome of 22 patients treated with DOACs (all four drugs were prescribed, but almost a half of patients received edoxaban) *vs* 19 patients treated with only traditional anticoagulation (*i.e.* LMWH/VKA). Authors reported better efficacy results in the DOAC cohort (64% of complete recanalization rate and 92% of overall transplant-free survival at 5 years) and a comparable risk of major spontaneous and major procedure-related bleedings. Even though the results presented are interesting, there are some general considerations about the heterogeneity of the study population to be highlighted[103].

Firstly, in the DOAC cohort 16 patients (72.7%) were already anticoagulated with traditional drugs; among these, 8 patients (50%) had already achieved a complete response at the time of switching to DOAC.

Secondly, among the 16 patients receiving DOACs it is not known the time from LMWH/VKA start to the switch to DOACs, so it is difficult to evaluate the actual efficacy or failure of DOACs in patients previously treated with traditional anticoagulation.

Lastly, the rate of objective response to the first-line anticoagulation therapy (6 patients with DOACs *vs* 37 patients with LMWH/VKA) was comparable (66.6% *vs* 67.5%, respectively)[103].

Another retrospective monocentric study, made by Sharma *et al*[104], has investigated the role of dabigatran (36 patients) following endovascular intervention for BCS compared to VKA (62 patients). Authors concluded that stent patency rate, mortality and bleeding complication rate were comparable between dabigatran and VKA groups at 6 and 12 mo[104].

Although results from the literature are limited, DOACs seem effective and safe in patients with BCS and international guidelines have consequently added these drugs as an option of treatment, but prospective studies are needed.

CONCLUSION

In the last few years, several studies have shown promising results in the use of DOACs for the treatment of SVT in term of efficacy and, above all, safety. Unfortunately, the majority of studies are retrospective, with small sample size and with extremely heterogeneous examined populations, not allowing to give strong recommendations about the use of DOACs in this setting. Moreover, there is no conformity among studies in dosage schedule, time of initiation and duration of treatment and bleeding event definition. In some cases, it is even not specified the DOAC used.

On the other hand, international guidelines have added this new class of drugs as an option of treatment, recognizing their potential role both in cirrhotic and non-cirrhotic patients with SVT. Although in some countries there are strict limitations in prescription, more and more physicians prescribe DOACs for SVT in their clinical practice worldwide.

Further studies and clinical trials are needed in order to increase the level of evidence in this field, but current knowledge on DOAC use is already changing the therapeutic scenario of SVT.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Angiotensin-converting enzyme 2 improves liver fibrosis in mice by regulating autophagy of hepatic stellate cells

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Abstract

BACKGROUND

Liver fibrosis is the common pathological process associated with the occurrence and development of various chronic liver diseases. At present, there is still a lack of effective prevention and treatment methods in clinical practice. Hepatic stellate cell (HSC) plays a key role in liver fibrogenesis. In recent years, the study of liver fibrosis targeting HSC autophagy has become a hot spot in this research field. Angiotensin-converting enzyme 2 (ACE2) is a key negative regulator of renin-angiotensin system, and its specific molecular mechanism on autophagy and liver fibrosis needs to be further explored.

AIM

To investigate the effect of ACE2 on hepatic fibrosis in mice by regulating HSC autophagy through the Adenosine monophosphate activates protein kinases (AMPK)/mammalian target of rapamycin (mTOR) pathway.

METHODS

Overexpression of ACE2 in a mouse liver fibrosis model was induced by injection of liver-specific recombinant adeno-associated virus ACE2 vector (rAAV2/8-ACE2). The degree of liver fibrosis was assessed by histopathological staining and the biomarkers in mouse serum were measured by Luminex multifactor analysis. The number of apoptotic HSCs was assessed by terminal deoxynucleoitidyl transferase-mediated dUTP nick-end labeling (TUNEL) and immunofluorescence staining. Transmission electron microscopy was used to identify the changes in the number of HSC autophagosomes. The effect of ACE2 overexpression on

autophagy-related proteins was evaluated by multicolor immunofluorescence staining. The expression of autophagy-related indicators and AMPK pathway-related proteins was measured by western blotting.

RESULTS

A mouse model of liver fibrosis was successfully established after 8 wk of intraperitoneal injection of carbon tetrachloride (CCl_4). rAAV2/8-ACE2 administration reduced collagen deposition and alleviated the degree of liver fibrosis in mice. The serum levels of platelet-derived growth factor, angiopoietin-2, vascular endothelial growth factor and angiotensin II were decreased, while the levels of interleukin (IL)-10 and angiotensin- (1-7) were increased in the rAAV2/8-ACE2 group. In addition, the expression of alpha-smooth muscle actin, fibronectin, and CD31 was down-regulated in the rAAV2/8-ACE2 group. TUNEL and immunofluorescence staining showed that rAAV2/8-ACE2 injection increased HSC apoptosis. Moreover, rAAV2/8-ACE2 injection notably decreased the number of autophagosomes and the expression of autophagy-related proteins (LC3I, LC3II, Beclin-1), and affected the expression of AMPK pathway-related proteins (AMPK, p-AMPK, p-mTOR).

CONCLUSION

ACE2 overexpression can inhibit HSC activation and promote cell apoptosis by regulating HSC autophagy through the AMPK/mTOR pathway, thereby alleviating liver fibrosis and hepatic sinusoidal remodeling.

Key Words: Angiotensin-converting enzyme 2; Hepatic stellate cells; Autophagy; Liver fibrosis; Portal hypertension; Mice

Core Tip: Liver fibrosis and cirrhosis are the common outcomes of most chronic liver diseases, and there is a lack of effective treatment at present. Angiotensin-converting enzyme 2 (ACE2), as the main target receptor for the coronavirus disease virus invasion into the human body, is one of the research hotspots. The involvement of autophagy in the activation mechanism of hepatic stellate cell (HSC) during liver fibrosis has attracted increasing attention. Our study found that ACE2 can inhibit the activation and proliferation of HSCs by regulating autophagy, and promote apoptosis of HSCs, providing new ideas for the treatment of liver fibrosis and hepatic sinusoidal remodeling.

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INTRODUCTION

Liver fibrosis and cirrhosis are the common pathological processes associated with the occurrence and development of various chronic liver diseases, and there is still a lack of effective prevention and treatment methods for these conditions. In liver fibrosis and cirrhosis, excessive deposition of extracellular matrix (ECM) in the liver and regenerating nodules compress blood vessels, resulting in structural changes. In addition, hepatic sinusoidal vasoconstriction and vascular remodeling cause functional changes that ultimately lead to increased intrahepatic vascular resistance and portal pressure [1]. Hepatic stellate cells (HSCs) are located in the perisinusoidal Disse space between liver sinusoidal endothelial cells (LSECs) and hepatocytes[2]. The vasomotion of hepatic sinusoids greatly affects intrahepatic blood flow and portal venous resistance, and HSCs and LSECs play key roles in increasing intrahepatic vascular resistance and portal venous pressure. Hepatic sinusoidal vascular remodeling occurs in hepatic fibrosis and is characterized by capillarization of the hepatic sinusoids and surrounded by more contractile HSCs[3]. HSC activation is a complex and coordinated process. After activation, HSCs begin to proliferate and release excess collagen, proteoglycan and other ECM components, which in turn cause changes in the intrahepatic structure; furthermore, HSCs acquire contractility, reducing the diameter of the hepatic sinusoids and increasing resistance, leading to liver fibrosis and portal hypertension[4-6].

Autophagy is a metabolic process in which eukaryotic cells eliminate disposable or potentially dangerous cytoplasmic material. It plays a critical role in cell development, differentiation, and homeostasis. In this process, some damaged proteins or organelles are wrapped by autophagic vesicles with a double membrane structure and sent to lysosomes (animals) or vacuoles (yeast and plants) for degradation and recycling[7]. Autophagy, as a cellular housekeeper, can eliminate defective proteins and organelles, clear intracellular pathogens, and prevent the accumulation of abnormal proteins. Therefore, autophagy plays an active role in the pathology of many diseases. Growing evidence suggests that an adequate autophagic response in hepatocytes and nonparenchymal cells (HSCs, LSECs, Kupffer cells) is critical for the physiological function of the liver[8]. During hepatic fibrogenesis, the study of the mechanism of autophagy involved in HSC activation has attracted increasing attention. Autophagy increases the degradation of lipid droplets in HSCs, providing energy for HSC activation[9,10]. A study showed that after reducing HSC autophagy in mice, HSC activation was inhibited, and the degree of liver fibrosis was alleviated[11]. In recent years, the study of liver fibrosis targeting HSC

autophagy has become a hot spot in this research field.

The renin-angiotensin system (RAS) is an important endocrine system that regulates vascular tone and water and electrolyte metabolism in the body. Our previous studies have confirmed that HSCs have local RAS, and activated HSCs increase the synthesis of angiotensin II (Ang II) in liver cirrhosis[12]. Under the action of angiotensin-converting enzyme 2 (ACE2), Ang II is converted to Ang- (1-7), which stimulates the Mas receptor to cause vasodilation. ACE2 is a key negative regulator of RAS, and studies have shown that it can inhibit liver fibrosis by degrading Ang II[13,14], but its specific molecular mechanism needs to be further explored. We confirmed that carvedilol could inhibit Ang II-induced HSC proliferation and contraction and improve liver fibrosis in mice[12]. The study also indicated that HSCs are the main cells expressing ACE2 in the liver. In addition, our study demonstrated that carvedilol could notably reduce HSC autophagy and inhibit HSC activation and proliferation[15]. It has been reported that ACE2 alleviates the severity of acute lung injury by inhibiting autophagy[16]. Therefore, we hypothesized that ACE2 could inhibit HSC activation and proliferation by regulating autophagy, thus improving hepatic sinusoidal remodeling and ultimately alleviating liver fibrosis and portal hypertension.

The Adenosine monophosphate activates protein kinases (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway is not only an important node in the intracellular energy metabolism monitoring system but also an important upstream pathway regulating autophagy. Studies have reported that ACE2 can improve vascular endothelial dysfunction in type 2 diabetic rats with insulin resistance by regulating the AMPK/mTOR pathway[17]. In addition, ACE2 was shown to effectively modulate the AMPK/mTOR signaling pathway in a mouse model of acute lung injury[16]. Our previous study confirmed that metformin could inhibit HSC proliferation, migration and angiogenesis through the Akt/mTOR and mTOR/hypoxia inducible factor-1α (HIF-1α) pathways[18]. In this study, we evaluated the effect of ACE2 on liver fibrosis in mice and demonstrated the molecular mechanism by which ACE2 regulates HSC autophagy through the AMPK/mTOR pathway to improve liver fibrosis and hepatic sinusoidal remodeling.

The aim of this study was to determine the effect of ACE2 on HSC activation, proliferation, apoptosis and liver fibrosis by regulating autophagy. This study will provide a new direction for the prevention and targeted treatment of liver fibrosis and portal hypertension.

MATERIALS AND METHODS

Mouse model of liver fibrosis

Forty adult male C57BL/6J mice (6-8 wk, 18-20 g) were purchased from the Experimental Animal Center of Shandong University (Jinan, China). The mice were housed in an air-conditioned room at a defined temperature (23-25 °C) for one week prior to the initiation of the experiments. All experimental protocols were approved by the Animal Care Committee of the Second Hospital, Cheeloo College of Medicine, Shandong University.

The liver fibrosis mouse model was established by intraperitoneal injection of carbon tetrachloride (CCl_4, 20%, 0.5 mL/100 g) twice a week for 8 wk. To evaluate the effect of ACE2 on liver fibrosis, the liver-specific recombinant adeno-associated viral vector rAAV-ACE2 (rAAV2/8-ACE2) was injected into the tail vein 4 wk after CCl_4 administration. Mice were randomly assigned to four groups (10 in each): Group 1, normal control (olive oil); Group 2, CCl_4-induced liver fibrosis (CCl_4); Group 3, rAAV2/8-ACE2 + CCl_4; and Group 4, rAAV2/8-ACE2 + CCl_4 + rapamycin (mTOR inhibitor). Rapamycin (2 mg/kg) was administered at the 6th week after the intraperitoneal injection of CCl_4.

The mice were dissected after anesthesia administration, and liver tissues were removed and partially stored at -80 °C. Another section was fixed in 4% paraformaldehyde and embedded in paraffin.

Cytokine Enzyme Linked Immunosorbent Assay and Luminex analysis

Mouse blood samples were centrifuged at 4 °C (3000 rpm) for 10 min, and the supernatant was collected. According to the manufacturer's instructions, the serum levels of platelet-derived growth factor BB (PDGF-BB), angiopoietin-2, vascular endothelial growth factor (VEGF), interleukin (IL)-10, Ang II and Ang- (1-7) were measured using Luminex multifactor assay kits and Enzyme Linked Immunosorbent Assay kits. The data were analyzed using Graph Pad Prism 8.0.

Histopathological evaluation

The paraffin-embedded liver tissue sections were morphologically evaluated based on hematoxylin and eosin (H&E) staining. The degree of liver fibrosis in mice was measured by Masson trichrome and Sirius red staining. According to the METAVIR scale, the degree of liver fibrosis was divided into four stages from 0 to 4 (0 - No fibrosis; 1 - Portal fibrosis; 2 - Periportal fibrosis; 3 - Bridging fibrosis; 4 - Cirrhosis). The quantity of collagen production in each group after Sirius red staining was analyzed using Image-Pro Plus 6.0 software.

Immunohistochemical staining

Liver tissue sections were deparaffinized, serially dehydrated in ethanol, and then incubated overnight with primary antibody at 4 °C after antigen retrieval. The primary antibodies used in the experiment included anti-alpha-smooth muscle actin (α-SMA) antibody (1:400, Abcam, United States), anti-fibronectin (FN) antibody (1:2000, Abcam, United States), and anti-CD31 antibody (1:2000, Abcam, United States). After incubation with the appropriate biotinylated secondary antibody for 30 min, the liver sections were stained with diaminobenzidine and hematoxylin. The positive staining areas appeared brownish yellow. The sections were observed under a light microscope, photographed, and then analyzed with Image-Pro Plus 6.0 software.

Transmission electron microscopy

Fresh liver tissue sections were immobilized in electron microscopy fixative (Servicebio, Wuhan, China) for 2 h. The specimens were then immobilized in osmic acid buffer and dehydrated in ethanol. Finally, the ultrathin sections were photographed using Transmission electron microscopy (TEM) (HT7800/HT7700, Hitachi, Tokyo, Japan) after staining with 2% uranium acetate in alcohol solution. The structure of autophagosomes in each group was observed by TEM.

Apoptosis detection by TUNEL and immunofluorescence staining

Apoptotic HSCs were localized with labeled nucleotides in TUNEL staining. The mouse liver sections were stained according to the in situ cell death detection kit (Roche, Germany) protocol. The sections were then incubated with an α-SMA primary antibody (1:500, Abcam, United States) and a CY3 goat anti-rabbit fluorescence secondary antibody (1:300, Servicebio, Wuhan, China). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and photographed under a fluorescence microscope. The relative number of apoptotic cells in each group was analyzed using Image-Pro Plus 6.0 software.

Multicolor immunofluorescence staining

Paraffin sections of mouse liver tissue were deparaffinized, subjected to antigen retrieval, and blocked with hydrogen peroxide and serum. The primary antibody, corresponding HRP-labeled secondary antibody, and fluorescently labeled tyramine were successively added. After microwave repair treatment, the first round of primary and secondary antibodies were eluted, and the fluorescently labeled tyramine was still attached to the target. When the second and third targets were detected, the previous steps were repeated for a new round of labeling and microwave repair processing. The fourth primary antibody and 594-labeled fluorescent secondary antibody were added, and the nuclei were then counterstained with DAPI. The slides were covered with anti-fade mounting medium. Finally, images were detected and collected with a slice scanner (pannoramic, 3Dhistech, Hungary). DAPI emits blue light; Fluorescein isothiocyanate (FITC-ACE2) emits green light; 647 (Desmin) is set to pink light; 594 (LC3) is set to purplish red light. The number of positive cells for each index was analyzed using Image-Pro Plus 6.0 software.

Western blot analysis

Mouse liver tissue proteins were extracted, and the concentration of each protein was determined. Equal amounts of protein samples were subjected to electrophoresis on 8%-12% sodium dodecylsulphate polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% nonfat dry milk for 1 h to block nonspecific sites and then incubated with the appropriate primary antibodies at 4 °C overnight. After incubation with the secondary antibody and membrane washing, the antibody-bound proteins were detected by chemiluminescence staining using an enhanced chemiluminescence assay kit (Millipore, United States). The density of each band was analyzed with ImageJ software.

Statistical analysis

The data are expressed as mean ± SD. Statistics were analyzed using GraphPad Prism 8.0 and SPSS 19.0 software. Statistical significance was determined by one-way ANOVA followed by LSD-*t* test. For all experiments, $P < 0.05$ was considered statistically significant.

RESULTS

Effect of ACE2 on CCl_4-induced liver injury and fibrogenesis

The effect of ACE2 on CCl_4-induced liver fibrosis was evaluated by H&E (Figure 1A), Masson trichrome (Figure 1B) and Sirius red staining (Figure 1C and D). Compared with those in the control group, inflammatory cell infiltration and fibrous tissue hyperplasia in the liver tissues of mice were increased after 8 wk of CCl_4 injection (METAVIR > F2) (Figure 1A). In the CCl_4 group, the liver architecture was widely disorganized, and the hepatic sinusoids could not be distinguished. In addition, notable collagen deposition and the formation of fibrous septa bridging the portal regions were observed in the CCl_4 group. However, fibrotic tissue and inflammatory cells in the rAAV2/8-ACE2 + CCl_4 group were markedly reduced compared with those in the CCl_4 and rapamycin groups (METAVIR ≤ F2) (Figure 1B-D). Masson trichrome and Sirius red staining showed that collagen deposition was significantly increased in mice treated with CCl_4 alone ($P < 0.05$). After rAAV2/8-ACE2 treatment, the degree of collagen deposition in the perisinusoidal spaces, interlobular septum and periportal zones was reduced ($P < 0.05$) (Figure 1B-D). The results indicated that rAAV2/8-ACE2 treatment could improve liver injury and fibrosis in mice, while rapamycin treatment increased the degree of liver fibrosis compared with that in the ACE2 overexpression group. This finding suggested that mTOR inhibitor could attenuate the antifibrotic effect of rAAV2/8-ACE2.

Analysis of serum biomarkers in mice with liver fibrosis

The levels of PDGF-BB, VEGF, angiopoietin-2, IL-10, Ang II and Ang- (1-7) in mouse serum were measured (Figure 2). PDGF signaling plays a vital role in HSC activation and angiogenesis[19]. VEGF and angiopoietin-2 are the most important regulators in the process of angiogenesis[20,21]. As a potential anti-inflammatory factor, IL-10 has been reported to inhibit the expression of many proinflammatory mediators[22]. The results showed that the levels of PDGF-BB, angiopoietin-2, VEGF, and Ang II in the CCl_4 group were notably higher than those in the normal control group ($P <$

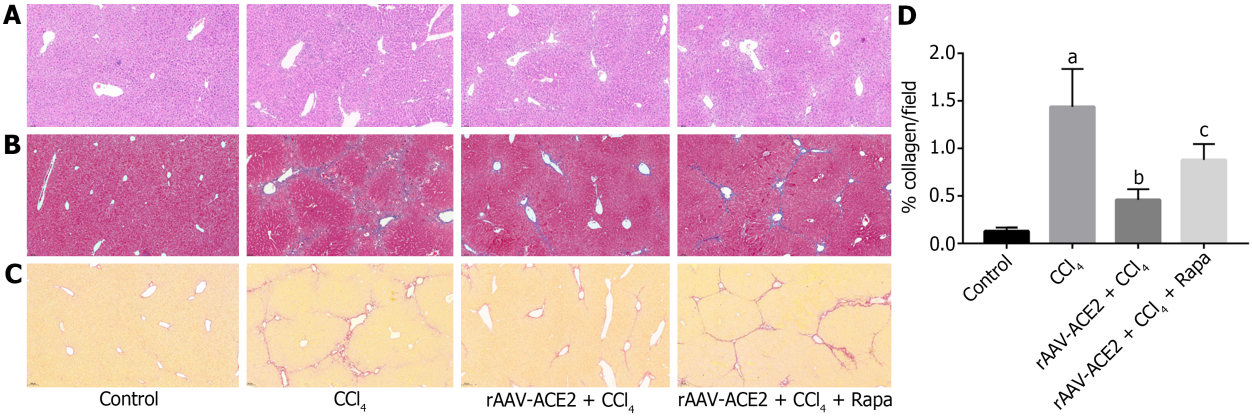

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Figure 1 Pathological changes in mouse liver tissues after rAAV-Angiotensin-converting enzyme 2 treatment (magnification × 100). A: Effect of rAAV-ACE2 treatment on liver fibrosis in mice was assessed by Hematoxylin and eosin staining; B: Effect of rAAV-ACE2 treatment on liver fibrosis in mice was assessed by Masson trichrome staining; C: Effect of rAAV-ACE2 treatment on liver fibrosis in mice was assessed by Sirius red staining; D: The quantity of collagen production in each group was analyzed using Image-Pro Plus 6.0. $^{a}P < 0.01$ *vs* Control; $^{b}P < 0.05$ *vs* CCl_4; $^{c}P < 0.05$ *vs* rAAV-ACE2 + CCl_4. Rapa: Rapamycin; ACE2: Angiotensin-converting enzyme 2.

