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[1]Passed away on October 20, 2007
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Telephone: +86-10-59080039
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Telephone: +86-10-59080039
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Spectrum of non-inflammatory bowel disease and non-infectious colitis

Ioannis E Koutroubakis

Ioannis E Koutroubakis, Department of Gastroenterology, University Hospital Heraklion, Crete, Greece
Author contributions: Koutroubakis IE wrote the manuscript.
Correspondence to: Ioannis E Koutroubakis, Professor, MD, PhD, Department of Gastroenterology, University Hospital Heraklion, PO Box 1352, 71110 Heraklion, Crete, Greece. ktjohn@her.forthnet.gr
Telephone: +30-2810-392253 Fax: +30-2810-542085
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Abstract

A variety of inflammatory diseases of the colon, which can be differentiated from inflammatory bowel disease (IBD) and infectious colitis by their clinical, endoscopic and histological characteristics, are reported as non-IBD and non-infectious colitis. These diseases include microscopic colitis, ischemic colitis, segmental colitis associated with diverticula, radiation colitis, diversion colitis, eosinophilic colitis and Behcet's colitis. The etiopathogenesis of most of these diseases remains obscure and the epidemiological data are rather limited. These conditions are often troublesome for the patient and are associated with diagnostic difficulties for the physician. In many cases the treatment is empirical and there is a need for future research using randomized controlled trials.

Key words: Diversion colitis; Ischemic colitis; Microscopic colitis; Radiation colitis; Segmental colitis

Peer reviewer: Elias A Kouroumalis, Professor, Department of Gastroenterology, Medical School, University of Crete, Heraklion, Crete, Greece

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This issue of *World Journal of Gastroenterology* contains a number of articles focusing on diagnosis and management of non-inflammatory bowel disease (IBD) and non-infectious colitis. This term includes a variety of inflammatory diseases of the colon, which can be differentiated from IBD and infectious colitis by their clinical, endoscopic and histological characteristics[1,2]. These diseases include microscopic colitides (collagenous and lymphocytic colitis), ischemic colitis, segmental colitis associated with diverticula (SCAD), radiation colitis, diversion colitis, eosinophilic colitis and Behcet' s colitis. The etiopathogenesis of most of these diseases remains obscure. Clinical presentations include chronic, watery diarrhoea, abdominal pain and intermittent rectal bleeding. Constitutional symptoms are typically absent and laboratory data are often non-specific. Colonoscopic evaluation and mucosal biopsy are essential in establishing these diagnoses and to exclude IBD and infectious colitis. Prognosis and responses to treatment are variable. In general these conditions are often troublesome for both the patient and the physician. Most of these diseases are uncommon; therefore, epidemiologic data and data from controlled trials are not readily available.

Experts for these diseases were invited to write clinical guidelines for the diagnosis and management of the most common and more important of these diseases, although the scarcity of original data for the recently characterized forms of colitis make this task rather difficult.

Ischemic colitis is the most common form of gastrointestinal ischemia and accounts for 1 in 1000 hospitalizations. However, due to its mild and transient nature the incidence of IC is believed to be underestimated[3]. Although frequent in the elderly, younger patients may also be affected. The first two articles[4,5] deal with the diagnosis and management of ischemic colitis and the diagnostic approach of chronic GI ischemia.

Segmental colitis (or diverticular colitis) has been defined as the chronic mucosal inflammation associated with diverticular disease. This condition, which is usually called segmental colitis associated with diverticulosis (SCAD), is mainly characterized by the involvement of the sigmoid colon with sparing of the rectum and proximal colon. SCAD often mimics IBD at endoscopic and histological examination[6]. Freeman[7] has recently reviewed the clinical, pathogenetic and therapeutic features of this disease.

Collagenous colitis and lymphocytic colitis are the two major conditions that are characterized by chronic

watery diarrhoea, without endoscopic or radiological lesions, but with histological abnormalities and are therefore considered as "microscopic colitis". Recent data suggests that the incidence of microscopic colitis is slightly less than the incidence of chronic idiopathic inflammatory bowel diseases (IBD)[8,9]. In their review Tysk *et al*[10] provide the current concepts on the diagnosis and management of microscopic colitis.

Radiation colitis has been known for years as an insidious and progressive iatrogenic disease that frequently develops 6 months to 5 years after regional radiotherapy for malignancy. Although improvements have been made in radiotherapy delivery, the incidence of radiation colitis is increasing. Kountouras *et al*[11] present an extensive review on the recent advances in the management and prophylaxis of radiation colitis.

The articles cited in this review of non-IBD and non-infectious colitis hopefully serve to remind us that there are a variety of inflammatory diseases of the colon. The articles aid in early diagnosis of these diseases and provide us with current therapeutic options, as well as future prospects.

Other diseases that are rather rare and not included in these articles are diversion colitis, eosinophilic colitis and Behcet's colitis.

Diversion colitis is a non-specific colonic inflammation following surgical diversion of the fecal stream away from the colorectal mucosa. Such surgery may be necessary in cases of colon cancer, trauma or inflammatory diseases. Diversion colitis is characterized histopathologically by a chronic lymphoplasmacytic inflammatory infiltrate, and the existence of lymphoid follicular hyperplasia is considered to be a hallmark feature[12]. The development of diversion colitis is attributed to a lack of short chain fatty acids (SCFA), normally produced from the breakdown of complex carbohydrates by resident bacteria. SCFA are the preferred energy substrate for colonocytes and are necessary for normal metabolism. Although most patients are asymptomatic, common symptoms are rectal bleeding, tenesmus and mucous discharge. It is observed in up to 91% of adults following diversion and it is usually mild or moderate but rarely severe (only in 4% of cases). The restoration of fecal continuity is the treatment of choice and is curative. However, prolonged diversion causes involution and atrophy of the segment leading to a poor functional outcome. Other possible treatment options are SCFA enemas (or 5-ASA enemas)[13,14].

Eosinophilic colitis is an etiologically obscure and rare colonic inflammation which can be associated with involvement of other sections of the gastrointestinal tract from esophagus to rectum in a diffuse or segmentary manner. An infiltrate of eosinophilic granulocytes is found to varying degrees in all wall layers. Eosinophilic gastroenteritis may involve any part of the gastrointestinal tract, however colonic involvement is usually confined to the right colon. The common clinical symptoms are acute colicky pain, diarrhoea, rectal bleeding and weight loss. A history of food intolerance or allergy is present in most of the patients and peripheral eosinophilia is present in 80% of cases. Colonoscopy is usually inconclusive but histology reveals an inflammatory infiltrate by eosinophils in the mucosal and submucosal layers. Treatment includes initially dietary manipulation and avoidance of specific foods, but in refractory cases, corticosteroids, immunosuppressants and sodium chromoglycate are effective although the published data on treatment of eosinophilic colitis are rather limited[15,16].

Behcet's disease is a chronic inflammatory disease characterized by systemic manifestations such as recurrent oral and genital ulcerations, ocular and cutaneous lesions, arthritis and vascular disease. Gastrointestinal involvement is rare; its frequency has been reported to be 3%-25%, with geographical differences[17]. Cases of Behcet's disease cluster along the ancient Silk Road, which extends from eastern Asia to the Mediterranean basin. In cases with ileocolonic involvement, it is often difficult to distinguish Behcet's disease from other inflammatory bowel diseases. Intestinal Behcet's disease commonly accompanies ulcerative lesions in the small and large bowel. The diagnosis of intestinal Behcet's disease, therefore, often depends on clinical manifestations of systemic Behcet's disease and intestinal ulcerative lesions. Treatment options include corticosteroids, azathioprine, or cyclosporine thalidomide and infliximab[18,19].

In conclusion, there is a wide variety of rarer causes of colitis included in the term non-IBD non-infectious colitis. The etiopathogenesis of most of these diseases remains obscure and the epidemiological data are rather limited. In many cases the treatment is empirical and there is a need for future research using randomized controlled trials.