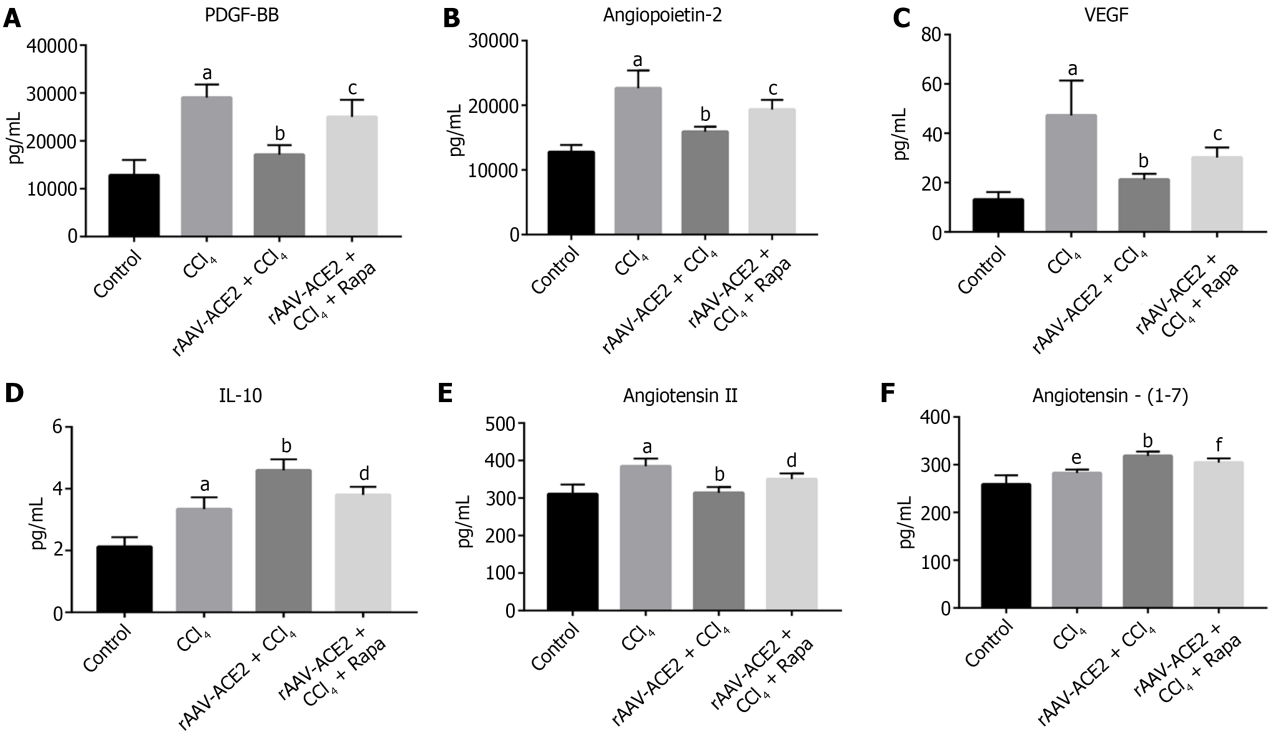

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Figure 2 Effect of rAAV-Angiotensin-converting enzyme 2 treatment on the levels of multiple biomarkers in mouse serum. A: The level of platelet-derived growth factor BB in each group was measured by Luminex assay; B: The level of angiopoietin-2 in each group was measured by Luminex assay; C: The level of vascular endothelial growth factor in each group was measured by Luminex assay; D: The level of interleukin-10 in each group was measured by Luminex assay; E: The level of angiotensin II in each group was measured by Enzyme Linked Immunosorbent Assay; F: The level of angiotensin- (1-7) in each group was measured by Enzyme Linked Immunosorbent Assay. $^{a}P < 0.001$ *vs* Control; $^{b}P < 0.001$ *vs* CCl_4; $^{c}P < 0.001$ *vs* rAAV-ACE2 + CCl_4; $^{d}P < 0.01$ *vs* rAAV-ACE2 + CCl_4; $^{e}P < 0.01$ *vs* Control; $^{f}P < 0.05$ *vs* rAAV-ACE2 + CCl_4. Rapa: Rapamycin; PDGF-BB: Platelet-derived growth factor BB; VEGF: Vascular endothelial growth factor; IL-10: Interleukin-10; ACE2: Angiotensin-converting enzyme 2.

0.001), and rAAV2/8-ACE2 injection reduced the expression of these cytokines ($P < 0.001$) (Figure 2A-C and E). In addition, the results demonstrated that the levels of IL-10 and Ang- (1-7) were higher in the rAAV2/8-ACE2 group than in the CCl_4 group ($P < 0.001$, $P < 0.001$), while rapamycin decreased the expression levels of these two cytokines ($P < 0.01$, $P < 0.05$) (Figure 2D and F). The results indicated that rAAV2/8-ACE2 treatment could inhibit HSC activation and angiogenesis in mice with liver fibrosis.

Effect of ACE2 on HSC activation and apoptosis

Effect of rAAV-ACE2 treatment on the expression of α-SMA, FN and CD31 in CCl_4-induced fibrotic mice was evaluated by immunohistochemistry staining (Figure 3A-C). α-SMA is a typical marker of HSC activation and proliferation. In the immunohistochemistry staining, α-SMA positive cells were distributed along the endothelium of hepatic sinusoids in the liver tissues of CCl_4-induced fibrotic mice. The number of α-SMA positive cells in the rAAV2/8-ACE2 treatment group was notably lower than that in the CCl_4 and rapamycin groups ($P < 0.05$, $P < 0.01$) (Figure 3A and D).

FN is the primary protein constituting the basement membrane, and CD31 is commonly used as a vascular endothelial marker. These proteins are rarely expressed in normal liver tissues. Immunohistochemical staining revealed that the expression of these proteins was increased in the CCl_4-induced liver fibrosis group ($P < 0.001$, $P < 0.001$) and decreased in the rAAV2/8-ACE2 treatment group ($P < 0.001$, $P < 0.001$). However, rapamycin increased the protein expression of FN and CD31 ($P < 0.01$, $P < 0.01$) (Figure 3B, C, E and F).

TUNEL and immunofluorescence staining were used to detect the number of apoptotic HSCs. Our results demonstrated that there were more apoptotic HSCs in the rAAV2/8-ACE2 treatment group than in the CCl_4 and rapamycin groups ($P < 0.05$, $P < 0.05$) (Figure 4).

The results showed that ACE2 overexpression inhibited HSC activation and induced HSC apoptosis in fibrotic mouse liver tissues, while the mTOR inhibitor attenuated the effect of rAAV2/8-ACE2 on HSCs.

Effect of ACE2 on HSC autophagy in mice

To further verify the effect of ACE2 on autophagy in liver fibrosis, a large number of autophagosomes were detected by ultrastructural analysis in the HSCs of mice in the CCl_4 group. TEM analysis showed that rAAV2/8-ACE2 injection decreased the number of autophagosomes in HSCs compared with that in the CCl_4 group ($P < 0.05$). However, autophagosomes were increased in the rAAV2/8-ACE2 + CCl_4 + rapamycin group ($P < 0.01$) (Figure 5A and B). The results of multicolor immunofluorescence staining demonstrated that the expression of the ACE2 protein was increased after rAAV2/8-ACE2 injection, and the expression of the autophagy protein LC3 was decreased compared with that in the CCl_4 group ($P < 0.01$). Treatment with rapamycin attenuated the inhibitory effect of ACE2 on LC3 protein expression ($P < 0.05$) (Figure 5C and D). These results suggested that ACE2 overexpression could reduce HSC activation and liver fibrosis by inhibiting HSC autophagy.

Effect of ACE2 on HSC autophagy and AMPK pathway proteins in mouse liver tissues

Autophagy is regulated by numerous autophagy-related genes, such as LC3 and Beclin-1. LC3II is a marker protein on the autophagosome membrane and is often considered an indicator of autophagy formation. As an autophagy-specific substrate, p62 interacts with LC3 to infiltrate into autophagosomes and is efficiently degraded by autophagolysosomes [23]. To determine the effect of ACE2 on HSC autophagy, we detected the expression of HSC autophagy-related indicators (LC3I, LC3II, Beclin-1) in the liver tissues of mice in each group by western blotting. Moreover, we verified the correlation of ACE2 with autophagy and the AMPK pathway by assessing the expression of AMPK pathway-related proteins (AMPK, p-AMPK, p-mTOR) and autophagy-related proteins (LC3I, LC3II, Beclin-1) in mouse liver tissues (Figure 6A and B). Compared with that in the control group, the p-AMPK/AMPK ratio was higher in the CCl_4 group ($P < 0.01$). However, the ratio of p-AMPK/AMPK in the rAAV2/8-ACE2-treated group was dramatically lower than that in the CCl_4 group ($P < 0.05$) (Figure 6A and C). In contrast, p-mTOR levels in mice in the rAAV2/8-ACE2-treated group were significantly higher than those in the CCl_4 group ($P < 0.01$) (Figure 6A and D). In addition, the results indicated that the protein levels of Beclin-1 and LC3II in the rAAV2/8-ACE2 + CCl_4 group were markedly reduced compared to those in the CCl_4 alone group ($P < 0.001$, $P < 0.05$) (Figure 6B, E and F). The m-TOR inhibitor (rapamycin) affected mTOR phosphorylation and the level of autophagy proteins in liver tissues. The present study showed that rapamycin abolished the effect of rAAV2/8-ACE2 on the expression of the autophagy proteins LC3I, LC3II and Beclin-1. Compared with those in the rAAV2/8-ACE2 group, the relative Beclin-1 and LC3II levels were increased by rapamycin treatment ($P < 0.05$, $P < 0.05$) (Figure 6B, E and F). The western blot results showed that ACE2 overexpression could inhibit the expression of autophagy-related proteins in mouse liver tissues through the AMPK/mTOR pathway.

DISCUSSION

Liver fibrosis has high morbidity and mortality worldwide, and it is a compensatory response to liver inflammation and injury caused by multiple pathogenic factors[24]. In liver fibrosis, excess fibrous ECM proteins, such as collagens I and III, are deposited in the Disse space of the hepatic sinusoids[25]. Changes in ECM composition induce LSECs to lose their fenestrae and form a basement membrane, a process known as hepatic sinusoidal capillarization[26]. The activation of HSCs plays a crucial role in the process of liver fibrosis. Upon activation due to liver injury, quiescent HSCs lose their retinoid droplets, exhibit increased α-SMA expression, and release large amounts of ECM, ultimately resulting in liver fibrosis[27].

ACE2 is expressed in human alveolar epithelial cells, esophageal epithelial cells, small intestinal epithelial cells, and vascular endothelial cells[28]. Our present study found that ACE2 was also expressed in liver HSCs. In recent years, the coronavirus disease 2019 (COVID-19) virus [severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)] that caused the outbreak has been proven to invade human alveolar epithelial cells mainly through ACE2[29]. SARS-CoV-2 infection can reduce ACE2 activity, leading to an imbalance in Ang II/ACE2 regulation[30]. ACE2, which is the main target receptor for SARS-CoV-2 invasion into the human body, is currently a research hotspot. A global registry study suggested that patients with chronic liver disease and cirrhosis had higher mortality after being infected with COVID-19[31]. The

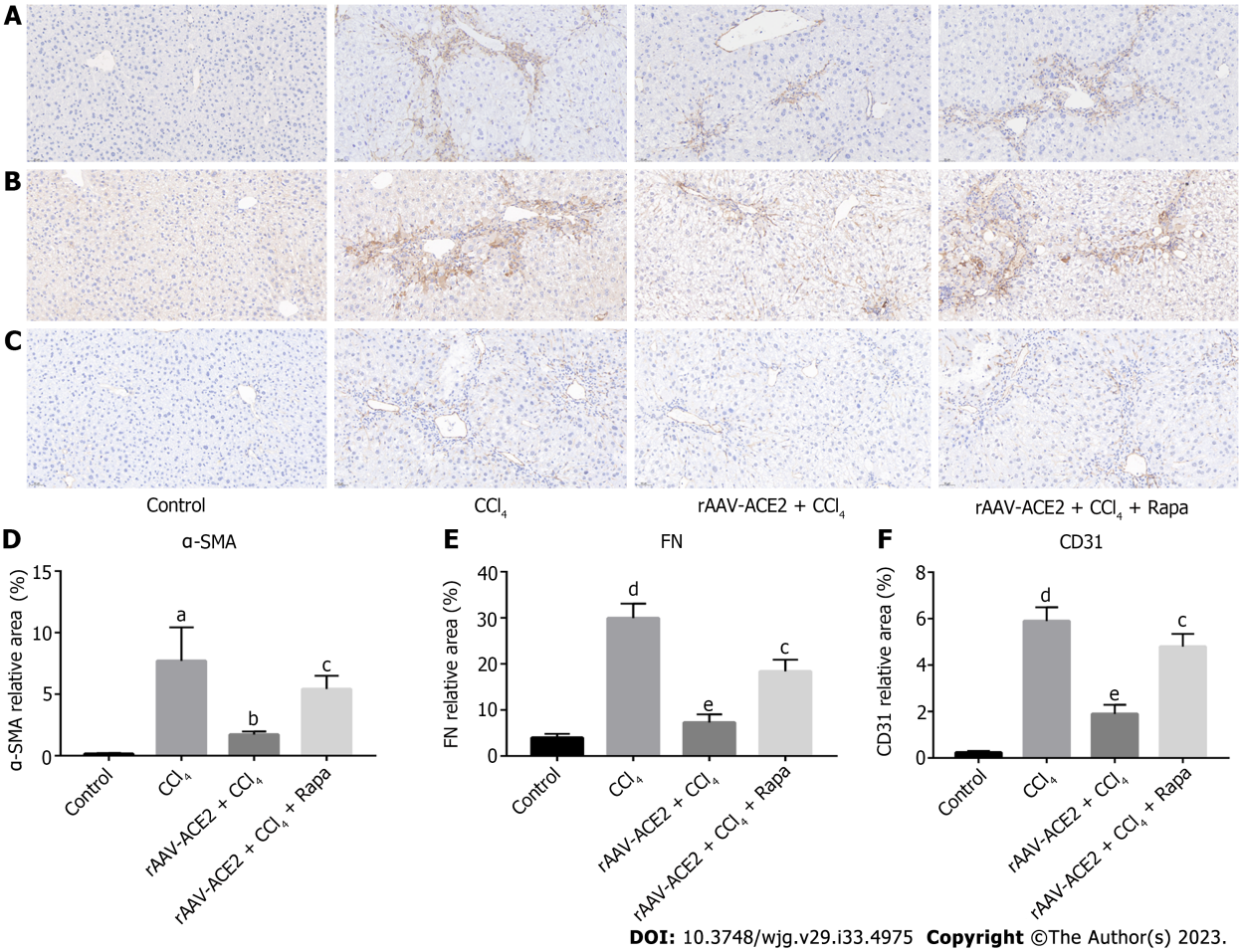

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Figure 3 Effect of rAAV-Angiotensin-converting enzyme 2 treatment on the expression of Alpha-smooth muscle actin, Fibronectin and CD31 in CCl_4-induced fibrotic mice was evaluated by immunohistochemistry staining (magnification × 200). A: The expression level of Alpha-smooth muscle actin in the liver tissues of each group was detected; B: The expression level of Fibronectin in the liver tissues of each group was detected; C: The expression level of CD31 in the liver tissues of each group was detected; D-F: The relative expression levels of the three proteins in each group were analyzed using Image-Pro Plus 6.0. $^{a}P < 0.01$ *vs* Control; $^{b}P < 0.05$ *vs* CCl_4; $^{c}P < 0.01$ *vs* rAAV-ACE2 + CCl_4; $^{d}P < 0.001$ *vs* Control; $^{e}P < 0.001$ *vs* CCl_4. Rapa: Rapamycin; α-SMA: Alpha-smooth muscle actin; FN: Fibronectin; ACE2: Angiotensin-converting enzyme 2.

baseline liver disease severity of patients with chronic liver disease and cirrhosis is closely related to the COVID-19-related incidence rates and mortality. Therefore, SARS-CoV-2 infection may exacerbate the degree of cirrhosis and portal hypertension in patients with chronic liver disease by reducing the activity of ACE2 in the liver.

ACE2 is an endogenous negative regulator that acts as a RAS "brake" to limit fibrogenesis through Ang II degradation and Ang- (1-7) formation. It was reported that the degree of liver fibrosis in ACE2 knockout mice increased after 21 d of bile duct ligation or chronic CCl_4 treatment[13]. In addition to its effect on the RAS, whether ACE2 can affect liver fibrosis through other mechanisms remains unclear. In our study, a liver fibrosis model was induced by the intraperitoneal injection of CCl_4 to investigate the effect of ACE2 on liver fibrosis by inhibiting autophagy. In addition, the liver-specific recombinant adeno-associated viral vector rAAV2/8-ACE2 was used in this study. ACE2 is specifically overexpressed in the liver with minimal systemic effects. Moreover, enhanced expression and activity of liver tissue-specific ACE2 can reduce local Ang II levels, increase local Ang- (1-7) levels, and minimize off-target effects[32].

Autophagy is the process of degrading defective proteins, damaged organelles, excess lipids and other harmful components in cells to maintain cellular components and homeostasis[33]. Autophagy levels are elevated in conditions of inflammation and oxidative stress, and excessive autophagy is involved in inflammatory and liver diseases[24]. Studies have demonstrated that inhibiting autophagy in HSCs reduces lipid droplet degradation, thereby preventing cell activation[9]. Autophagosome is composed of a small portion of the cytoplasm surrounded by double membranes. The digested substances are various components contained in the cytoplasm, such as mitochondria and fragments of endoplasmic reticulum, and the contents are degraded by fusion with lysosomes. Autophagy generally refers to macroautophagy, which includes two consecutive stages of autophagosome formation and degradation[15]. It has been reported that IL-10 inhibits oxidative stress-induced HSC autophagosome formation, HSC activation and liver fibrosis through the mTOR/STAT3 signaling pathway[34]. The TEM results indicated that the number of autophagosomes in the rAAV2/8-ACE2-treated group was decreased. To explore the relationship between ACE2 and autophagy, we detected the expression of the autophagy proteins LC3I, LC3II and Beclin-1 in the liver tissues of mice in each group. The results indicated that ACE2 overexpression effectively inhibited autophagy during mouse liver fibrosis.

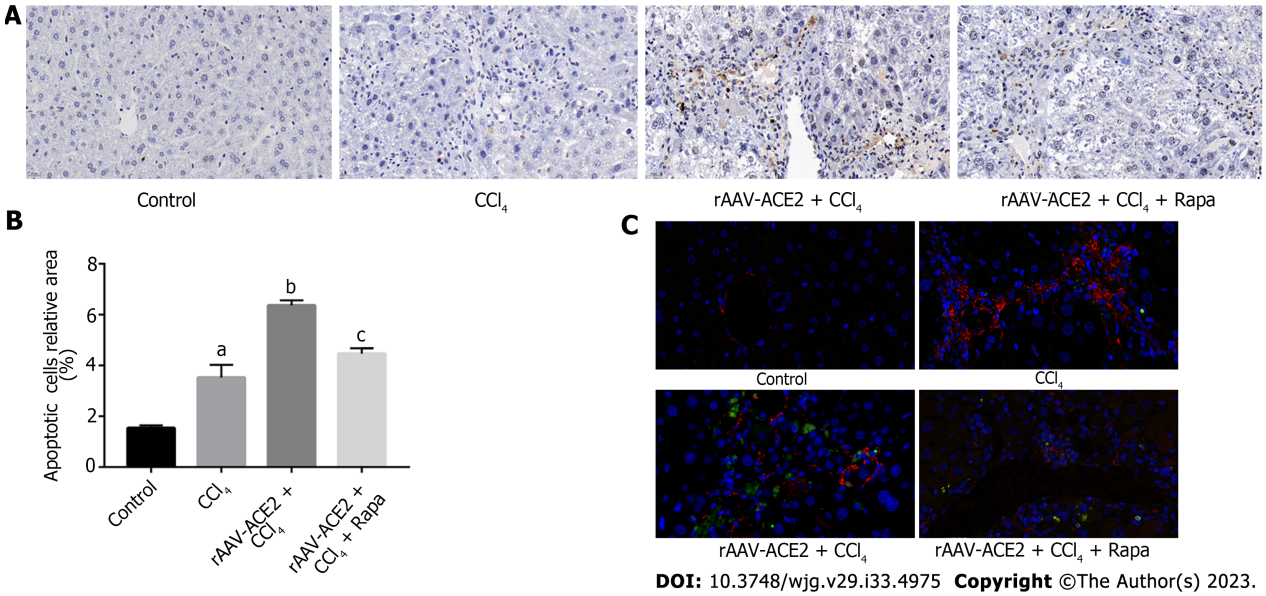

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Figure 4 Effect of rAAV-Angiotensin-converting enzyme 2 treatment on hepatic stellate cell apoptosis. A: Transferase-mediated dUTP nick-end labeling (TUNEL) staining (magnification × 400); B: The relative number of apoptotic cells in each group was analyzed using Image-Pro Plus 6.0. $^{a}P < 0.05$ *vs* Control; $^{b}P < 0.05$ *vs* CCl_4; $^{c}P < 0.05$ *vs* rAAV-ACE2 + CCl_4; C: TUNEL and Alpha-smooth muscle actin (α-SMA) immunofluorescence co-localization staining (magnification × 630). The nuclei stained by DAPI were blue, FITC luciferin-labeled apoptotic cells were green, and α-SMA displayed red. Rapa: Rapamycin; ACE2: Angiotensin-converting enzyme 2; DAPI: 4',6-diamidino-2-phenylindole; FITC: Fluorescein isothiocyanate.

Autophagy regulation is intricately associated with signaling pathways such as the AMPK/mTOR pathway. AMPK can inhibit mTORC1 activity by activating the TSC1/TSC2 protein heterodimer[35,36]. mTORC1 negatively regulates the initiation of autophagy through phosphorylation at Ser757 of ULK1 upon activation[36]. Compared with that in the CCl_4 group, the p-AMPK/AMPK ratio was decreased ($P < 0.05$), while the relative expression of p-mTOR was increased in the rAAV2/8-ACE2 group ($P < 0.01$). The results showed that ACE2 overexpression could influence the AMPK/mTOR signaling pathway. We treated mice with an m-TOR inhibitor (rapamycin), which effectively inhibited m-TOR phosphorylation. The findings of the study indicated that ACE2 overexpression could inhibit HSC autophagy in mouse liver tissues through the AMPK/mTOR pathway. The results suggested that the AMPK/mTOR signaling pathway was an important node for ACE2 to regulate HSC autophagy.

Pathological staining showed the successful establishment of a mouse model of liver fibrosis after 8 wk of intraperitoneal injection of CCl_4. rAAV2/8-ACE2 injection alleviated collagen deposition and fibrosis in the liver tissues of mice. We further investigated the mechanism by which ACE2 overexpression alleviated liver fibrosis. When liver injury occurs, HSCs are activated and proliferate, and the demand for intracellular energy increases. At this time, blocking autophagy can impair HSC activation and fibrotic activity[10]. α-SMA is an important indicator for evaluating HSC activation and proliferation. In the present study, rAAV2/8-ACE2 injection inhibited α-SMA expression and HSC activation. In addition, apoptosis plays a vital role in the proliferation, differentiation and death of HSCs, and HSC apoptosis is the key to reversing liver fibrosis[37]. TUNEL and immunofluorescence staining showed that rAAV2/8-ACE2 injection increased HSC apoptosis. Our previous study demonstrated a complex relationship between autophagy and apoptosis, and the inhibition of autophagy could induce HSC apoptosis[15]. Therefore, our findings indicated that ACE2 overexpression could alleviate liver fibrosis by regulating autophagy to inhibit HSC activation and promote apoptosis.

Intrahepatic angiogenesis and sinusoidal remodeling play an important role in the development of hepatic fibrosis and portal hypertension[38]. The inhibition of pathological angiogenesis can alleviate liver fibrosis. LSEC capillarization is associated with the accumulation of interstitial collagen in the Disse space of hepatic sinusoids and is the main pathological change in liver fibrosis[26]. The reversal of LSEC capillarization has been reported to promote HSC quiescence[39]. During cirrhosis, angiogenesis-related cytokines and receptors expressed in HSCs, such as VEGF, PDGF, and angiopoietin, can induce HSC migration, angiogenesis, and collagen production[40]. Our study demonstrated that the levels of VEGF, angiopoietin-2 and PDGF-BB were elevated in liver fibrosis, resulting in increased angiogenesis. rAAV2/8-ACE2 injection inhibited the expression levels of these angiogenesis-related factors. Therefore, the results indicated that ACE2 overexpression could effectively attenuate intrahepatic angiogenesis, thus alleviating hepatic sinusoidal resistance.

In the present study, adeno-associated viral vector technology, pathological staining, multifactor analysis, multicolor immunofluorescence staining, TEM and other advanced techniques were used to comprehensively explore the relationship and mechanism among ACE2, autophagy and liver fibrosis. However, there are still some limitations of this study. Whether ACE2 affects HSC autophagy and liver fibrosis through other pathways needs to be further explored. This study provides a new theoretical basis for the targeted treatment of liver fibrosis and portal hypertension, and its clinical application needs further research.

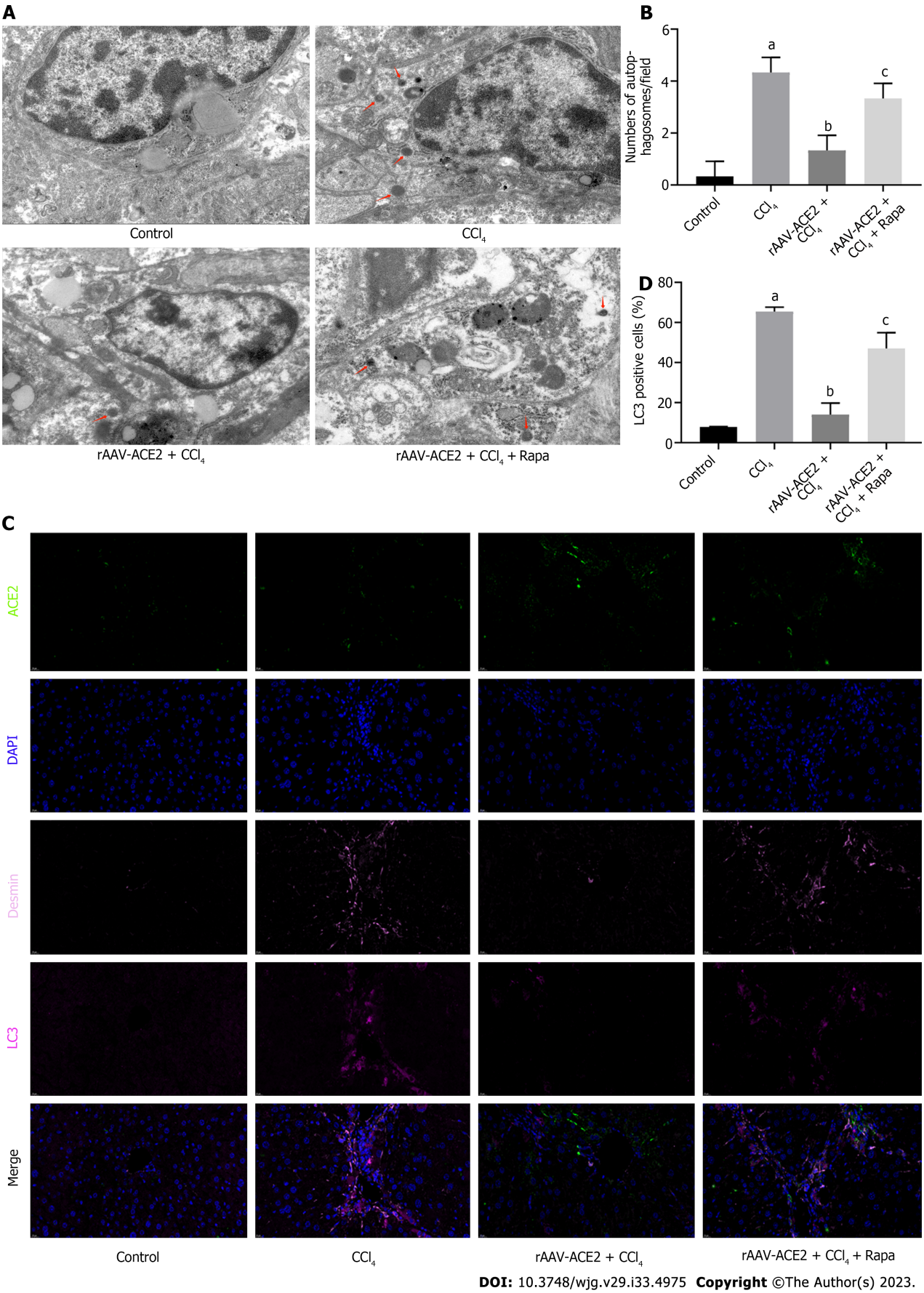

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Figure 5 Effect of rAAV-Angiotensin-converting enzyme 2 administration on hepatic stellate cell autophagy in mouse liver tissues. A: Effect of rAAV-ACE2 administration on hepatic stellate cell autophagosome formation in CCl_4-induced fibrotic mice was observed by Transmission electron microscopy (magnification × 7000). As shown, red arrows indicated autophagosomes; B: The number of autophagosomes in each group was quantified. $^aP < 0.01$ *vs*

Control; [b]*P* < 0.05 *vs* CCl_4; [c]*P* < 0.01 *vs* rAAV-ACE2 + CCl_4; C: Effect of rAAV-ACE2 administration on the expression of autophagy protein LC3. Multicolor immunofluorescence staining for ACE2 (green), autophagic LC3 (Purplish red), desmin (pink), nuclear DAPI (blue), and merged signals in mouse liver tissues (magnification × 400); D: The number of LC3 positive cells in each group was analyzed using Image-Pro Plus 6.0 software. [a]*P* < 0.001 *vs* Control; [b]*P* < 0.01 *vs* CCl_4; [c] *P* < 0.05 *vs* rAAV-ACE2 + CCl_4. Rapa: Rapamycin; DAPI: 4',6-diamidino-2-phenylindole; ACE2: Angiotensin-converting enzyme 2.

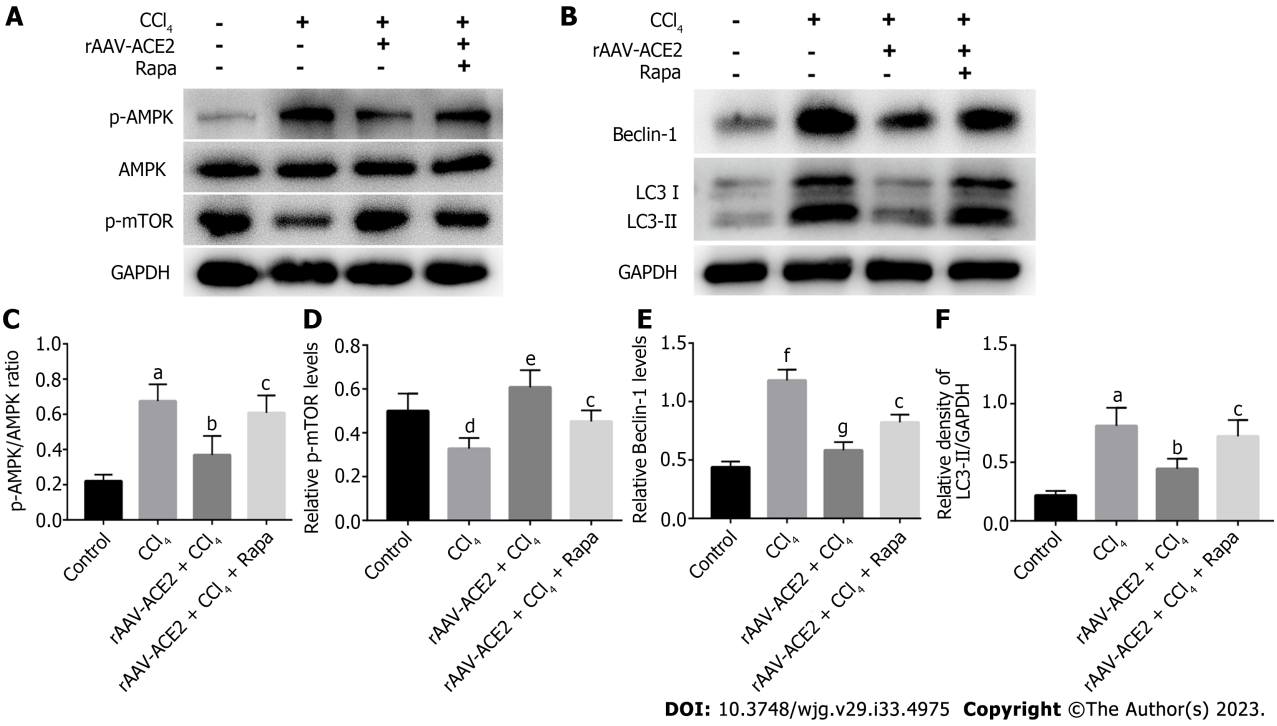

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Figure 6 Effect of rAAV-Angiotensin-converting enzyme 2 treatment on the expression of adenosine monophosphate activates protein kinases signaling pathway proteins and autophagy-related proteins in mouse liver tissues. A: The expression levels of p-AMPK, AMPK and p-mTOR were detected by western blotting; B: The expression levels of autophagy markers Beclin-1, LC3I and LC3II were detected by western blotting; C-F: The relative protein levels in each group were analyzed using ImageJ. [a]*P* < 0.01 *vs* Control; [b]*P* < 0.05 *vs* CCl_4; [c]*P* < 0.05 *vs* rAAV-ACE2 + CCl_4; [d]*P* < 0.05 *vs* Control; [e]*P* < 0.01 *vs* CCl_4; [f]*P* < 0.001 *vs* Control; [g]*P* < 0.001 *vs* CCl_4. Rapa: Rapamycin; p-mTOR: p-mammalian target of rapamycin; AMPK: Adenosine monophosphate activates protein kinases; ACE2: Angiotensin-converting enzyme 2.

CONCLUSION

In summary, the study indicates that autophagy plays a crucial role in HSC activation and liver fibrosis. ACE2 overexpression can inhibit HSC activation and promote apoptosis by regulating HSC autophagy, thereby alleviating liver fibrosis and hepatic sinusoidal remodeling. Our study also demonstrates that the AMPK/mTOR pathway is involved in the effect of ACE2 on autophagy. This study may provide new ideas for exploring the molecular mechanism by which ACE2 inhibits liver fibrosis and hepatic sinusoidal remodeling.

ARTICLE HIGHLIGHTS

Research background

Liver cirrhosis is a hallmark of end-stage chronic liver disease, which leads to millions of deaths each year. At present, the treatment options for liver fibrosis and cirrhosis are limited and often ineffective. Angiotensin-converting enzyme 2 (ACE2)-driven protective renin-angiotensin system (RAS) provides an effective therapeutic target for liver fibrosis. In addition, the study of liver fibrosis targeting hepatic stellate cell (HSC) autophagy has attracted more and more attention.

Research motivation

In addition to its effect on the RAS, whether ACE2 can affect liver fibrosis through other mechanisms remains unclear. Moreover, how to enhance the expression and activity of tissue-specific ACE2 to avoid its potential off-target effect is a problem to be solved. Using a suitable and efficient gene delivery system to achieve tissue-specific overexpression of ACE2 has pointed out a new direction for the targeted treatment of liver fibrosis.

Research objectives
The aim of this study is to determine the effect of ACE2 on HSC activation, proliferation, apoptosis and liver fibrosis by regulating autophagy. This study provides new ideas for exploring the molecular mechanism by which ACE2 inhibits liver fibrosis and hepatic sinusoidal remodeling.

Research methods
In this study, a mouse model of liver fibrosis was constructed, and adeno-associated viral vector technology, pathological staining, multifactor analysis, multicolor immunofluorescence staining, transmission electron microscopy, TUNEL apoptosis assays, western blot analysis and other experimental methods were used to comprehensively explore the relationship and mechanism among ACE2, autophagy and liver fibrosis.

Research results
In vivo experiments showed that rAAV2/8-ACE2 treatment could inhibit HSC activation and angiogenesis, induce HSC apoptosis, and alleviate HSC proliferation and liver fibrosis by inhibiting HSC autophagy. This study also demonstrated that ACE2 overexpression could inhibit HSC autophagy in mouse liver tissues through the Adenosine monophosphate activates protein kinases (AMPK)/mammalian target of rapamycin (mTOR) pathway. The completion of this study provides new ideas for the prevention and targeted treatment of liver fibrosis and portal hypertension.

Research conclusions
The study demonstrates that autophagy plays a crucial role in HSC activation and liver fibrosis. ACE2 overexpression can inhibit HSC activation and promote apoptosis by regulating HSC autophagy through the AMPK/mTOR pathway, thereby alleviating liver fibrosis and hepatic sinusoidal remodeling.

Research perspectives
The pathogenesis of liver fibrosis and cirrhosis is a complex process involving the interaction of various growth factors, cytokines, and vasoactive substances. We need further clinical research to improve patient treatment outcomes through advanced technologies such as drug carrier-targeted HSC-specific therapies.

FOOTNOTES

Author contributions: Wu Y, Xu WH, and Zhang CQ designed and coordinated the study; Wu Y, Sun JT, and Yin AH performed the experiments, acquired and analyzed data; Xu WH and Zhang CQ interpreted the data; Wu Y wrote the manuscript; All authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Diabetes exacerbates inflammatory bowel disease in mice with diet-induced obesity

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Abstract

BACKGROUND

The increased prevalence of inflammatory bowel disease (IBD) among patients with obesity and type 2 diabetes suggests a causal link between these diseases, potentially involving the effect of hyperglycemia to disrupt intestinal barrier integrity.

AIM

To investigate whether the deleterious impact of diabetes on the intestinal barrier is associated with increased IBD severity in a murine model of colitis in mice with and without diet-induced obesity.

METHODS

Mice were fed chow or a high-fat diet and subsequently received streptozotocin to induce diabetic-range hyperglycemia. Six weeks later, dextran sodium sulfate was given to induce colitis. In select experiments, a subset of diabetic mice was treated with the antidiabetic drug dapagliflozin prior to colitis onset. Endpoints included both clinical and histological measures of colitis activity as well as histochemical markers of colonic epithelial barrier integrity.

RESULTS
In mice given a high-fat diet, but not chow-fed animals, diabetes was associated with significantly increased clinical colitis activity and histopathologic markers of disease severity. Diabetes was also associated with a decrease in key components that regulate colonic epithelial barrier integrity (colonic mucin layer content and epithelial tight junction proteins) in diet-induced obese mice. Each of these effects of diabetes in diet-induced obese mice was ameliorated by restoring normoglycemia.

CONCLUSION
In obese mice, diabetes worsened clinical and pathologic outcomes of colitis *via* mechanisms that are reversible with treatment of hyperglycemia. Hyperglycemia-induced intestinal barrier dysfunction offers a plausible mechanism linking diabetes to increased colitis severity. These findings suggest that effective diabetes management may decrease the clinical severity of IBD.

Key Words: Inflammatory bowel disease; Type 2 diabetes; Obesity; Intestinal barrier; Hyperglycemia; Colitis in mice; Tight junction proteins

Core Tip: Metabolic syndrome affects many patients with inflammatory bowel disease (IBD). This study used mouse models of colitis to investigate how diabetes and obesity interact to impair intestinal barrier function and exacerbate IBD outcomes, highlighting the deleterious impact of sustained hyperglycemia on intestinal barrier integrity. We showed that diabetic hyperglycemia impairs the colonic mucin barrier and tight junction protein abundance in the setting of diet-induced obesity, which corresponds to worse clinical and histopathological IBD outcomes. These findings are important because as more patients with IBD are affected by obesity and/or diabetes, it is imperative to understand how these disease processes interact.

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), and type 2 diabetes (T2D) are among the most challenging and costly medical disorders in modern society. Each is a chronic condition with no permanent medical cure that is increasing in prevalence globally and is associated with significant patient morbidity and economic cost[1,2]. IBD is an autoimmune condition affecting the gastrointestinal tract. The pathogenesis of IBD is multifactorial, involving genetic predisposition, immunologic abnormalities, alterations in gut microbiota, and environmental factors, particularly exposure to a Western diet[3-5]. The pathogenesis of T2D involves insulin resistance and loss of pancreatic β cell function related to risk factors including obesity, visceral adiposity, and exposure to high-fat and high-sugar diets[6,7].

There is increasing evidence that these two disease processes may be linked. Recent national cohort studies have shown that patients with IBD are at increased risk of developing T2D, even after controlling for multiple risk factors including steroid exposure, age, and body mass index[8,9]. Furthermore, the development of T2D in patients with IBD is a predictor of poor disease-related outcomes, with several studies showing higher rates of IBD-related hospitalizations in patients with IBD[10,11] as well as disease flares in patients with either CD or UC and increased IBD-related surgeries in patients with CD[11].