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TOPIC HIGHLIGHT

Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, *Series Editor*

Diagnosis and management of microscopic colitis

Curt Tysk, Johan Bohr, Nils Nyhlin, Anna Wickbom, Sune Eriksson

Curt Tysk, Johan Bohr, Nils Nyhlin, Anna Wickbom, Department of Medicine, Division of Gastroenterology, Örebro University Hospital and School of Health and Medical Sciences, Örebro University, SE-701 85 Örebro, Sweden
Sune Eriksson, Department of Pathology, Örebro University Hospital, SE-701 85 Örebro, Sweden
Author contributions: All authors contributed in writing the article.
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Correspondence to: Curt Tysk, Professor, Department of Medicine, Division of Gastroenterology, Örebro University Hospital, SE-701 85 Örebro, Sweden. curt.tysk@orebroll.se
Telephone: +46-19-6021000 Fax: +46-19-6021774
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Abstract

Microscopic colitis, comprising collagenous and lymphocytic colitis, is characterized clinically by chronic watery diarrhea, and a macroscopically normal colonic mucosa where diagnostic histopathological features are seen on microscopic examination. The annual incidence of each disorder is 4-6/100 000 inhabitants, with a peak incidence in 60-70-year-old individuals and a noticeable female predominance for collagenous colitis. The etiology is unknown. Chronic diarrhea, abdominal pain, weight loss, fatigue and fecal incontinence are common symptoms, which impair the health-related quality of life of the patient. There is an association with other autoimmune disorders such as celiac disease, diabetes mellitus, thyroid disorders and arthritis. Budesonide is the best-documented short-term treatment, but the optimal long-term strategy needs further study. The long-term prognosis is good and the risk of complications including colonic cancer is low.

Key words: Microscopic colitis; Collagenous colitis; Lymphocytic colitis; Chronic diarrhea; Budesonide

Peer reviewer: David S Rampton, Professor, Centre for Gastroenterology, Institute of Cell and Molecular Science, Queen Mary School of Medicine and Dentistry, London E1 2AD, United Kingdom

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INTRODUCTION

Chronic diarrhea, reported in 4%-5% of individuals in Western populations, is a common cause for consulting a physician in general practice or in internal medicine, and for referral to a gastroenterologist[1]. Microscopic colitis (MC), previously regarded as rare, and certainly overlooked, has now emerged as a common cause of chronic diarrhea especially in elderly women. The condition is characterized clinically by chronic watery diarrhea, and a macroscopically normal or almost normal colonic mucosa, where microscopic examination of mucosal biopsies reveals characteristic histopathological changes[2]. MC comprises the two entities collagenous colitis (CC) and lymphocytic colitis (LC), which have indistinguishable clinical presentations but are separated by histopathological characteristics. This review will highlight epidemiology, clinical features, diagnosis and management of MC.

EPIDEMIOLOGY

CC and LC, first described in 1976[3] and in 1989[4], respectively, have mostly been reported from European or North American centers, but the disease is found worldwide[5-10]. Currently, epidemiological data have been reported from seven different regions (Table 1)[5,6,11-17]. Long-term epidemiological data from Sweden and US since the 1980s show a rising incidence, which seems to have levelled off during the last study periods in the Swedish study. Whether the increasing incidence figures are an artefact, reflecting an increased awareness and improved diagnosis of the condition, or in fact represents a true rise is at present unknown. MC may be diagnosed in 10%-20% of cases investigated for chronic

Table 1 Annual incidence/100 000 inhabitants in population-based epidemiological studies of CC and LC[5,6,11-17]

Region and study period	CC	LC
Örebro, Sweden 1984-1988	0.8	
Örebro, Sweden 1989-1993	2.7	
Örebro, Sweden 1993-1995	3.7	3.1
Örebro, Sweden 1996-1998	6.1	5.7
Örebro, Sweden 1999-2004	5.2	5.5
Terassa, Spain 1993-1997	2.3	3.7
Iceland 1995-1999	5.2	4.0
Olmsted County, USA 1985-1989	0.3	0.5
Olmsted County, USA 1990-1993	1.6	1.0
Olmsted County, USA 1994-1997	3.9	6.4
Olmsted County, USA 1997-2001	6.2	12.9
Lothian, UK 1998-2003	0.8	
Tayside, UK 1999-2004	1.1	0.6
Calgary, Canada 2002-2004	4.6	5.4

watery diarrhea[5].

CC mainly affects middle-aged women with a peak incidence around 65 years of age, and the female:male ratio is about 7:1 (Figure 1)[6,18]. However, the disease can occur in all ages, including children[19]. In LC, the peak incidence is in the same age group as CC, but the female predominance is less pronounced with a female:male ratio of 2-3:1 (Figure 1)[20].

CLINICAL PRESENTATION

The clinical symptoms of CC and LC are similar and the diseases cannot be differentiated on clinical grounds. Both disorders cause chronic or recurrent non-bloody, watery diarrhea, often associated with nocturnal diarrhea, diffuse abdominal pain, and weight loss, which may be substantial[18,20,21]. Although some patients may suffer from severe diarrhea, serious dehydration is rare. Fatigue, nausea and fecal incontinence are other associated symptoms and the disease may significantly impair quality of life in the affected patient[22,23].

The onset of disease can be sudden and mimic infectious diarrhea[18,20]. The clinical course is often chronic relapsing and benign. Severe complications are rare, although there are reports of colonic perforation in CC[24-26]. No increased risk of colorectal cancer has been reported in CC[27]. A few cases with concomitant lymphoproliferative disorders and CC have been presented but further studies are required to assess if there is an increased risk[28].

Some patients may have mild symptoms that may be misinterpreted as irritable bowel syndrome[29]. Morphological findings of LC have been reported even in constipated or asymptomatic patients[30]. The natural history of the condition in these patients is unknown.

Patients with MC often have concomitant autoimmune diseases[18,20,21]. The most common are thyroid disorders, celiac disease, diabetes mellitus and rheumatoid arthritis. The occurrence of such associations, reported in up to 40%-50% of patients in some cases, is variable depending on the study, and differences between LC and CC with respect to

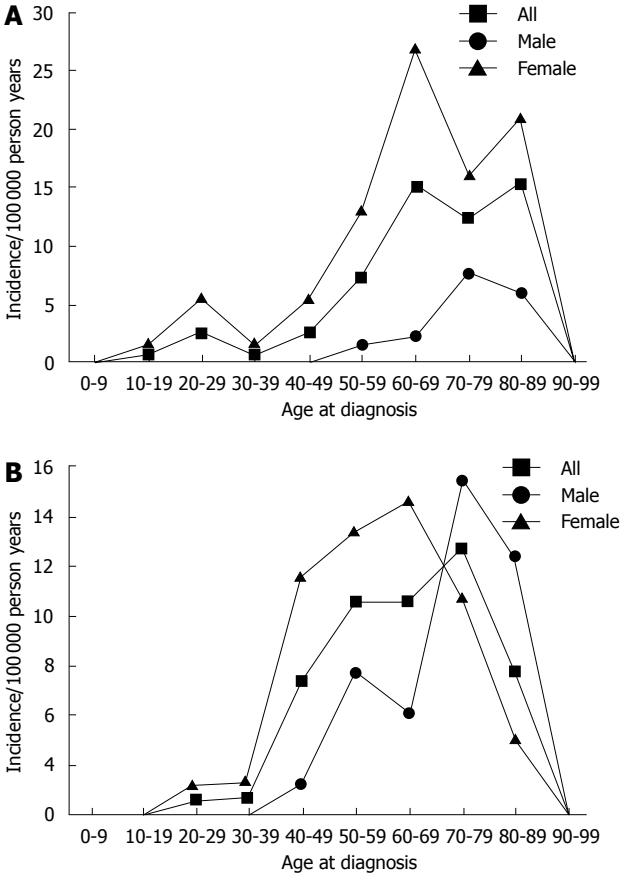

Figure 1 Age- and sex-specific incidence of CC (A) and LC (B). Reprinted with permission from *Gut* 2004; 53: 346-350[5].

associated conditions have been described[18,20,21,31]. Bile acid malabsorption can often co-exist with MC and lead to worsening of symptoms[32]. An interchange between ulcerative colitis or Crohn's disease and MC has been reported occasionally[33,34]. Whether this merely is a chance association of two fairly common disorders occurring in the same individual, or results from a common genetic predisposition or shared immunological pathways remains unknown.