Obesity also constitutes a potential link between T2D and IBD. T2D is strongly associated with obesity, with the majority of T2D patients being either overweight or obese[12,13]. Obesity also affects up to 40% of adult IBD patients, with an additional 20%-40% of patients being overweight[14]. Comorbid obesity in IBD patients has been associated with higher rates of surgical complications and more severe disease that may be less responsive to standard medical therapies [15,16]. Consumption of obesity-inducing high-fat diets (HFD) is a well-known risk factor for developing IBD[17-19], and in preclinical rodent studies, diet-induced obesity (DIO) caused by consuming a HFD worsens clinical and histological IBD outcomes[20,21]. However, the mechanisms underlying the relationship among DIO, T2D, and the development of IBD are unknown.

Notably, both IBD and metabolic syndrome (of which T2D and DIO comprise two of the principle components) are associated with an altered gut microbiome, chronic systemic inflammation, and intestinal barrier dysfunction[4,22,23]. Specifically, the role of increased gut permeability leading to enhanced influx of microbial products from the gut lumen into systemic circulation has been implicated in the pathogenesis of metabolic syndrome and its many complications[22]. Increased gut permeability is also associated with active IBD, as confocal laser endomicroscopic assessment of fluorescent leakage across the intestinal barrier is increased in symptomatic patients with IBD (both CD and UC) compared to

healthy controls and asymptomatic IBD patients[24].

Currently, the mechanisms that link intestinal barrier dysfunction in diabetes, obesity, and IBD remain poorly understood. Recent work indicates that diabetic hyperglycemia may drive intestinal barrier impairments resulting in increased risk for enteric infections[25]. However, the extent to which intestinal barrier dysfunction contributes to the increased risk of IBD complications in patients with comorbid T2D and what role DIO may play in this effect is unknown.

The current work was undertaken to determine the pathogenic role of diabetes to exacerbate intestinal inflammation in a mouse model of IBD with or without coexisting DIO, highlighting intestinal barrier disruption as a mechanism that links diabetes and IBD.

MATERIALS AND METHODS

Animals

Eight-week-old C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME, United States). All animals were group-housed under specific pathogen-free conditions in a temperature-controlled environment (14:10 h lights on/off cycle; lights on at 7:00 am) in cages containing a maximum of five animals. Mice were either fed standard laboratory chow (5053 PicoLab® Rodent Diet 20; LabDiet, St. Louis, MO, United States) throughout the study or placed on an HFD (D12492; Research Diets, New Brunswick, NJ, United States) at 8 wk of age for a period of 9 subsequent weeks to induce DIO. All procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Washington. Body weight (BW) and food intake were recorded at least twice weekly throughout the experiment.

Streptozotocin-induced diabetes

Mice were randomly chosen to be placed on a HFD for 9 wk to induce DIO (n = 30) or maintained on standard chow (n = 20) and then received five consecutive daily intraperitoneal injections of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, United States) at a low dose (40 mg/kg BW) to induce diabetes-range hyperglycemia (STZ-diabetes; random blood glucose levels ≥ 250 mg/dL) or sodium citrate vehicle control (Veh) at an equivalent dose (40 mg/kg)[26]. Mice were chosen randomly for the treatment groups, anesthetized with isoflurane during STZ injections, and placed immediately back into their home cage afterwards. Following STZ administration, random blood glucose levels were recorded at least twice weekly throughout the experiment.

Dextran sodium sulfate-induced colitis

Six weeks following STZ or Veh administration, 2% dextran sodium sulfate (DSS) (36-50 kDa; MP Biochemicals, Santa Ana, CA, United States) dissolved in autoclaved water or untreated autoclaved water (Veh) was provided in drinking water for 7 d, with 4-8 animals per experimental group. Animals were acclimated to the medicated water delivery system for 1 wk prior to DSS course in their home cage, with daily water and food intake recorded. To control for the observation that diabetic mice consumed more water leading up to the DSS course, paired water administration was performed during the DSS course. Disease activity index (DAI) scores were calculated daily based on the sum of the percentage of BW lost (0-4), degree of rectal bleeding (0-4), and consistency of stools (0-4)[27].

Diabetes treatment with a sodium-glucose cotransporter-2 inhibitor

A subset of C57BL/6J male mice (n = 23) was placed on an HFD to induce DIO and then received either STZ or sodium citrate Veh as described above. After 6 wk of sustained hyperglycemia, the sodium-glucose cotransporter-2 inhibitor (SGLT2i) dapagliflozin was added to the drinking water for randomly selected groups at a dose of 25 mg/kg based on ideal BW (0.03 kg) to ameliorate hyperglycemia for 3 wk in total[28]. During the last week, 2% DSS was added to drinking water for select experimental groups as detailed above. Results were compared to mice that received drinking water without dapagliflozin. There were 7-8 animals in each experimental group.

Tissue collection

Mice were sacrificed on day 7 of the DSS course by euthanasia with an anesthetic overdose. Blood, colon, small intestine, liver, mesenteric fat, spleen, and fecal samples were collected. The spleen weight was recorded. Sections of liver, spleen, and mesenteric fat were stored either fresh frozen at -80 °C or placed into 10% zinc-buffered formalin (ZBF) for fixation and histological processing. The entire colon was removed from the surrounding mesentery and excised, and the length was measured from the end of the cecum to the end of the rectum. The colon was flushed with 0.1 M phosphate-buffered saline (PBS). A small section of proximal colon was cut and flash frozen at -80 °C, and the remaining colon was fixed in ZBF for histological examination. The small intestine was divided in half and flushed with PBS. Pieces of the proximal and distal small bowel were flash frozen at -80 °C, and the remaining tissue was placed into ZBF. Tissue collection and assessment of intestinal barrier integrity were performed 7 wk after STZ administration, limiting any direct toxic effect of intraperitoneal injection of STZ on the colon.

Histopathology

After the 3 d fixation in ZBF, the intestines were cut longitudinally, swiss-rolled, pinned, and placed in 70% ethanol. Colon, proximal small bowel, and distal small bowel were paraffin embedded, sectioned at 4 μm thickness, deparaffinized, and stained with either hematoxylin and eosin or Alcian blue (AB) to stain mucins by the University of

Washington Diabetes Research Center Cellular and Molecular Imaging Core. Using a modified protocol from Wirtz *et al* [29], histopathologic scoring of the extent of tissue damage was performed by a blinded pathologist, taking into account distribution of tissue damage [0 = none, 1 = focal (less than 3 sites), 2 = moderate (3-5 sites), 3 = diffuse (> 5 sites)], severity of tissue damage (0 = none, 1 = isolated focal epithelial damage, 2 = mucosal erosions and ulcerations, 3 = extensive damage deep into the bowel wall), lamina propria inflammatory cell infiltration (0 = infrequent, 1 = increased, some neutrophils, 2 = submucosal presence of inflammatory cell clusters, 3 = transmural cell infiltrations), and presence of chronic or active inflammation (0 = none, 1 = active, 2 = chronic).

Brightfield microscopy of AB-stained colonic tissues was performed using the Keyence BZ-X800 microscope (Keyence Corp. of America, Itasca, IL, United States). Four random images at × 20 magnification were taken from each animal of intact colonic tissue, avoiding any ulceration of the epithelium, by a blinded independent investigator. Percent area of AB staining was performed using Fiji open source imaging software specific for AB imaging analysis (Color Deconvolution, Vector: AB & H, Color 1). Threshold limits were set (0, 207) and used to measure the percent area of staining across the region of interest selected (four representative crypts per image). This process was repeated for four distinct images per animal, and values were averaged per animal.

Immunofluorescence

Colon sections mounted on slides were deparaffinized with xylene, and antigen-retrieval in 10 mmol/L sodium citrate, pH 6.0 was performed. Sections were incubated in 0.1 M PBS followed by 0.2% Triton X-100 in PBS, then blocked in 2% donkey serum in 0.05% Triton X-100 at 37 °C for 1 h, and finally incubated overnight with rabbit anti-E-cadherin (24E10; Cell Signaling Technology, Danvers, MA, United States). Sections were washed, incubated for 2 h with Alexa 555-conjugated donkey anti-rabbit antibody, and then stained with DAPI.

Immunofluorescence images were captured using the Keyence BZ-X800 microscope, and four random images of intact colonic tissue at × 20 magnification were taken from each animal. Percent area of E-cadherin staining in the epithelium was performed using Fiji open source imaging software, building on prior published immunohistochemistry protein quantification methods[30]. Fluorescent images (E-cadherin: 555) were converted to 8-bit images, threshold staining limits were set (E-cadherin: 9, 255), and the region of interest outlining the entire epithelium captured in the × 20 image was selected. Percent area values were recorded for each image, repeated for a total of four images per animal, and then averaged.

Statistical analysis

Data from individual experiments including blood glucose, DAI, colon length, and spleen length data were shown as dot plots representing data from individual animals, and bar graphs represented mean ± standard error of the mean. AB and E-cadherin data were also presented as mean values ± standard error of the mean. Student's *t* test was used to compare the means in two groups, and one-way analysis of variance was used to compare multiple groups, using GraphPad Prism 5 (GraphPad software, La Jolla, CA, United States). Animals were not excluded from the study unless otherwise indicated. All differences were considered statistically significant at $P < 0.05$.

RESULTS

Effects of STZ-diabetes and DSS-induced colitis on BW, blood glucose, food intake, and water consumption in DIO mice

As a first step, we characterized the effects of STZ and DSS administration either alone or in combination on BW, blood glucose, food intake, and water intake in DIO mice. Mice were fed an HFD for 9 wk to induce DIO and then given either Veh or STZ to induce diabetes. After 6 wk, both STZ and Veh groups were subsequently exposed to either DSS or control drinking water (Veh), generating four study groups (Veh/Veh, STZ/Veh, Veh/DSS, and STZ/DSS). In terms of BW, all mice developed DIO after 9 wk of a HFD leading up to STZ administration, with no significant differences in BW between each group (mean BW pre-STZ: Veh/Veh 45.9 ± 3.9 g, Veh/STZ 45.4 ± 1.6 g, Veh/DSS 45.02 ± 5.0 g, STZ/DSS 44.4 ± 5.2 g; $P = 0.924$) (Figure 1A). Following STZ administration, BW decreased relative to Veh-treated controls but stabilized prior to DSS administration (Figure 1A). While BW was maintained in Veh-treated non-diabetic (Veh/Veh) and STZ-diabetic (STZ/Veh) mice, BW declined in both Veh/DSS and STZ/DSS groups throughout the 7-d DSS course but to a greater extent in STZ/DSS mice (Figure 1A and B).

As expected, STZ administration induced diabetes-range hyperglycemia across all groups relative to Veh-treated controls (30-d pre-DSS random blood glucose mean level: STZ 333 ± 31 mg/dL *vs* Veh 167 ± 10 mg/dL; $P < 0.0001$) (Figure 1C). Notably, there was a significant reduction in the blood glucose level during DSS course in the STZ/DSS group (Figure 1C and D), which was associated with a reduction in food and water intake during DSS administration (Figure 1E and F). This reduction in blood glucose during DSS course was less pronounced in non-diabetic DSS-treated mice (Veh/DSS, Figure 1C and D), as the decrease in food and water intake during the DSS course was similarly less significant in these mice (Figure 1E and F). Paired water administration between Veh/DSS-treated and STZ/DSS-treated groups ensured that hyperglycemic mice did not consume more DSS-treated water (Figure 1F).

Diabetic hyperglycemia worsens clinical and pathological outcomes of DSS colitis in DIO mice

We next determined the effects of STZ-hyperglycemia on clinical and histopathological outcomes of DSS colitis in DIO mice. Diabetic STZ/DSS mice had a more rapid onset of DSS colitis, preceding the onset of clinical colitis symptoms in

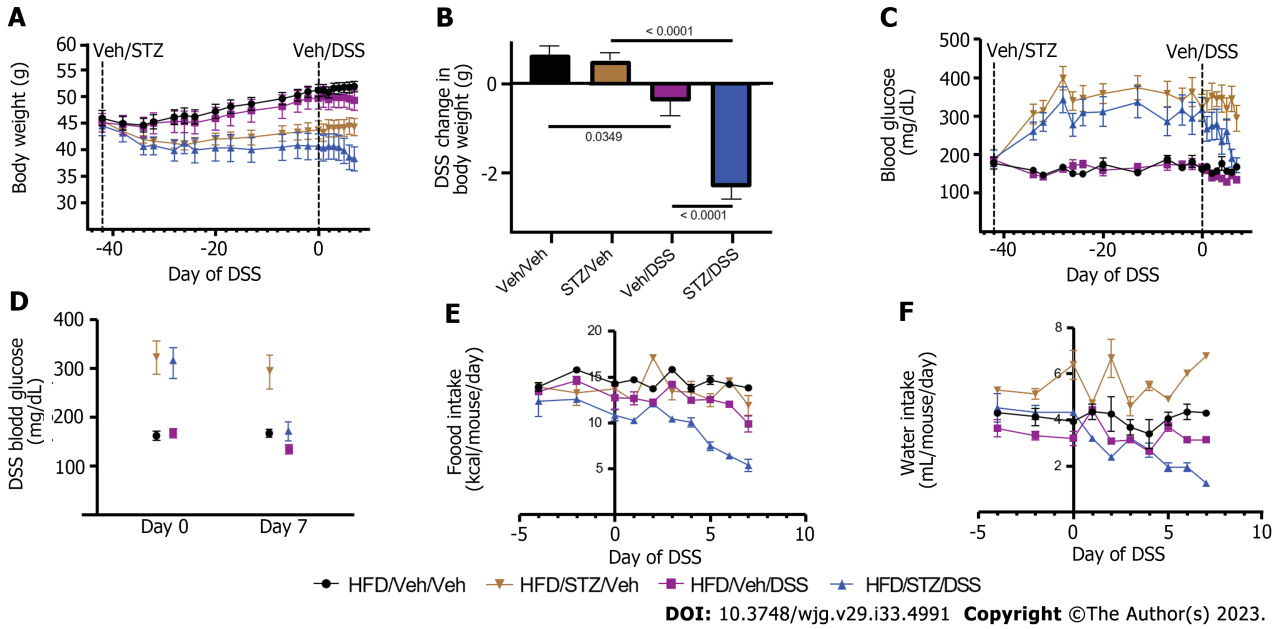

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Figure 1 Body weight, blood glucose, food intake, and water consumption in diabetic diet-induced obese mice. A: Body weight; B: Change in body weight during dextran sodium sulfate (DSS) course; C: Blood glucose; D: Change in blood glucose during the DSS course; E: Food intake; F: Water consumption in high-fat diet-fed, diet-induced obese mice that received either vehicle (Veh) or streptozotocin to induce hyperglycemia and subsequently received Veh or DSS in the drinking water for 7 d to induce colitis. *n* = 7-8 per group, mean ± standard error of the mean. DSS: Dextran sodium sulfate; Veh: Vehicle; STZ: Streptozotocin.

Veh/DSS mice by 2 d (day 2 *vs* day 4 following DSS) (Figure 2A and B), and their DAI scores were significantly higher throughout the entire course of DSS (from days 2-7; Figure 2A and B, $P < 0.05$ on days 2 and 5, $P < 0.0001$ on days 3, 4, 6, and 7), such that by the end of the DSS course (day 7), the mean DAI score was significantly higher in diabetic STZ/DSS mice compared to normoglycemic Veh/DSS controls (Figure 2B). As disease activity was undetectable in groups that did not receive DSS (Veh/Veh and STZ/Veh), we concluded that STZ-diabetes does not independently cause colitis symptoms (Figure 2A).

In murine models of IBD, colonic length serves as a pathologic marker of disease severity, as it shortens in response to mural inflammation in DSS colitis[20]. In STZ/DSS mice, the mean colon length at the time of sacrifice on day 7 was significantly shorter compared to Veh/DSS mice (Figure 2C). Spleen weight serves as another pathologic marker of disease severity in DSS colitis, as its weight increases in response to systemic inflammation[20]. Our finding that the spleen-to-BW ratio was significantly higher in STZ/DSS mice than Veh/DSS mice (Figure 2D) indicated greater systemic inflammation in the former group. Consistent with this interpretation, histologic damage scores assessing the degree of colonic inflammation and tissue injury were also significantly higher in STZ/DSS mice than in Veh/DSS mice (Figure 2E-G). Collectively, these findings indicated that in DIO mice, STZ-diabetes both hastens the onset of and worsens the clinical and histopathological severity of DSS-induced colitis.

Effect of hyperglycemia on the intestinal barrier in IBD in DIO mice

To investigate the mechanism by which STZ-hyperglycemia mitigates IBD outcomes in the setting of DIO, we examined the effect of STZ with and without DSS exposure on intestinal barrier function. The mucous layer of the colon and the tight junction proteins in the colonic epithelial layer are key components of the barrier that defend against pathogen entry into the bloodstream. The former is composed of mucins secreted by goblet cells in colonic crypts. By coating the colonic epithelial layer, it limits exposure of the epithelium to luminal contents[31,32]. When this mucous layer is thinned in disease states, its protective properties are diminished, placing the epithelium at greater risk of injury[31,32].

To assess the degree of mucous layer thinning, we stained postmortem colonic tissue for mucins and goblet cells with AB. Our findings showed that in DIO mice, hyperglycemia (STZ/Veh) was associated with significantly reduced AB staining compared to non-diabetic controls (Veh/Veh) (Figure 3A-C). We further showed that at the end of the DSS course in DSS-treated mice, STZ-diabetes was associated with significantly decreased colonic AB staining and sampled from areas of the colon with intact epithelium (Figure 3C). Therefore, diabetes was independently found to impair the colonic mucin barrier, with no significant effect of DSS alone when looking at areas of intact bowel without active colonic inflammation. Loss of the colonic mucin barrier, therefore, could play a causal role in the effect of diabetes to increase the rapidity of onset and severity of DSS colitis in the setting of DIO.

Tight junction proteins are an additional key component of the protective barrier of the colon, as they help to regulate the permeability of the epithelial layer[33]. Changes in the composition and concentration of tight junction proteins that occur in many disease states including IBD are associated with a more permeable epithelial barrier[33-35]. In the setting of DIO, we found that the abundance of the colonic epithelial tight junction protein E-cadherin, a key determinant of epithelial barrier integrity, was significantly decreased in hyperglycemic mice (STZ/Veh)[36] compared to non-diabetic

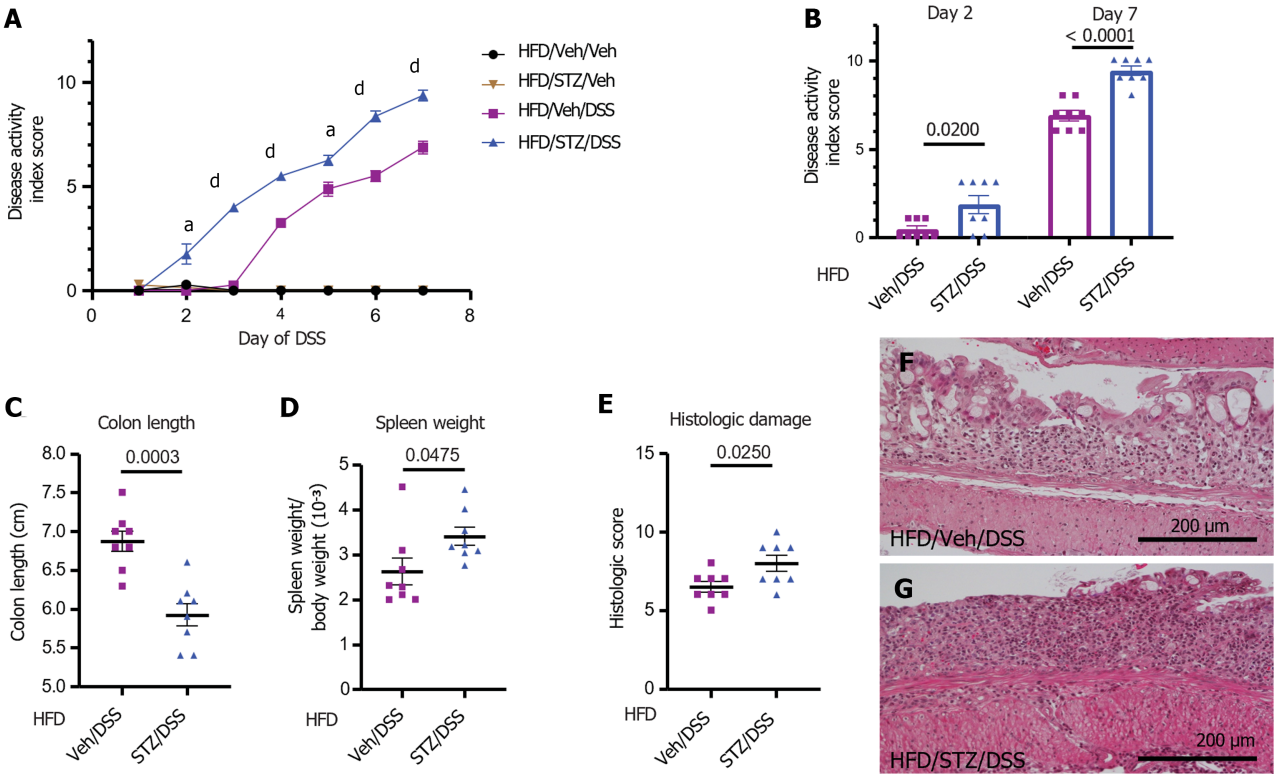

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Figure 2 Clinical and pathological outcomes of dextran sodium sulfate colitis in diabetic diet-induced obese mice. A: Clinical disease activity index scores in high-fat diet (HFD)-fed mice that received either vehicle (Veh) or streptozotocin (STZ) to induce diabetes prior to the dextran sodium sulfate (DSS) course; B: Quantification on days 2 and 7; C: Colon length; D: Spleen weight-to-body weight ratio on day 7 of DSS; E: Histologic scoring of DSS damage in colonic tissues from HFD-fed normoglycemic Veh and hyperglycemic STZ mice treated with DSS; F: Representative × 20 images of normoglycemic Veh mice; G: Hyperglycemic STZ mice. n = 7-8 per group, mean ± standard error of the mean. In (A), $^{a}P < 0.05$, $^{d}P < 0.0001$ and comparison noted in (A) is between HFD/Veh/DSS and HFD/STZ/DSS groups for days 2-7 of DSS. DSS: Dextran sodium sulfate; Veh: Vehicle; STZ: Streptozotocin; HFD: High-fat diet.

controls (Veh/Veh) (Figure 3D-F). In the presence of DSS exposure, STZ-diabetes was also associated with a significant decrease of colonic E-cadherin staining (Figure 3F) when superimposed on DIO. Combined with the decrease of the protective mucous layer noted above, these data suggest that epithelial barrier integrity is impaired by diabetic hyperglycemia. Importantly, the degree of hyperglycemia correlated inversely with both AB staining and E-cadherin staining in all DIO mice regardless of exposure to STZ or DSS (Figure 3G and H). Furthermore, in DSS-treated groups, the amount of AB and E-cadherin staining correlated inversely with DAI scores on day 7 (Figure 3I and J). Finally, the degree of hyperglycemia correlated directly with IBD activity (Figure 3K). Taken together, these findings supported a mechanism whereby hyperglycemia increased the onset and severity of DSS colitis by decreasing the expression of key tight junction proteins and diminishing the colonic mucins that help maintain gut epithelial barrier integrity.