ETIOLOGY AND PATHOGENESIS OF MUCOSAL INFLAMMATION

The cause of MC is multifactorial and largely unknown. CC and LC are presently considered to represent specific mucosal responses in predisposed individuals to various noxious luminal agents. As CC and LC have many clinical similarities and share histopathological features, except for the subepithelial collagen layer found in CC, it has been discussed whether LC and CC are in fact the same disease seen in different stages of development. Conversion of LC to CC or *vice versa* has been reported. However, conversion is seen infrequently and this fact, together with the observed difference in sex ratio, makes it more likely to consider CC and LC as two separate but related entities.

Data on the mucosal inflammation in MC are limited. In the epithelium, mainly CD8+ T lymphocytes are

found that carry the α/β form of the T-cell receptor, and in the lamina propria there are mainly CD4+ T lymphocytes[35]. By means of segmental colorectal perfusion, increased luminal levels of eosinophilic cationic protein (ECP), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been found in CC[36-38]. By immunohistochemistry, others have verified increased mucosal levels of VEGF that are not affected following therapy with budesonide[39]. A study of cytokines in MC found a TH1 mucosal cytokine profile with interferon γ, tumor necrosis factor (TNF)α and interleukin-15 as the predominantly up-regulated cytokines[40]. Using Ussing chamber technology, transcellular and paracellular mucosal permeability has been found to be increased in patients with CC[41,42]. The excess subepithelial collagen in CC may be caused by an imbalance of collagen turnover. An increased collagen synthesis is supported by the finding of an increase in the number or the activity of myofibroblasts[43]. Among degrading enzymes, matrix-metalloproteinases (MMPs) have a central role that is regulated by tissue endogenous inhibitors of metalloproteinases (TIMPs)[44]. Impaired collagen degradation in CC is supported by the finding of restricted MMP-1 RNA expression and increased TIMP expression[45].

GENETICS

A familial occurrence of MC has been reported, but the role of genetic factors still remains largely unknown[46-49]. Human leukocyte antigen (HLA) studies have shown an association between MC and HLA-DQ2 or DQ1/3, and recently an association has reported between MC and HLA-DR3-DQ2 haplotype and with TNF2 allele carriage, irrespective of the presence of concomitant celiac disease[50,51]. Variants of the MMP-9 gene have been reported to be associated with CC[52]. No association with NOD2/CARD15 polymorphisms and susceptibility to CC has been found[53].

LUMINAL FACTORS

The mucosal inflammation with an increased number of intraepithelial T lymphocytes has suggested that MC may be caused by an immunological response to a luminal agent in predisposed individuals. This theory is supported by the observation that diversion of the fecal stream by an ileostomy normalizes or reduces the characteristic histopathological changes in CC[54]. After closure of the ileostomy, recurrence of symptoms and histopathological changes occur.

Drug-induced MC

There are several reports on drug-induced MC and a strong likelihood of association has been found with acarbose, aspirin, Cyclo3 Fort, non-steroidal anti-inflammatory drugs, lanzoprazole, ranitidine, sertraline and ticlopidine[55]. Assessment of concomitant drug use in patients with MC is therefore important to identify and consider withdrawal of drugs that might cause or worsen the condition.