Hyperglycemia fails to worsen DSS-colitis symptoms or impair the intestinal barrier in chow-fed mice

To investigate the contribution made by DIO to the deleterious effects of STZ-diabetes on IBD outcomes in mice, we repeated the above experiments in chow-fed mice. Mice were given low-dose STZ (or Veh) to induce T2D-range hyperglycemia 6 wk prior to DSS. In contrast to HFD-fed mice (mean BW 45.1 ± 4.1 g) in the above experiments, chow-fed mice maintained normal BWs leading up to STZ administration (Figure 4A; mean BW 30.1 ± 2.1 g). STZ administration induced hyperglycemia leading up to the DSS course, which was comparable to that observed in mice fed an HFD (Figure 4B). During the course of DSS, in contrast to STZ-diabetic DIO mice, STZ-diabetic chow-fed mice (Chow/STZ/DSS) did not exhibit a more rapid onset or greater degree of severity of colitis following DSS administration compared to chow-fed mice without STZ-diabetes (Chow/Veh/DSS) (Figure 4C). Chow/Veh/DSS mice had a slightly shorter colon length on day 7 of DSS compared to Chow/STZ/DSS mice (Figure 1D), but there were no significant differences in spleen weight between both DSS-treated groups (Figure 1E).

Notably, neither AB mucin staining (Chow/Veh/Veh *vs* Chow/STZ/Veh, $P = 0.761$; Chow/Veh/DSS *vs* Chow/STZ/DSS, $P = 0.994$) (Figure 4) nor E-cadherin tight junction protein staining (Chow/Veh/Veh *vs* Chow/STZ/Veh, $P = 0.863$; Chow/Veh/DSS *vs* Chow/STZ/DSS, $P = 0.999$) (Figure 4G) was impacted by STZ administration among chow-fed mice, irrespective of DSS administration. Thus, non-obese, chow-fed mice were protected from the deleterious effects of STZ-diabetes on both colitis severity and markers of gut epithelial permeability. These findings indicated that hyperglycemia exacerbates DSS-induced colitis only in the setting of DIO.

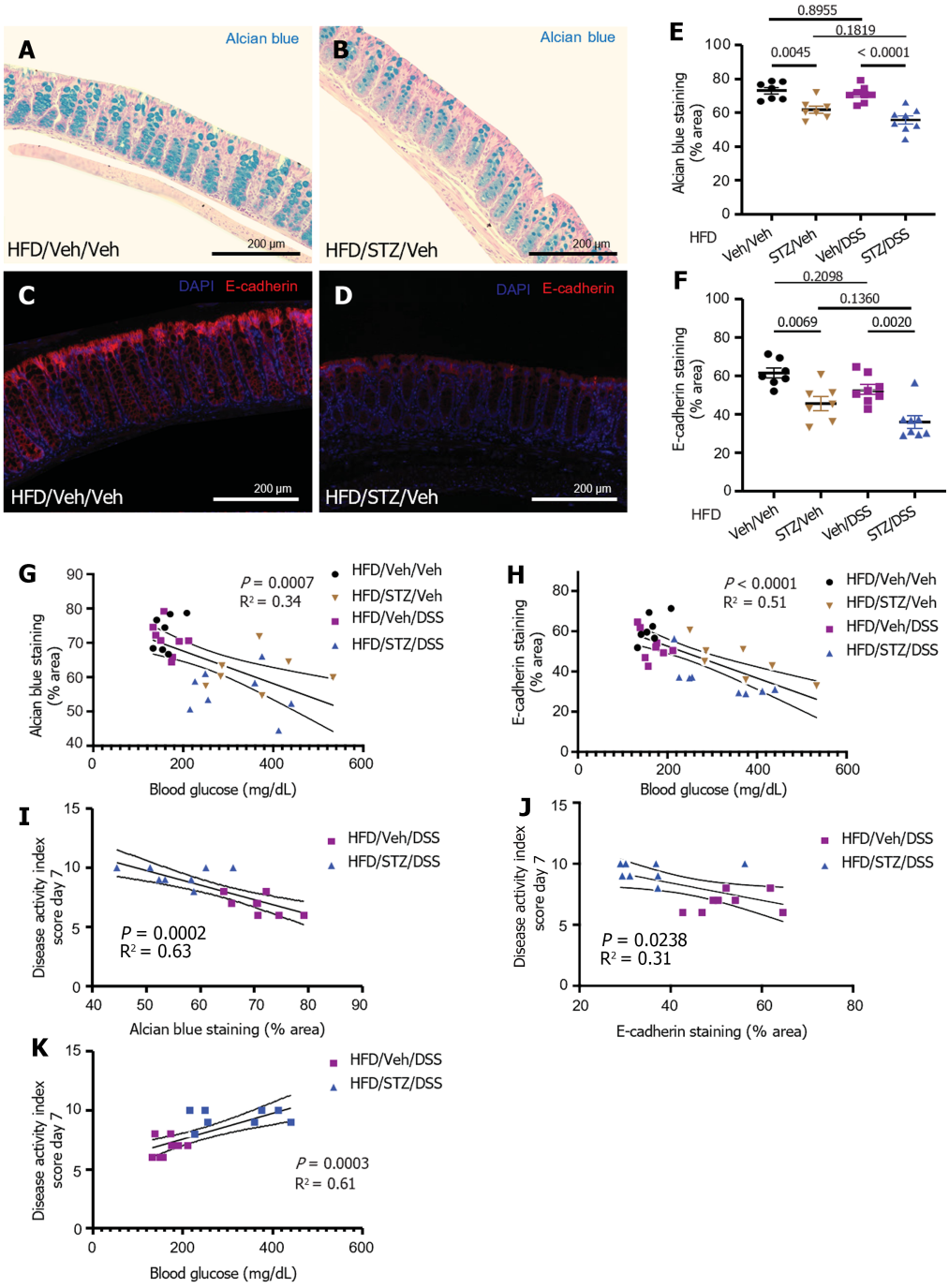

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Figure 3 Colonic mucin barrier and tight junction protein abundance in diabetes and dextran sodium sulfate colitis in diet-induced obese mice. A: Alcian blue (AB) staining highlighted the colonic mucin barrier in normoglycemic mice; B: AB staining highlights the colonic mucin barrier in hyperglycemic mice; C: AB staining in areas of the colon with intact epithelium was quantified for each treatment group that received vehicle (Veh)/streptozotocin (STZ) and/or Veh/dextran sodium sulfate (DSS). D: Tight junction protein E-cadherin was highlighted by immunohistochemical staining in the colons of normoglycemic mice; E: Tight junction protein E-cadherin is highlighted by immunohistochemical staining in the colons of hyperglycemic mice; F: E-cadherin abundance in areas of the colon with intact epithelium was quantified for each experimental treatment group; G: Correlation of degree of hyperglycemia with AB staining; H: Correlation of degree of hyperglycemia with E-cadherin staining; I: Correlation of AB staining with clinical colitis disease severity in DSS-treated groups; J: Correlation of E-cadherin staining with clinical colitis disease severity in DSS-treated groups; K: Correlation of degree of hyperglycemia with colitis disease severity in DSS-treated groups. *n* = 7-8 per group, mean ± standard error of the mean. DSS: Dextran sodium sulfate; Veh: Vehicle; STZ: Streptozotocin; HFD: High-fat diet.

Restoring normoglycemia attenuates the deleterious effect of DSS treatment on colitis severity and intestinal barrier integrity

To determine the impact of hyperglycemia per se, independent of other elements of the diabetic state generated by STZ, on intestinal barrier function and colitis outcomes, we repeated the study with DIO mice treated with STZ and DSS, with the additional step of administering the SGLT2i dapagliflozin at a dose that normalizes glycemia to a subgroup of these mice prior to DSS administration[28]. As before, mice were given an HFD to induce DIO (mean BW 49.7 ± 6.2 g)

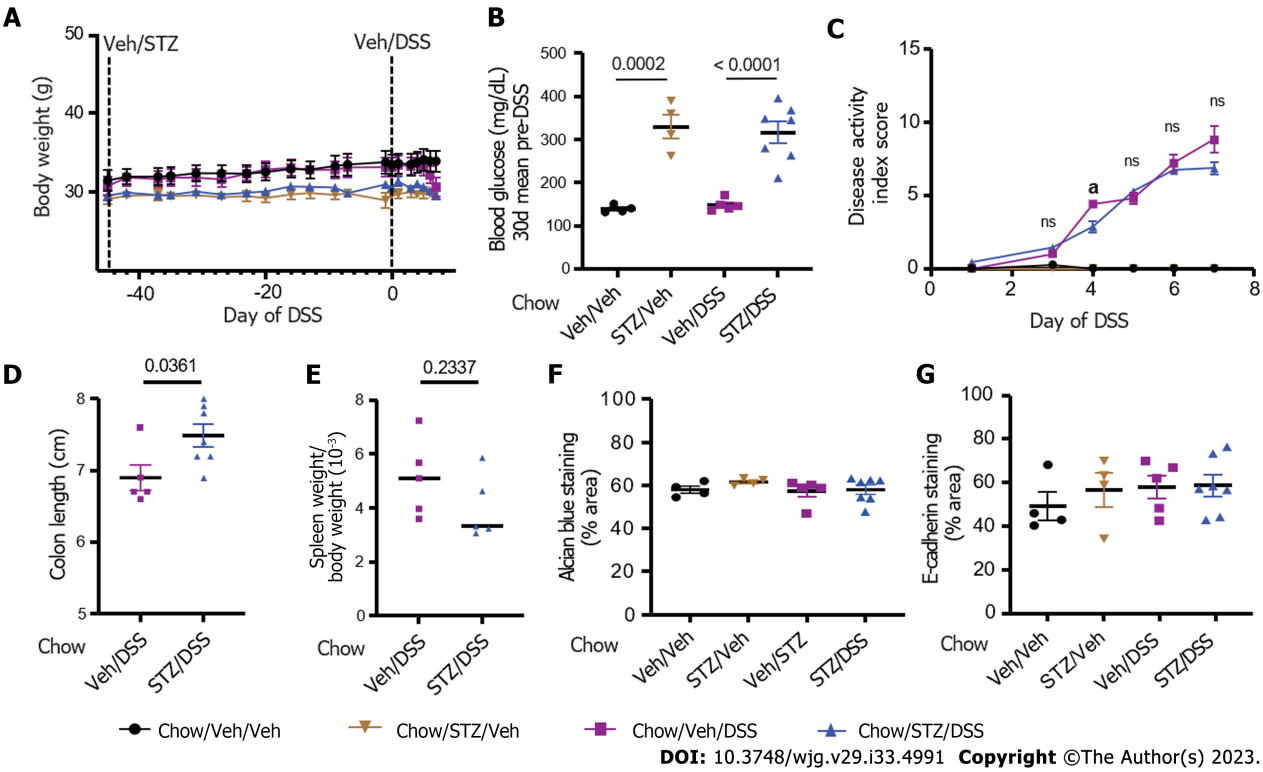

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Figure 4 Body weight, blood glucose, clinical disease activity, histopathologic disease activity, and intestinal barrier integrity in diabetic chow-fed mice. A: Body weight; B: Blood glucose in chow-fed non-obese mice that received either vehicle (Veh) or streptozotocin (STZ) to induce diabetes and were then given dextran sodium sulfate (DSS) to induce colitis; C: Clinical DSS colitis disease activity scores in normoglycemic and hyperglycemic mice; D: Colon length; E: Spleen weight-to-body weight ratios; F: Colonic Alcian blue staining; G: Tight junction protein E-cadherin staining quantified from areas with intact colonic epithelium. n = 4-7 per group, mean ± standard error of the mean. $^{a}P < 0.05$, and comparison noted in (C) is between chow/Veh/DSS and chow/STZ/DSS groups for days 3-7 of DSS. DSS: Dextran sodium sulfate; Veh: Vehicle; STZ: Streptozotocin.

(Figure 5A) and were then given either Veh or STZ to induce diabetic-range hyperglycemia (mean post-STZ blood glucose levels: Veh 173.6 ± 15.8 *vs* STZ 282.5 ± 68.6; $P = 0.0007$) (Figure 5B). After 6 wk of sustained hyperglycemia post-STZ treatment, the SGLT2i dapagliflozin (or Veh) was added to the drinking water, which resulted in euglycemic blood glucose levels equivalent to non-STZ treated control levels (mean 10 d pre-DSS blood glucose levels: Veh/Veh/DSS 156.8 ± 11.7 mg/dL *vs* STZ/SGLT2i/DSS 172.8 ± 17.5 mg/dL; $P = 0.793$) (Figure 5B and C) and significantly lower than STZ-treated mice who did not receive dapagliflozin (STZ/Veh/DSS 242.1 ± 72.8mg/dL *vs* STZ/SGLT2i/DSS 172.8 ± 17.5 mg/dL; $P = 0.022$).

After 2 wk of SGLT2i (or Veh) exposure, DSS was added to drinking water for the last week to induce colitis in each group. Similar to our earlier observations in DIO mice (Figure 1), DSS administration caused a significant reduction in BW (Figure 5A) and blood glucose levels in all groups (Figure 5B and C). Paired DSS water administration was performed, and total DSS consumption did not differ significantly between groups (Figure 5D). As previously noted (Figure 2A), the onset of colitis symptoms was accelerated in hyperglycemic STZ/Veh/DSS mice with DIO compared to normoglycemic Veh/Veh/DSS mice, becoming evident by day 2 compared to day 3 (Figure 5E and F). This more rapid onset of symptoms was mitigated by SGLT2i treatment, as STZ/SGLT2i/DSS mice manifested clinical colitis on day 3 instead of day 2 (Figure 5E and F). While symptoms of clinical colitis severity were comparable between groups from days 3-5 (Figure 5E), STZ/Veh/DSS mice demonstrated significantly worse DAI scores on both days 6 and 7 of DSS administration than either non-STZ treated mice (Veh/Veh/DSS) or STZ-treated mice receiving SGLT2i (STZ/SGLT2i/DSS) (Figure 5F).

Similar outcomes were observed when histochemical parameters of epithelial barrier were assessed in areas of intact colon in DSS-treated mice. The effect of untreated hyperglycemia to decrease AB mucins in DIO mice (compared to non-diabetic controls) in intact areas of colonic epithelium was fully reversed by dapagliflozin treatment (Figure 6A and B). Moreover, the amount of colonic AB staining correlated inversely with the degree of hyperglycemia across the three groups (based on 1-wk average blood glucose levels prior to DSS) (Figure 6C). E-cadherin abundance similarly was decreased by STZ treatment and was partially reversed by SGLT2i administration, although this did not achieve statistical significance (Figure 6D and E, $P = 0.050$). The abundance of the colonic tight junction protein E-cadherin reached near significance with an inverse correlation to blood glucose levels as well (Figure 6F). These findings strengthened the conclusion that in DIO mice, hyperglycemia is the primary driver of both STZ-induced colonic epithelial barrier disruption and colitis severity.

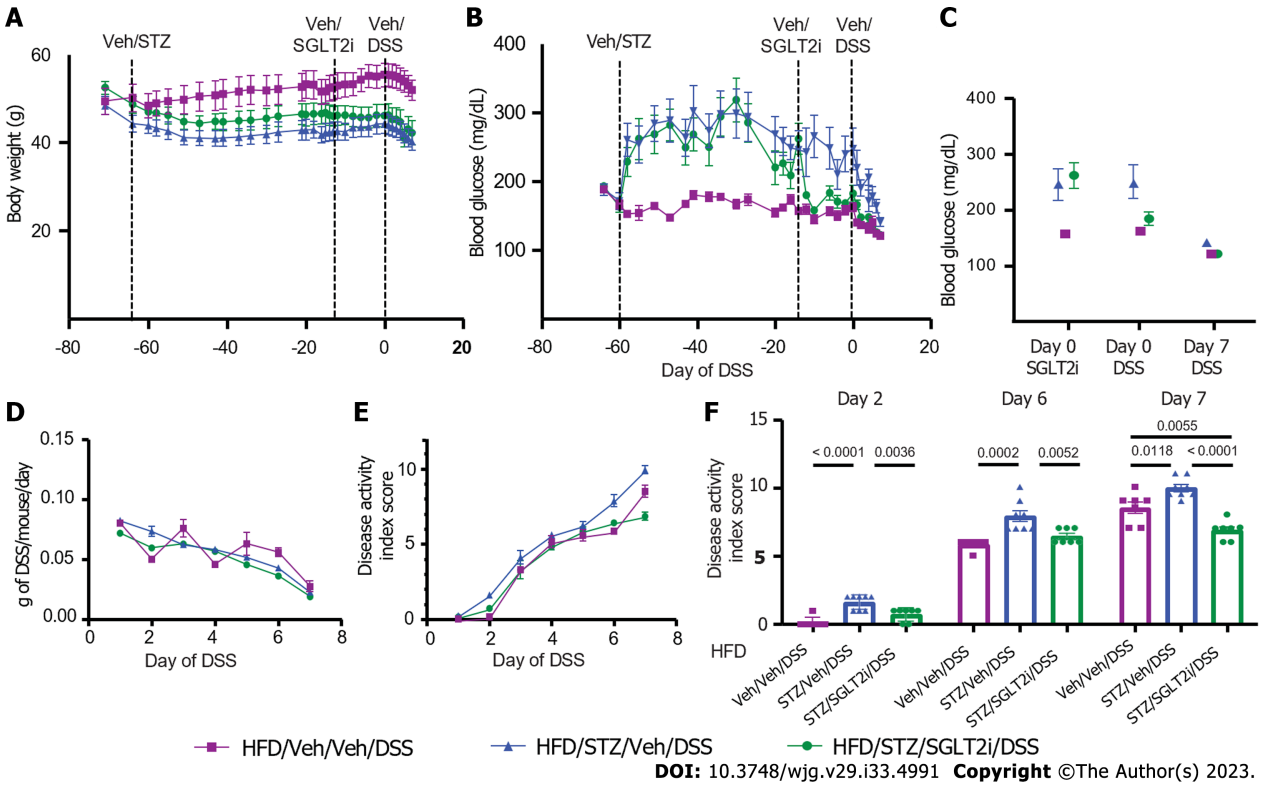

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Figure 5 Body weight, blood glucose, and dextran sodium sulfate colitis activity in diabetic diet-induced obese mice on antidiabetic treatment. A: Body weight; B: Blood glucose; C: Change in blood glucose in high-fat diet-fed mice that received either vehicle (Veh) or streptozotocin to induce diabetes and were then treated with the sodium-glucose cotransporter-2 inhibitor dapagliflozin or Veh, prior to dextran sodium sulfate (DSS) colitis onset; D: Amount of DSS consumed during the DSS course; E: DSS colitis disease activity index scores through the 7 d course of DSS; F: DSS colitis disease activity index scores quantified on days 2, 6, and 7. *n* = 7-8 per group, mean ± standard error of the mean. DSS: Dextran sodium sulfate; Veh: Vehicle; STZ: Streptozotocin; HFD: High-fat diet.

DISCUSSION

In the current work, we determined whether diabetic hyperglycemia exacerbates colitis severity in a murine model of IBD. Our findings demonstrated that diabetic hyperglycemia both accelerates the onset and worsens the clinical and pathological outcomes of DSS colitis. Interestingly, each of these effects of diabetes on IBD outcomes was detected in mice with DIO but not in chow-fed, non-obese mice. Furthermore, we show that the severity of IBD increases directly in relation to the degree of hyperglycemia and that reversal of hyperglycemia with an antidiabetic medication eliminated this effect of diabetes. Taken together, these findings support a model whereby the combination of obesity and hyperglycemia predisposes to more severe outcomes of intestinal inflammation in IBD.

As a first step towards understanding the mechanisms by which diabetes and DIO influence IBD outcomes, we sought to investigate the effect of diabetes on the intestinal barrier, focusing in particular on the colonic mucin layer and tight junction proteins. Previous work had suggested that diabetic hyperglycemia impairs intestinal barrier function and increases intestinal permeability, although how this might impact IBD pathology had not been studied[25]. Importantly, it has been reported that symptomatic patients with both UC and CD have increased *in vivo* intestinal permeability[24] and decreased intestinal mucins and goblet cell depletion compared to healthy controls[37]. Additionally, in IBD colon organoid cultures, tight junction proteins are significantly reduced[38]. In our current work, we reported that STZ-induced diabetic hyperglycemia significantly impairs these two components of intestinal barrier integrity in the setting of DIO. Furthermore, the amount of mucins and tight junction protein staining correlated inversely with colitis disease activity. This supported a model in which the diabetic state acts injuriously on the intestinal barrier, making the intestines more susceptible to DSS-induced colitis. It is notable that diabetes on its own is insufficient to cause IBD pathology, but when exposed to a chemical colitic agent, the presence of diabetes and associated impaired intestinal barrier function significantly worsens IBD outcomes.

Our next goal was to determine the contribution of DIO on the effect of diabetes to exacerbate DSS-induced intestinal inflammation. Obesity is present in the vast majority of T2D patients[12,13], and patients with IBD have similar rates of obesity compared to the general population[39]. Comorbid obesity has been associated with higher hospitalization rates, more active disease, and a higher prevalence of perianal disease in patients with CD[40]. In patients with UC, elevated body mass index is associated with an increased risk of biologic therapy treatment failure[41]. Furthermore, HFD exposure exacerbates IBD outcomes in preclinical rodent models[20,21]. Conversely, severe hyperglycemia seen in uncontrolled, insulin-deficient T1D has a deleterious effect on intestinal barrier function independent of obesity[25]. To determine the contribution of DIO on the ability of a more modest, physiologic degree of hyperglycemia mimicking T2D

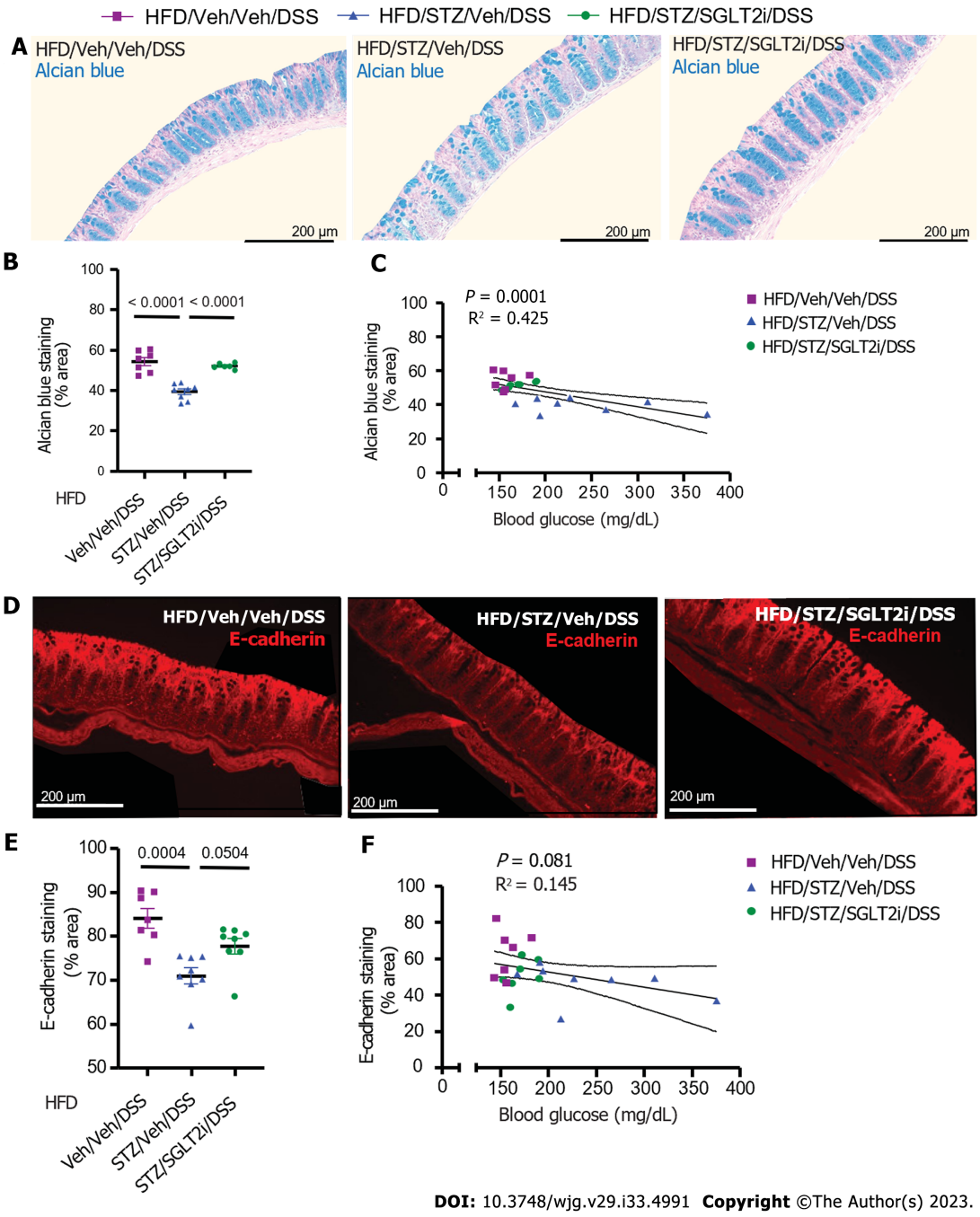

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Figure 6 Colonic mucin barrier and tight junction protein abundance in diet-induced obese mice on antidiabetic treatment with dextran sodium sulfate colitis. A: Alcian blue (AB) staining highlighted the colonic mucin barrier in high-fat diet (HFD)-fed mice administered either vehicle (Veh) or streptozotocin (STZ), followed by antidiabetic treatment with Veh or a sodium-glucose cotransporter-2 inhibitor (SGLT2i) prior to the dextran sodium sulfate (DSS) course; B: AB staining following the DSS course in areas of the colon with intact epithelium was quantified for each treatment group that received Veh/STZ or Veh/SGLT2i; C: Correlation of degree of hyperglycemia with AB staining; D: Tight junction protein E-cadherin staining in the same HFD-fed, DSS-treated mice that received Veh/STZ and Veh/SGLT2i; E: E-cadherin abundance following the DSS course in areas of the colon with intact epithelium; F: Correlation of degree of hyperglycemia with E-cadherin abundance. *n* = 7-8 per group, mean ± standard error of the mean. DSS: Dextran sodium sulfate; Veh: Vehicle; STZ: Streptozotocin; HFD: High-fat diet; SGLT2i: Sodium-glucose cotransporter-2 inhibitor.

to impact intestinal barrier pathology and IBD outcomes, we tested whether low-dose STZ would worsen DSS colitis in DIO mice fed an HFD compared to non-obese, chow-fed mice. We reported here that the effect of modest hyperglycemia to impair intestinal barrier function and therefore influence IBD outcomes is dependent on coexisting DIO. One potential explanation for these findings involves an effect of the HFD exposure on the gut microbiome, shifting its composition to a more mucin-degrading and proinflammatory profile[42]. When superimposed on this change of gut flora and the state of chronic inflammation in obesity, we observed clear-cut, deleterious effects of diabetes on both intestinal permeability and colitis disease activity. Further studies are warranted to determine the exact contribution that the HFD consumption makes to worsen IBD outcomes independent of obesity.

Next, we determined the specific contribution made by hyperglycemia *per se* to the effect of diabetes on intestinal barrier pathology. To this end, we sought to normalize the blood glucose level without reversing other aspects of the uncontrolled diabetic state (*e.g.*, insulin deficiency, elevated circulating levels of ketone bodies and free fatty acids). This goal was achieved by administering an SGLT2i, which selectively normalizes glycemia without impacting insulin levels or other aspects of the diabetic state. We reported that restoring normoglycemia *via* SGLT2i administration after STZ treatment modestly improved clinical colitis outcomes. Notably, the onset of clinical colitis was delayed in mice treated with SGLT2i compared to hyperglycemic mice, and the severity of colitis at the end of the DSS course was significantly improved compared to hyperglycemic mice.

Reversal of hyperglycemia after STZ treatment with an SGLT2i resulted in significant improvement in the colonic mucin barrier, with no difference between non-STZ treated normoglycemic mice and mice treated with STZ who then received SGLT2i. The abundance of tight junction proteins also tended to improve with normalization of the blood glucose level, although it did not reach statistical significance. From these findings, we inferred that the effect of diabetes to influence intestinal barrier pathology and IBD outcomes appears to be dependent, or at least heavily reliant, on hyperglycemia. This conclusion was further supported by our findings that the degree of intestinal barrier dysfunction and colitis disease severity varied directly with the degree of hyperglycemia. These findings heightened the importance of studies to evaluate the impact of effective diabetes treatment in patients with IBD, especially given recent evidence that patients with IBD are at increased risk of developing T2D[8,9], that comorbid T2D predicts poorer IBD outcomes[11], and that high-fat, obesogenic diets are associated with a higher incidence of IBD[17-19].

The growing patient population affected by T2D, obesity, and IBD creates a compelling rationale for continued efforts to understand shared mechanisms between these disease processes, particularly in light of evidence that comorbid T2D or obesity negatively affects IBD outcomes in patients[10,11,40,41]. It is also imperative to understand how treatments for each of these conditions affect the others, particularly as corticosteroids are a mainstay of treatment to induce remission in both UC and CD and are known to both exacerbate hyperglycemia in patients with pre-existing T2D and to precipitate hyperglycemia in patients with no prior diabetes diagnosis[43]. These considerations underscore the need for clinicians to consider how these disease processes and their respective treatments affect their patients and highlight the need to investigate the potential role of antidiabetic medications in IBD management prior to hyperglycemia onset or exacerbation among those at risk.

CONCLUSION

In mice with DIO, diabetic hyperglycemia disrupts the intestinal barrier integrity and is associated with more severe clinical and pathological outcomes of colitis, highlighting the potential translational importance of ensuring optimal diabetes management in IBD patients.

ARTICLE HIGHLIGHTS

Research background

Emerging epidemiologic evidence links type 2 diabetes (T2D) and obesity to inflammatory bowel disease (IBD). However, evidence to determine the exact mechanisms by which obesity and/or diabetes influence IBD outcomes is limited. This study uses mouse models of colitis to investigate how diabetes and obesity interact to impair intestinal barrier function and exacerbate IBD outcomes, highlighting the deleterious impact of sustained hyperglycemia on intestinal barrier integrity.

Research motivation

Patients with IBD are at an increased risk of developing T2D, which serves as a predictor of poor outcomes in IBD. The rates of comorbid obesity in IBD are increasing as well, and obesity is related to a more severe IBD phenotype. As more patients with IBD are affected by obesity and/or T2D, it is imperative to understand how these disease processes interact and how treatments for each condition may impact the other.

Research objectives

In this study, we used murine models of colitis to determine the effect of T2D-range hyperglycemia on IBD outcomes and intestinal barrier function with and without coexisting diet-induced obesity (DIO).

Research methods

Mice were fed standard chow or a high-fat diet to induce DIO and then given streptozotocin (STZ) to induce sustained T2D-range hyperglycemia. Mice were then given dextran sodium sulfate (DSS) to induce colitis. Body weight and blood glucose levels were compared as well as clinical colitis scores and histopathologic assessment of intestinal injury. The effects of hyperglycemia and DIO on intestinal barrier function were interrogated by comparing colonic mucins and tight junction protein abundance. To highlight the role of hyperglycemia itself, a sodium-glucose cotransporter-2 inhibitor was subsequently used to selectively reverse hyperglycemia prior to DSS course.

Research results

In the setting of DIO, STZ-diabetes significantly worsened clinical and histopathological outcomes of DSS colitis in mice. This effect was associated with a significant reduction in the colonic mucin barrier and tight junction protein abundance and was ameliorated by the use of a sodium-glucose cotransporter-2 inhibitor to reverse hyperglycemia prior to colitis onset. Together, these findings highlighted the deleterious effect of diabetic hyperglycemia on the intestinal barrier as a mechanism by which diabetes and obesity interact to affect IBD outcomes.

Research conclusions

This study reported the novel finding that diabetic hyperglycemia disrupted intestinal barrier integrity in the setting of DIO and exacerbated DSS colitis outcomes in mice. Given the increased prevalence of T2D in patients with IBD and the negative impact of comorbid obesity on IBD outcomes, it is imperative to understand how these disease processes interact.

Research perspectives

These findings have significant translational relevance, and future research can expand on them by determining whether strict glycemic control in patients with T2D and IBD is associated with improved IBD outcomes.

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FOOTNOTES

Author contributions: Francis KL, Schwartz MW, and Scarlett JM conceived the study; Francis KL and Scarlett JM carried out the experiments and were responsible for the data collection, analysis, and interpretation; Hu SJ and Krutzsch CA participated in the data collection and image analysis; Pacheco MC was responsible for the pathologic analysis of tissues; Francis KL, Scarlett JM, Schwartz MW, Alonge KM, Pacheco MC, Morton GJ, Hu SJ, and Krutzsch CA participated in the study design and data interpretation; Francis KL was responsible for drafting the article; Morton GJ, Alonge KM, Schwartz MW, Pacheco MC, Scarlett SJ, Krutzsch CA, and Scarlett JM were responsible for revising and editing the manuscript.

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ORIGINAL ARTICLE

Basic Study

Novel deformable self-assembled magnetic anastomosis ring for endoscopic treatment of colonic stenosis *via* natural orifice

Miao-Miao Zhang, Guang-Bin Zhao, Han-Zhi Zhang, Shu-Qin Xu, Ai-Hua Shi, Jian-Qi Mao, Jing-Ci Gai, Yu-Han Zhang, Jia Ma, Yun Li, Yi Lyu, Xiao-Peng Yan

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Abstract

BACKGROUND

Although endoscope-assisted magnetic compression anastomosis has already been reported for colonic anastomosis, there is no report on a single-approach operation using the natural orifice.

AIM

To design a deformable self-assembled magnetic anastomosis ring (DSAMAR) for colonic anastomosis for use in single-approach operation and evaluate its feasibility and safety through animal experiments.

METHODS

The animal model for colonic stenosis was prepared by partial colonic ligation in

eight beagles. The magnetic compression anastomosis of their colonic stricture was performed by endoscopically assisted transanal implantation of the DSAMAR. The anastomotic specimen, obtained 2 wk after the operation, was observed by both the naked eye and a light microscope.

RESULTS

The DSAMAR was successfully inserted into the proximal end of colon stenosis through the anus. The DSAMAR of seven dogs was successfully transformed into rings, while that of the remaining dog was removed after the first deformation failed. The rings were successfully retransformed after optimization. All animals underwent colonic anastomosis using the DSAMAR. No device-related or procedure-related adverse events were observed. The colostomy specimens of the experimental dogs were obtained 2 wk after the operation. Both gross and histological observations showed good anastomotic healing.

CONCLUSION

The DSAMAR is a safe and feasible option for the treatment of colon stenosis. Its specific deformation and self-assembly capability maximize the applicability of the minimally invasive treatment.

Key Words: Magnetic surgery; Magnamosis; Colonic stenosis; Natural orifice; Endoscopy

Core Tip: By combining magnetic compression anastomosis with endoscopic technology, we could design a deformable self-assembled magnetic anastomosis ring (DSAMAR) to perform magnetic compression anastomosis with only a single channel. We then verified the feasibility of magnetic compression anastomosis for the recanalization of colonic stenosis through animal experiments. The results showed that minimally invasive treatment of colonic stenosis can be achieved using the DSAMAR.

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INTRODUCTION

Benign colonic stenosis is a disease with various causes, including post-colectomy anastomotic stenosis[1], transcatheter arterial embolization[2], and acute pancreatitis[3]. Benign colonic stenosis is generally treated by endoscopic balloon dilatation (EBD)[4] and surgery[5]. EBD is widely performed in clinics because of its effectiveness, technical safety, and ease of process. However, in some cases, sufficient dilatation is not achieved because of the formation of stiff fibrosis tissue at the stenosis site, which is often resistant to balloon pressure[6]. Open surgery, laparoscopic stenosis resection, and bypass anastomosis are the traditional treatment strategies for benign colonic stenosis. Although a good therapeutic effect can be obtained through surgery, it is no longer the first choice of clinicians because of trauma and various perioperative adverse events.

Magnetic compression anastomosis or magnamosis, a novel technique, can be used for managing digestive tract anastomosis[7-9], vascular anastomosis[10,11], pathologic fistula[12], and therapeutic fistula[13]. The use of magnamosis along with endoscopy for treating gastrointestinal stenosis has been reported[14]. The cylinder or circle is the most commonly used shape of the magnet in such cases. In endoscopic magnetic compression anastomosis, the daughter and parent magnets are placed at the two opposite ends of the narrow digestive duct. The patient must have two channels: A natural orifice and the digestive tract fistula[14,15]. However, some patients only have a natural orifice, making magnetic compression anastomosis very difficult to perform. Therefore, for successful magnamosis, the magnet must be designed in a way that allows efficient optimization of the operation path. The design of the magnet must meet two conditions: first, the magnet should be able to pass through the narrow digestive cavity; and second, the magnet should be able to create wide anastomoses.

This study aims to design a novel deformable self-assembled magnetic anastomosis ring (DSAMAR) that can be placed at the natural orifice of the body. It also explores the feasibility of the DSAMAR in digestive tract anastomosis using dogs as experimental animals.

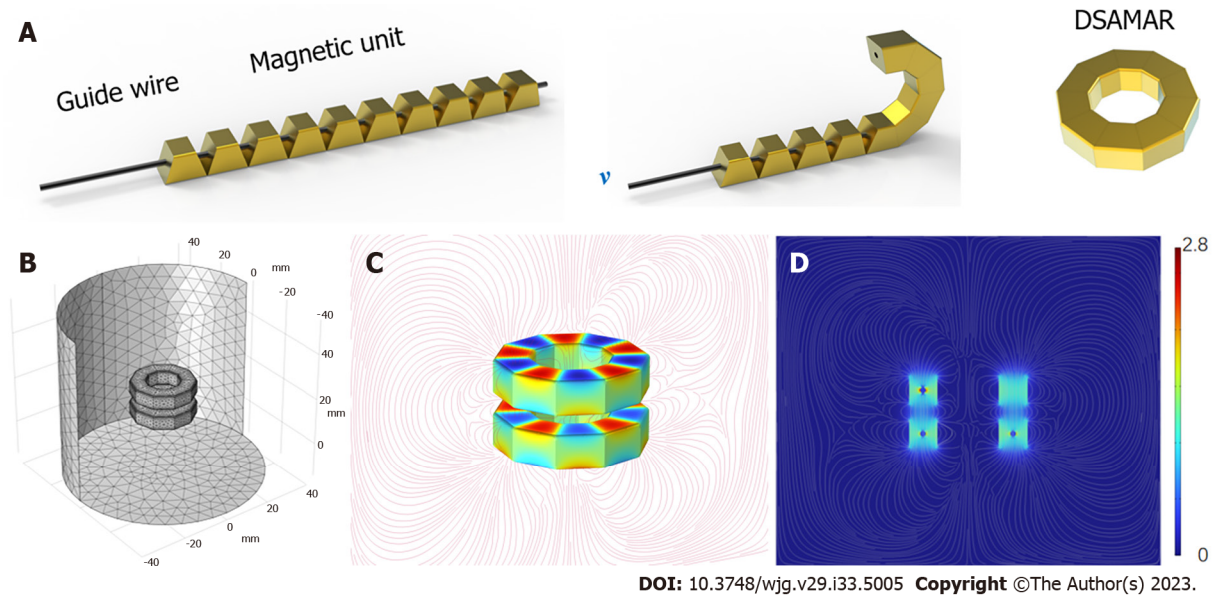

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Figure 1 A schematic of the deformable self-assembled magnetic anastomosis ring. A: Transformation of the deformable self-assembled magnetic anastomosis ring from a linear structure to a ring; B to D: Computer simulation of magnetic flux density. DSAMAR: Deformable self-assembled magnetic anastomosis ring.