Infection

An infectious cause has been suspected, especially in patients with a sudden onset of disease. An association with MC and *Campylobacter jejuni, Yersinia enterocolitica* or *Clostridium difficile* has been reported occasionally[56-59]. LC shares many features with "Brainerd diarrhea", which refers to outbreaks of acute watery diarrhea with long duration, first reported among 122 residents of Brainerd, Minnesota, USA[60]. Colonic biopsies of these patients show epithelial lymphocytosis similar to LC, but no crypt distortion or epithelial destruction[61]. Investigations of several outbreaks of Brainerd diarrhea have established an incubation period of 10-30 d and median duration of illness of 16 mo[62]. Although an infectious agent is thought to be the cause of Brainerd diarrhea, no microorganism has yet been identified. Furthermore, a seasonal pattern of onset of LC[20,63] may support an infectious cause. However, in most cases of MC with a sudden onset, stool cultures remain negative.

Bile acids

Bile acid malabsorption can coexist with MC, which leads to worsening of symptoms. Concurrent bile acid malabsorption was found in 27%-44% of patients with CC and in 9%-60% of patients with LC[32,64,65]. These observations are the rationale for recommendations on bile acid binding treatment in MC. The treatment is especially effective in patients with concomitant bile acid malabsorption, but improvement has also been shown in patients without bile acid malabsorption.

Autoimmunity

The association with other autoimmune diseases such as thyroid disease, celiac disease, diabetes mellitus or arthritis has suggested an autoimmune process. However, no specific autoantibody or marker has been identified.

Nitric oxide (NO)

Colonic NO production is greatly increased in active MC caused by upregulation of inducible nitric oxide synthase (iNOS) in the colonic epithelium[66-69]. A major transcriptional inducer of iNOS gene expression is the transcription factor nuclear factor-κB (NF-κB). In active CC, colonic mucosal NF-κB has been found to be activated in epithelial cells but not in lamina propria macrophages, in contrast to ulcerative colitis[70]. The levels of NO are correlated to clinical and histological disease activity[67]. NO has been suggested to be involved in the pathophysiology of diarrhea in CC, as infusion in the colon of N^G-monomethyl-L-arginine, an inhibitor of NOS, reduced colonic net secretion by 70% and the addition of L-arginine, a precursor of NO synthesis, increased colonic net secretion by 50%[68]. Further