MATERIALS AND METHODS

Ethical statement

The experimental protocol was approved by the Committee for Ethics of Animal Experiments of Xi'an Jiaotong University (license No. 2022-1451). Eight beagles (male = 4, female = 4) were acquired from the Laboratory Animal Center of the Xi'an Jiaotong University (Xi'an, China) as experimental animals. The research protocol and all the experimental procedures were conducted strictly in accordance with the Guidelines for the Care and Use of Experimental Animals issued by the Xi'an Jiaotong University Medical Center.

DSAMAR

The DSAMAR consists of 10 trapezoidal magnetic units assembled in the opposite order of the N and S poles of the adjacent magnetic units. The long-axis direction of each magnetic unit contains a 1-mm diameter round hole that allows the stainless steel guide wire to pass through. Owing to the opposite N–S polarity of each magnetic unit, passing 10 sequentially arranged trapezoidal magnetic units through a hard guide wire and forming a linear arrangement is possible. When the guide wire is slowly drawn out, the inclined planes of the adjacent magnetic units attract each other and are sequentially arranged and assembled into a DSAMAR (Figure 1A) (Video 1). The magnetic unit is processed by N50-sintered neodymium-iron-boron, and its surface is coated with titanium nitride. Each magnetic unit of a DSAMAR weighs 1.25 g, the maximal magnetic field intensity at its compression surfaces is 480 mT, and the suction of two DSAMARs at zero distance can reach 184 N. The magnetic flux density can be calculated by computer simulations (Figure 1B-D).

Study design

This study aimed to verify the feasibility of the DSAMAR, an innovative surgical method. Hence, no control group was used. Eight beagles (age: > 1 year; weight: 12–15 kg) were used as experimental animals. Their operation time, postoperative adverse events, survival rate, and magnetic-ring-expel time were recorded. One month after the operation, the anastomosis was achieved and its healing was observed both by the naked eye and a light microscope.

Colonic stenosis model

The beagles were given a slag-free liquid diet 2 d before surgery. In addition, they were fasted and their water intake was restricted 6 h before surgery. They were anesthetized by an intravenous injection of 3% pentobarbital sodium (1 mL/kg) and fixed in the supine position. Repeat enemas with soapy water were conducted to clean the intestines. A 14Fr gastric tube was inserted into the colon through the anus. An approximately 10-cm-long incision in the middle of the lower abdomen was made into the abdominal cavity. The descending colon was exposed and ligated on the 14Fr gastric tube with a 1-0 silk thread in the middle part of the descending colon. The gastric tube was removed and a colon stenosis model was formed. The diameter of the colon in the narrow segment was approximately 4.5 mm. The abdomen was then closed layer by layer. The colon stenosis model was evaluated both by colonoscopy and colonography (Figure 2).

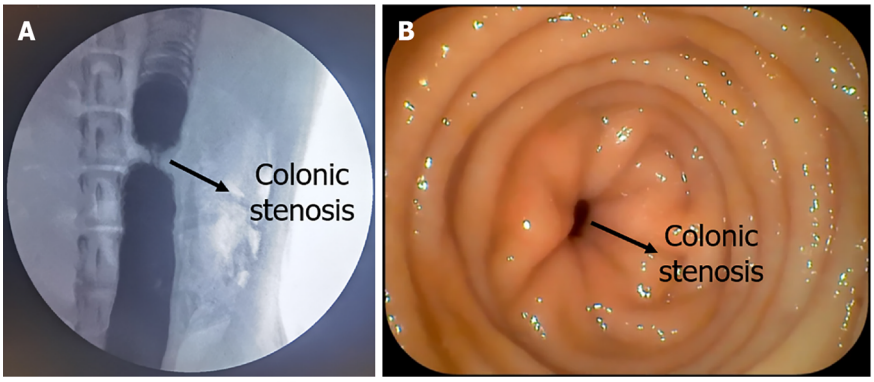

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Figure 2 Colonic stenosis model. A: Colonic stenosis as seen by colonography; B: Colonic stenosis as seen by colonoscopy.

Endoscopic procedures

First, transanal colonoscopy was performed to determine the location and extent of colon stenosis. A guide wire was inserted through the colonoscopy biopsy hole. Under X-ray, the head end of the guide wire was passed through the colon stenosis segment. The position of the head end of the guide wire was maintained, and then the colonoscope was withdrawn (Figure 3A). Through the other end of the guide wire, 10 trapezoidal magnetic units were successively inserted in the opposite order of the N and S poles of the adjacent magnetic units. Again, under X-ray, a 5Fr push tube was used to push the magnetic units along the guide wire to make them enter the proximal end of the stenosis colon (Figure 3B and C). The magnetic units were then slowly pushed out of the guide wire one by one and transformed into a ring in the colon according to a predetermined plan. Then, the guide wire and the push tube were removed. Another DSAMAR, self-assembled in vitro, was inserted through the anus. Under X-ray, a colonoscope was used to push the magnet to the distal end of the stenosis colon. At this time, the two DSAMARs could automatically attract and compress the colon stenosis (Figure 3D and E). The two DSAMARs were then placed in the colon.

Postoperative care

All dogs were managed in a single cage after emergence from anesthesia. Pethidine hydrochloride (1 mg/kg) was intramuscularly injected every 12 h for 3 d after the operation for analgesia. The dogs were given intravenous nutritional support until the DSAMAR detached from the anus. The time that the magnets took to get expelled from all dogs was recorded. The dogs resumed oral feeding after the DSAMAR was expelled. Colonoscopy and colonography were performed to evaluate the patency of the colon.

Tissue harvest and analysis

All dogs were euthanized 2 wk after the operation, and their colonic anastomosis specimens were obtained. The healing of anastomosis was observed by the naked eye. The anastomotic specimen was then soaked overnight in 10% formalin for fixation. Then, the specimen was embedded in paraffin and a 4-μm-thick section from the anastomosis was prepared. The sections were stained with hematoxylin and eosin (H&E) and Masson trichrome and examined under a bright-field microscope.

Statistical analysis

SPSS statistical 20.0 software was used for data analysis. The quantitative data of normal distribution were described by mean ± SD, while those of non-normal distribution were described by the median. Differences between the groups were compared by an independent sample *t*-test or a nonparametric test. $P < 0.05$ indicated a significant difference.

RESULTS

Procedural parameters

The colonic stenosis model was successfully prepared in all dogs (success rate = 100%). In seven of the eight beagles, the DSAMAR was successfully deformed only once after implantation in the proximal colon. In the remaining dog, the DSAMAR did not form during deformation, but it was successfully deformed after the removal of the magnetic ring and its re-implantation. The distal colon magnet was placed without any issue, and the DSAMARs at both ends of the stenosis were automatically attracted to each other. The endoscopic procedures (except for the model-preparation time) took 20.88 ± 5.69 min to complete, which was within the acceptable range (15–32 min). The average expulsion time of the magnets was 6.13 ± 0.99 d, again within the accepted range (5–8 d). The data related to animal experiments are listed in Table 1. The discharged DSAMARs are shown in Figure 4A-C. Colonoscopy and colonography were performed after the discharge of the magnet and a good patency of the colon was observed (Figure 4D and E).

Table 1 Data related to animal experiments

Animal	Male/female	Weight (kg)	Operation time (min)	DSAMAR expelled time (d)	Device failure (Y/N)	Device-related adverse events (Y/N)
No. 1	M	12.5	26	5	N	N
No. 2	F	12.0	18	6	N	N
No. 3	F	14.5	32	7	Y[1]	N
No. 4	M	12.0	15	6	N	N
No. 5	F	13.5	18	5	N	N
No. 6	M	14.0	20	8	N	N
No. 7	M	12.0	22	6	N	N
No. 8	F	15.0	16	6	N	N

[1]The deformable self-assembled magnetic anastomosis ring was not formed during deformation; instead, it was successfully deformed after the removal of the magnetic ring and its re-implantation.
DSAMAR: Deformable self-assembled magnetic anastomosis ring; M: Male; F: Female; Y: Yes; N: No.

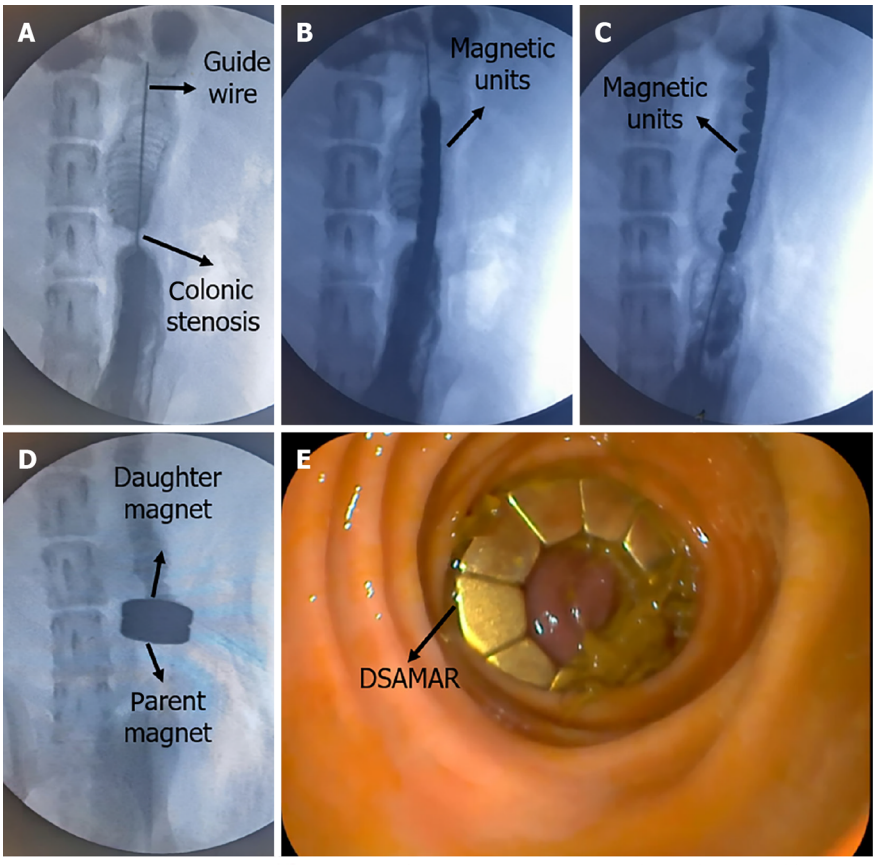

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Figure 3 Animal experiment process. A: Hard guide wire passes through the stenosis into the proximal colon; B: Linear deformable self-assembled magnetic anastomosis ring (DSAMAR) passes through the narrow segment of the colon; C: DSAMAR enters the proximal colon completely; D: Magnetic rings at both ends of the narrow colon were attracted to each other; E: DSAMAR in the distal colon as seen by colonoscopy.

Survival rate and postoperative adverse events

The survival rate of all dogs was 100% in 2 wk after surgery. The dogs were generally in good condition after surgery, with no intestinal bleeding, perforation, obstruction, and other adverse events. After the dogs resumed normal feeding, they all excreted formed feces.

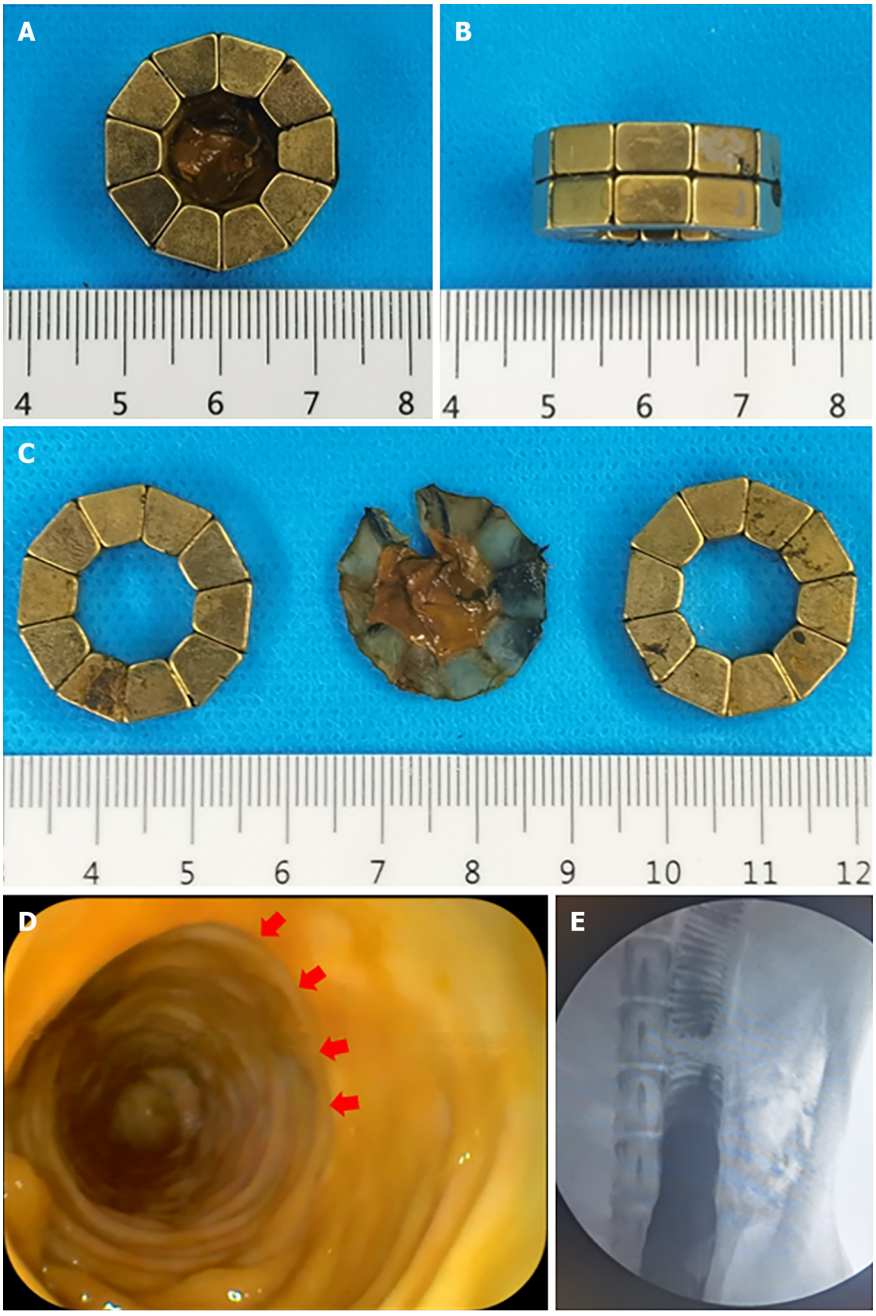

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Figure 4 Magnetic compression anastomosis was established. A to C: Deformable self-assembled magnetic anastomosis rings expelled from the body; D: Magnetic compression anastomosis as seen by colonoscopy; E: Colonography showed good patency of the colon.

Gross and histological appearance of anastomosis

The experimental dogs were euthanized 2 wk after surgery, and their colonic anastomosis gross specimens were obtained by laparotomy. The colonic stenosis was not observed by the naked eye. The mucosa of the magnetic compression anastomosis was smooth and flat (Figure 5A and B). H&E and Masson staining showed good continuity and mucosal healing of the anastomotic mucosa (Figure 5C and D).

DISCUSSION

The DSAMAR along with endoscopy is a feasible treatment for gastrointestinal stenosis. Magnetic compression anastomosis is a safe technique for digestive tract anastomosis[7-9]. Non-surgical digestive tract anastomosis can be achieved when magnetic compression anastomosis is combined with endoscopy. Although most of the cases have been reported in clinical settings[14-16], the safety and feasibility of magnetic compression anastomosis are beyond doubt. To the best of our knowledge, the DSAMAR is the only magnet that can enter the narrow intestine through the natural orifice and perform the self-assembly anastomosis, which greatly increases the potential application of the magnamosis

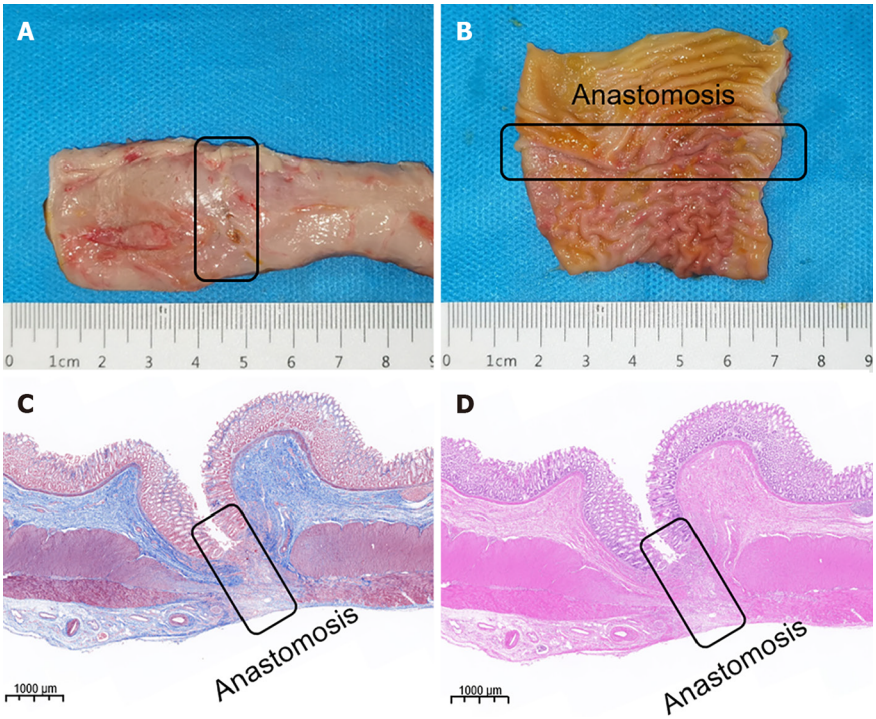

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Figure 5 Magnetic compression anastomosis specimen. A: Serous surface of the anastomosis; B: Colonic anastomosis seen on mucosal surface; C: Masson's staining of anastomosis; D: Hematoxylin and eosin staining of anastomosis.

technology.

The special deformation mode of the DSAMAR allows the magnet to pass through the narrow section of the digestive tract with a minimal cross-section. The DSAMAR then deforms and self-assembles into a ring, with a slimming deformation ratio of up to 1:16.35. This creates suitable conditions for the implantation of a magnet ring through the single channel of the natural orifice. Approximately 100% success rate of the deformation self-assembly of the DSAMAR *in vitro* can be achieved. Only one of the eight dogs had an unplanned deformation of the DSAMAR when failed to form a ring. This happened because the colon was not inflated enough to provide sufficient space for the DSAMAR to deform. In this case, the DSAMAR was re-inserted into the proximal colon and inflated by a colonoscope to fully expand the colon, which helped successfully complete the deformable self-assembly.

The result of animal experiments provides the best proof of the potential clinical transformation of the DSAMAR. The gross specimen and histological observation of the colonic anastomosis in dogs showed that the DSAMAR established a good anastomosis, which was not significantly different from that achieved using the conventional magnetic ring of digestive tract anastomosis reported previously. Different degrees of the narrowing of the human digestive tract after magnetic compression anastomosis have been reported, consistent with the results of previous studies[17-18]. There may be still some differences in the long-term anastomosis effect of magnetic compression anastomosis in humans and animals. This phenomenon needs to be further investigated through clinical studies with larger samples. Nevertheless, our study not only confirms the advantages of magnamosis, but also fully affirms the ingenious design of the DSAMAR. Compared with the currently available magnamosis rings, the DSAMAR can further optimize the magnetic compression anastomosis of colorectal anastomosis stenosis in postoperative patients with esophageal stenosis (non-atresia) and no fistula. This is because the DSAMAR uses only the natural orifice of a patient to successfully place the magnet.