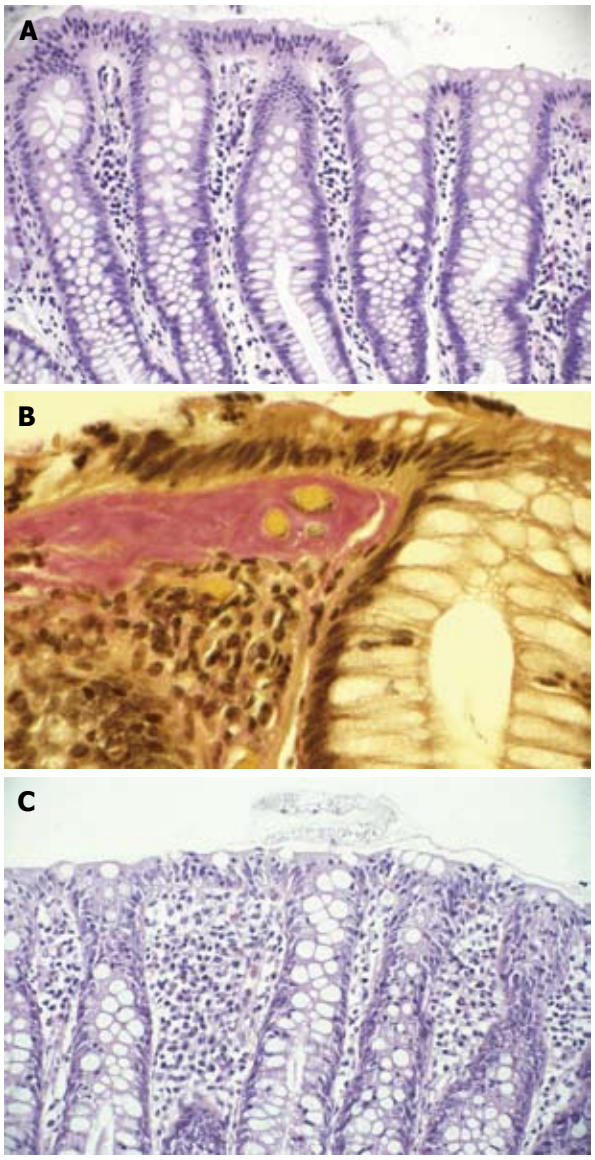

Figure 2 Biopsy from colon. A: normal colonic mucosa (H&E stain); B: typical findings of CC-increased subepithelial collagen layer, inflammation of lamina propria and epithelial cell damage with intraepithelial lymphocytes (Van Gieson's stain); C: typical findings of LC-epithelial cell damage with intraepithelial lymphocytes and inflammation in the lamina propria (H&E stain).

support for NO being involved in the pathogenesis of CC comes from therapeutic studies. Treatment with budesonide, in contrast to placebo, has resulted in a significant reduction of iNOS mRNA that is correlated with clinical and histopathological improvement[71].

Secretory or osmotic diarrhea

The exact mechanism of diarrhea in MC has not been clarified fully. In CC, diarrhea has been regarded as secretory and caused by reduced net absorption of Na^+ and Cl^- ions caused by epithelial cell lesions, and the thickened collagenous layer as a co-factor that causes a diffusion barrier, and by additional active Cl^- secretion[72]. Fasting, on the other hand, seems to reduce diarrhea, which indicates an osmotic component in some patients as well[73].

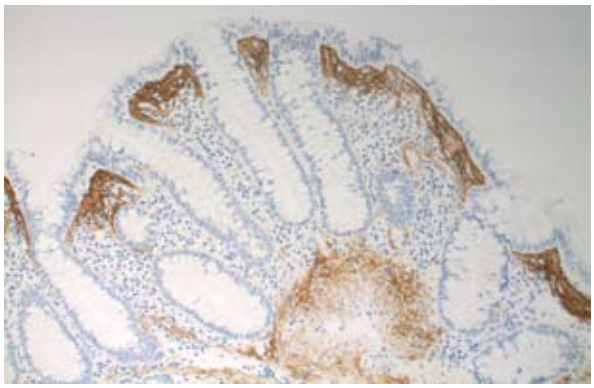

Figure 3 Tenascin immunostaining in CC.

DIAGNOSIS

Diagnosis of MC relies solely on typical microscopic changes seen in colonic mucosal biopsies[74]. In CC, a thickening of the subepithelial collagen layer is seen together with a chronic mononuclear inflammation in the lamina propria, and epithelial cell damage, with an occasionally increased number of intraepithelial lymphocytes (Figure 2). The thickened subepithelial collagen layer in CC is $\geqslant$ 10 μm in well-orientated sections, in contrast to a normal basal membrane of $<$ 3 μm. The thickening of the collagen layer may be variable and is most prominent in the ascending or transverse colon, and may be absent in biopsies from the sigmoid colon or rectum, which emphasizes the importance of obtaining biopsies from the proximal colon when diagnosing CC[75]. Generally, the histopathological changes are restricted to the large bowel, but a thickened collagen layer has infrequently been found in the stomach, duodenum or terminal ileum. In addition to conventional histological staining, the use of tenascin immunostaining has been suggested in uncertain cases of CC (Figure 3)[43,76].

The diagnostic features of LC (Figure 2) are an increased number of intraepithelial lymphocytes ($\geqslant$ 20/100 surface epithelial cells), in conjunction with surface epithelial cell damage and infiltration of lymphocytes and plasma cells into the lamina propria, but the collagen layer is normal, in contrast to CC[74]. In uncertain cases, immunostaining of CD3+ T lymphocytes facilitates the assessment of intraepithelial lymphocyte count (Figure 4).

Barium enema and colonoscopy are usually normal, although subtle mucosal changes can be seen such as edema, erythema and abnormal vascular pattern[18,20]. Tears of colonic mucosa have occasionally been seen during colonoscopy, which might be a sign of increased risk of colonic perforation during the procedure[26,77-79]. In the future, the use of confocal laser microscopy may enable *in vivo* diagnosis of MC[80-82].