This study has certain limitations also. The animal experiments only verified the application of the DSAMAR for the treatment of colonic stenosis. The animal sample size was small and the observation time of anastomosis was also quite short. We plan to verify the feasibility of the application of the DSAMAR in esophageal and duodenal stenoses in future studies and further observe the long-term patency of magnetic compression anastomosis. In previous studies on animal experiments[19], the colonic stenosis model was prepared by the partial ligation of silk thread, which was different from that used clinically. It may lead to differences in the anastomosis effect of the clinical application compared to that observed with animal experiments.

CONCLUSION

This study demonstrated the feasibility and safety of the DSAMAR for the treatment of digestive tract stenosis through the natural orifice by conducting animal experiments. Because of its unique deformable self-assembly, the DSAMAR can be used to handle complex gastrointestinal stenosis cases. In our next studies, we plan to further optimize the design of the DSAMAR and improve the endoscopic operation process through more animal experiments. It is expected to be used

in clinical practice in the near future.

ARTICLE HIGHLIGHTS

Research background

Magnetic compression anastomosis, or magnamosis, has been widely used for anastomotic recanalization of gastrointestinal stenosis. At present, the magnets applied to the digestive tract lumen anastomosis are usually cylindrical or circular.

Research motivation

In order to achieve magnetic anastomosis recanalization through a single-access natural orifice, we designed a deformable self-assembled magnetic anastomosis ring (DSAMAR). It is expected to provide a more minimally invasive treatment method for patients with colonic stenosis.

Research objectives

To verify the feasibility and safety of using DSAMAR to recanalize colonic stenosis through the single-approach natural orifice.

Research methods

Colonic stricture models were established in 8 beagle dogs. DSAMAR was used to achieve magnamosis recanalization of colonic stenosis and evaluate the formation of colonic anastomosis.

Research results

The results showed that the use of DSAMAR for recanalization of colonic stenosis was feasible and the anastomosis formation was good.

Research conclusions

The feasibility and safety of using DSAMAR to achieve colonic stenosis recanalization through a single-approach natural orifice were verified through animal experiments.

Research perspectives

The design of DSAMAR is ingenious. In addition to colon stenosis, it can also be used for minimally invasive treatment of digestive tract stenosis such as esophageal stricture and duodenal obstruction. It could provide a more minimally invasive treatment for patients with gastrointestinal stenosis.

FOOTNOTES

Author contributions: Lyu Y and Yan XP designed and coordinated the study; Zhang MM, Zhao GB, Zhang HZ, Xu SQ, Mao JQ, Zhang YH, Ma J, Li Y, Yan XP performed the research and acquired the data; Zhang MM, Zhao GB, Zhang HZ analyzed the data; Shi AH, Zhao GB, Zhang MM tested and analyzed the magnetic test; Zhang MM, Zhao GB, and Yan XP wrote the manuscript; Yan XP and Lyu Y conceived of the study and contributed to the study design, the interpretation of the results, and the critical revision of the manuscript; all authors read and approved the final manuscript.

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Institutional review board statement: The experimental protocol was approved by the Committee for Ethics of Animal Experiments of Xi'an Jiaotong University (No. 2022-1451).

Institutional animal care and use committee statement: The study protocol and all experimental procedures were carried out strictly in accordance with the Guidelines for Care and Use of Experimental Animals issued by the Xi'an Jiaotong University Medical Center. This experimental study was approved by the Experimental Ethics Committee of Xi'an Jiaotong University (No. 2022-1451).

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Data sharing statement: The authors declare that the data supporting the findings of this study are available within the article or from the corresponding authors upon request.

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CASE REPORT

Carcinoid syndrome caused by a pulmonary carcinoid mimics intestinal manifestations of ulcerative colitis: A case report

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Abstract

BACKGROUND

Pulmonary carcinoids are rare, low-grade malignant tumors characterized by neuroendocrine differentiation and relatively indolent clinical behavior. Most cases present as a slow-growing polypoidal mass in the major bronchi leading to hemoptysis and pulmonary infection due to blockage of the distal bronchi. Carcinoid syndrome is a paraneoplastic syndrome caused by the systemic release of vasoactive substances that presents in 5% of patients with neuroendocrine tumors. Due to such nonspecific presentation, most patients are misdiagnosed or diagnosed late and may receive several courses of antibiotics to treat recurrent pneumonia before the tumor is diagnosed.

CASE SUMMARY

We report the case of a 48-year-old male who presented with cough, dyspnea, a history of recurrent pneumonitis, and therapy-refractory ulcerative colitis that completely subsided after the resection of a pulmonary carcinoid.

CONCLUSION

We report and emphasize pulmonary carcinoid as a differential diagnosis in patients with nonresponding inflammatory bowel diseases and recurrent pneumonia.

Key Words: Carcinoid syndrome; Paraneoplastic syndrome; Pulmonary carcinoid; Neuroendocrine tumor; Ulcerative colitis; Case report

Core Tip: Pulmonary carcinoids are rare neuroendocrine tumors that may cause a paraneoplastic syndrome. We report the case of a 48-year-old man with a history of recurrent pneumonitis and therapy-refractory ulcerative colitis that was completely resolved after resection of a pulmonary carcinoid. Pulmonary carcinoid should be considered in the differential diagnosis of patients with unresponsive inflammatory bowel disease and recurrent pneumonia.

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INTRODUCTION

Pulmonary carcinoids are rare, slow-growing neuroendocrine tumors (NETs) that account for 1%-2% of all primary lung cancers[1]. NETs arise from enterochromaffin or Kulchitsky cells lining the gastrointestinal tract (58%) and respiratory system (25%)[2]. Bronchial carcinoids commonly present with recurrent pneumonitis, cough, fever, and hemoptysis due to their central origin and hypervascularity[1]. Rarely, patients may present with features of carcinoid syndrome, a term applied to a collection of symptoms that are associated with the secretion of vasoactive substances by carcinoid tumors[3, 4]. The main symptoms of carcinoid syndrome are cutaneous flushing, diarrhea, bronchospasms and, ultimately, carcinoid heart disease[3]. Carcinoid syndrome is predominantly associated with NETs that arise from the midgut in the setting of extensive liver metastases but may be present in patients with bronchial carcinoids[3]. Early detection is crucial because surgical excision of the tumor is the only curative approach and the main determinant of the prognosis[2].

CASE PRESENTATION

Chief complaints

A 48-year-old white man with a medical history of recurrent pneumonitis and therapy-resistant ulcerative colitis over 10 years presented with fever and cough with sputum production for 10 d. He reported resting dyspnea and pain over the right thoracic wall.

History of present illness

Under antibiotic therapy with moxifloxacin, no clinical improvement was reported. A chest roentgenogram showed an effaced right costo-phrenic angle and nonvisualization of the right hilar shadow suggestive of right lower lobe collapse (Figure 1).

Imaging examinations

Bronchoscopy revealed complete occlusion of the right bronchus intermedius due to a smooth-walled vascular mass with intact overlying epithelium. A computed tomography (CT) scan revealed a well-defined round-to-oval, smoothly marginated tumor measuring 43 mm × 56 mm in size located endobronchially in the proximal right main stem bronchus causing complete collapse of the right middle and lower lobe (Figure 2). A PET-CT scan showed a low metabolically active mass in the lower lobe, consistent with a carcinoid tumor, and metabolically active prominent and probably reactive mediastinal and hilar lymph nodes.

FINAL DIAGNOSIS

The definitive diagnosis of typical bronchial carcinoid was confirmed in the transbronchial biopsy of the mass.

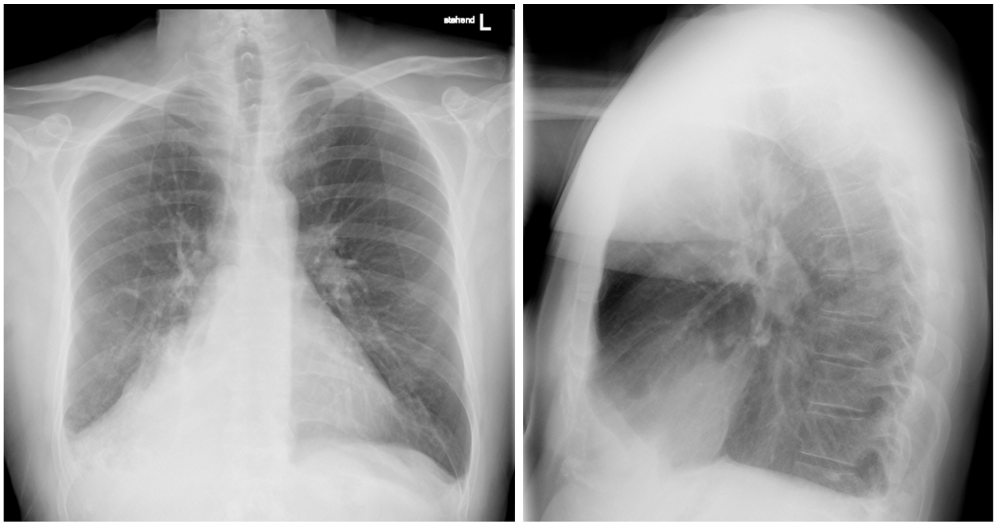

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Figure 1 The initial chest roentgenogram showing an unclear visualization of the right hilar shadow suggestive of right lower lobe collapse with minimal right-sided pleural effusion.

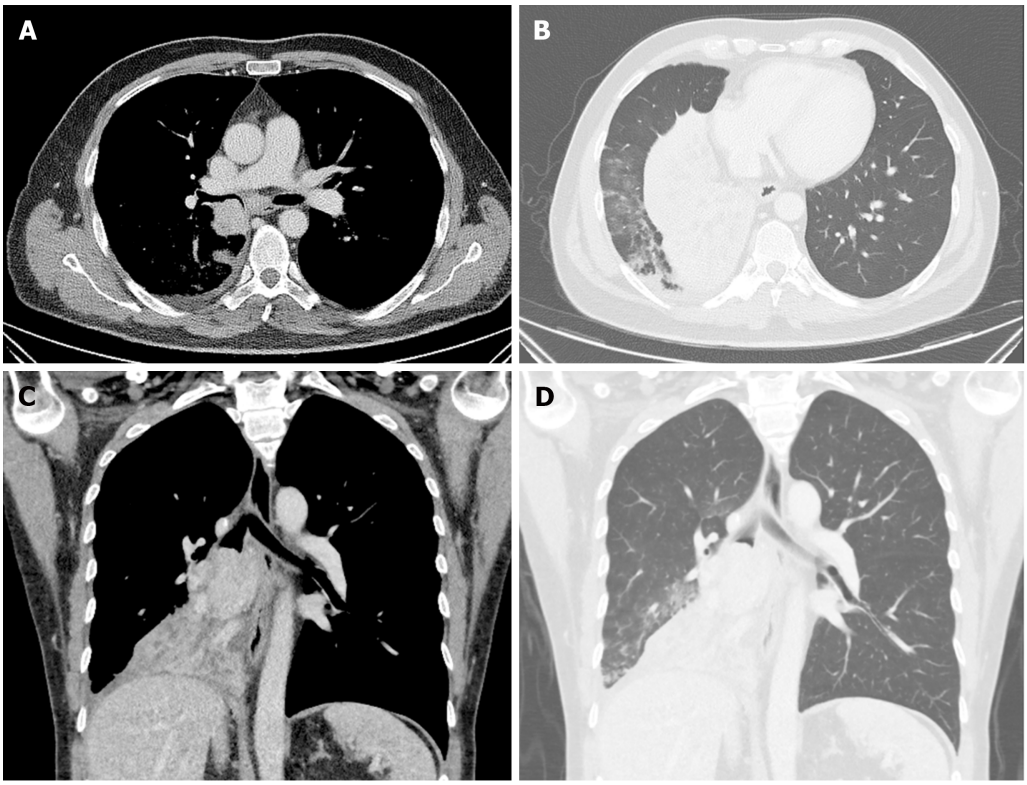

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Figure 2 Preoperative computed tomography scan. A and B: Mediastinal window during the phase of contrast shows a well-defined 43 mm × 56 mm round bordered intraluminal growth in the right bronchus intermedius occluding the airway; C and D: Lung window shows near complete collapse of the right middle lobe with no aeration post obstruction.

TREATMENT

All pulmonary carcinoid tumors should be treated as malignancies, as the only curative approach is a margin-free surgical resection. A right middle and lower sleeve lobectomy with bronchoplastic repair and radical lymphadenectomy were performed. Histopathologic examination of the resected tumor (Figure 3) revealed a solid growth pattern of uniform tumor cells with granular chromatin. The histologic appearance of the resection specimen was consistent with the transbronchial biopsy specimen. Here, the mitotic rate was under 1%, with fewer than 2 mitoses in 2 mm^2, and necrosis was not detected. Strong immunohistochemical positivity for synaptophysin and chromogranin was detected, and

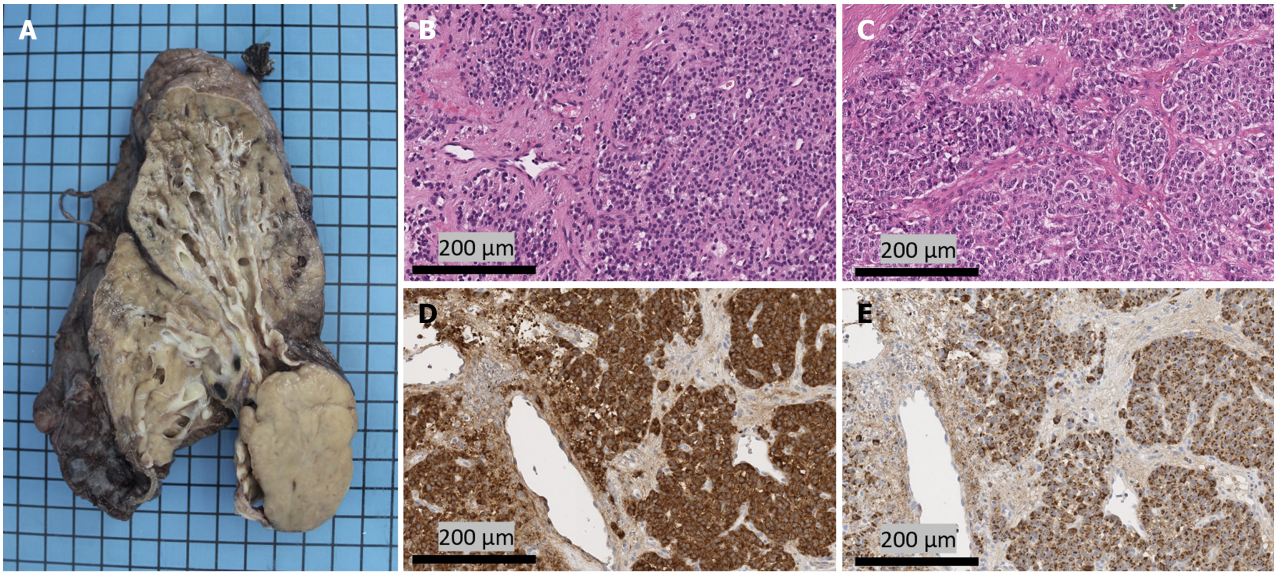

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Figure 3 Surgical specimen. A: Macroscopic appearance of a solid, well-circumscribed intraluminal tumoral lesion in the right main stem bronchus; B and C: Representative images of tumor cells revealing round nuclei, granular chromatin and large eosinophilic cytoplasm (H&E); D: Immunohistochemistry showing positivity to chromogranin (Scale 1:100); E: The tumor demonstrates high synaptophysin expression.

somatostatin receptor subtype 2a showed strong membranous expression (score 3+). All of these features lead to the diagnosis of a typical carcinoid of the lung[5].

The patient had no postoperative complications. He was discharged home 14 d after the operation and was followed up at 3 mo, 6 mo, 10 mo, and 36 mo. On a DOTATATE PET scan performed 3 mo after the operation, there was no evidence of any lymphadenopathy or distant metastasis. At 36 mo after the operation, the patient was stable, with no evidence of recurrence in the DOTATATE PET scan. Unexpectedly, after tumor resection and in the absence of any therapy, the patient presented complete clinical remission of ulcerative colitis.

Long-standing therapy-resistant ulcerative colitis was first diagnosed in 2009. Initially, combined therapy with rectal and oral mesalazine was prescribed; however, the patient reported further persistent bloody diarrhea with acute disease flares approximately twice a year. On colonoscopy, pronounced erythema in the rectum with fibrin deposits and granularity of the mucosal surface was observed. All findings were consistent with the diagnosis of ulcerative colitis. The diagnosis of ulcerative proctitis was ultimately confirmed in the step biopsies. The addition of topical steroids did not relieve the symptoms of colitis. Furthermore, the patient reported an increased frequency of bowel movements accompanied by rectal tenesmus. After three months of remission induction therapy with high-dose systemic steroids, mild disease control was achieved; however, any attempt at dose reduction was not tolerated by the patient. For this reason, immunomodulatory therapy with azathioprine was initiated, which led to a notable alleviation of the symptoms and disease remission. Due to alterations in both ALT and AST levels, azathioprine therapy was replaced by 6-mercaptopurin in combination with systemic steroids. The intestinal manifestations responded moderately to this therapy, and the steroids were reduced over time. The therapy with 6-mercaptopurin was carried out until two weeks before the lung operation with a milder course of ulcerative colitis.

OUTCOME AND FOLLOW-UP

Remarkably, after resection of the pulmonary carcinoid, the patient presented an unaltered bowel habit with no need for therapy. In light of the clinical evolution of the intestinal symptoms, it is questionable whether the clinical manifestations of ulcerative colitis were exacerbated by the pulmonary carcinoid or whether they were part of a carcinoid syndrome.

DISCUSSION

Here, we report a case of a young adult with an over 10-year history of ulcerative colitis and concurrent pneumonitis episodes who was diagnosed with pulmonary carcinoid. After complete resection of the pulmonary carcinoid, the intestinal symptoms completely subsided with no need for further therapy even after three years of follow up. The presence of a 56 mm measuring pulmonary carcinoid suggests[6] that the carcinoid was already present at the time of the patient's ulcerative colitis diagnosis. Pulmonary carcinoids are rare neoplasms that are able to synthesize and secrete neuroendocrine peptides into the central circulation, causing many symptoms, such as flushing or diarrhea. Taken together, these facts and the timely evolution of the condition indicated that the intestinal manifestations in this patient

seemed to be exacerbated by the pulmonary carcinoid or even the result of a carcinoid syndrome. To our knowledge, this is the first reported case of pulmonary carcinoid with this clinical presentation. Interestingly, studies in mice have shown that serotonin increases the susceptibility to experimental colitis[7].

The diagnosis of pulmonary carcinoids is often overlooked even in symptomatic patients due to low clinical suspicion and the various ways in which pulmonary carcinoids can present[8,9]. Apart from symptoms such as chest pain, atelectasis or pleural effusion, common pulmonary manifestations are hemoptysis, postobstructive pneumonitis, and dyspnea, especially in centrally located tumors, as in our case[1]. Carcinoids can also be associated with a variety of hormonally induced systemic symptoms due to gastrin, vasoactive intestinal peptide, serotonin, or histamine production, and this condition is termed carcinoid syndrome[9]. The most commonly encountered symptoms are flushing of the face, severe, debilitating diarrhea, and bronchospasms[10]. Metastatic tumor spread in the liver circumvents the hepatic inactivation of these substances and leads to the development of carcinoid syndrome. Rarely, bronchial and ovarian carcinoids can release hormones directly into the systemic circulation, thereby producing symptoms in the absence of liver metastasis[4]. Carcinoid syndrome-associated diarrhea is, as in our patient, unspecific and usually described as intermittent and sporadic. It is often accompanied by mild abdominal cramping and may become continuous when complicated by bacterial overgrowth[3]. This somewhat atypical course may explain the delayed diagnosis of NETs, which can sometimes occur after many years of unspecific symptoms that result in patients being diagnosed with an advanced incurable disease.

Typical bronchial carcinoids rarely metastasize and have an excellent prognosis even when regional lymph nodes are involved[2]. Surgery has been reported to provide a 5- and 10-year survival rate of > 90% for typical pulmonary carcinoids and is the treatment of choice[4].

CONCLUSION

In conclusion, the present case highlights that localized pulmonary carcinoids can potentially cause a carcinoid syndrome even in the absence of liver metastasis. Furthermore, intestinal manifestations, among other paraneoplastic symptoms, can precede symptoms of bronchial obstruction and superinfection. Thus, in patients with a history of recurring pneumonitis, the differential diagnosis of patients with persistent diarrhea and abdominal cramping despite optimum medical treatment should include pulmonary carcinoids[2]. A strong clinical suspicion aided by radiological examinations, including chest CT scans, enables the timely and accurate diagnosis of pulmonary carcinoids, which is crucial for early surgical resection[1].

FOOTNOTES

Author contributions: Mota-Reyes C and Hoffmann H designed the study; all co-authors analyzed the data and wrote the manuscript.

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