Laboratory tests are non-diagnostic and only non-specific abnormalities such as moderately elevated C-reactive protein, erythrocyte sedimentation rate, or mild anemia are found. Stool tests reveal no pathological microorganisms, but fecal calprotectin can be slightly elevated[83].

Table 2 Data from four randomized, placebo-controlled trials of oral budesonide in CC and LC

Author year	Number of cases	Dosage	Clinical response budesonide *vs* placebo	Histological response budesonide *vs* placebo	Adverse events
Collagenous colitis					
Baert *et al*[91] 2002	28	9 mg/d Budenofalk 8 wk	Improvement: 8/14 *vs* 3/14 ($P = 0.05$)	Reduction of lamina propria inflammation in 9/13 *vs* 4/12 ($P < 0.001$) No difference in collagen layer	Mild No difference between treatment groups
Miehlke *et al*[93] 2002	45	9 mg/d Entocort 6 wk	Remission: 15/23 *vs* 0/22 ($P < 0.0001$)	Improvement in 17/23 *vs* 5/22 ($P < 0.01$) No difference in collagen layer	Mild 38% *vs* 12% $P = 0.052$
Bonderup *et al*[92] 2003	20	9 mg/d Entocort 8 wk	Response: 10/10 *vs* 2/10 ($P < 0.001$)	Reduction of overall inflammation ($P < 0.01$) and of collagen layer in sigmoid colon ($P < 0.02$)	None
Lymphocytic colitis					
Miehlke *et al*[95] 2007	41	9 mg/d Budenofalk 6 wk	Remission: 18/21 *vs* 8/20 ($P = 0.004$)	Response in 11/15 *vs* 4/12 ($P = 0.04$)	Mild No difference between treatment groups

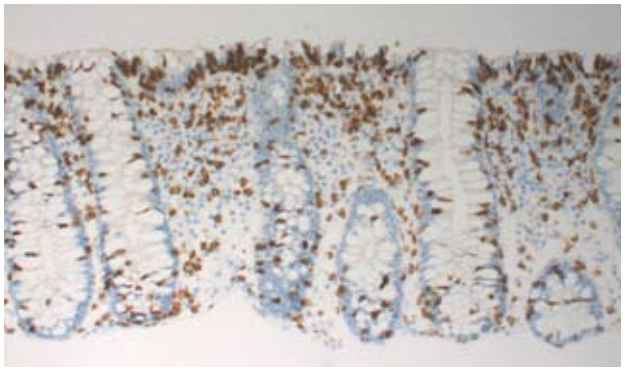

Figure 4 Immunostaining of CD3+ T lymphocytes in LC.

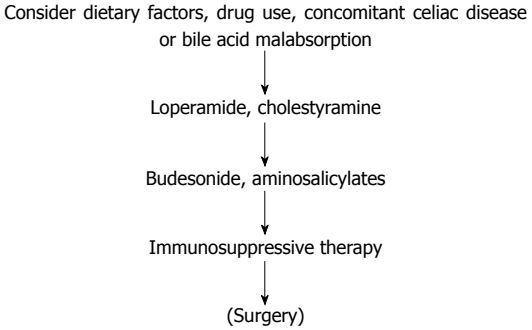

Figure 5 Treatment algorithm for MC.

ATYPICAL MC

In addition to CC and LC, other rare subtypes of MC have been described including MC with giant cells[84,85], paucicellular LC[86], cryptal LC[87], pseudomembranous CC[88], MC with granulomatous inflammation[89], and MC not otherwise specified[74]. The clinical features of these conditions are similar to those of classical MC, but histopathological appearance differs. Further studies are required to address the relationship and clinical significance of these atypical forms of MC[90].

THERAPY AND PROGNOSIS

A careful assessment of concomitant drug use and dietary factors such as excess use of caffeine, alcohol and dairy products that might worsen the condition is important. Concomitant bile acid malabsorption or celiac disease should be considered. In the patient with mild symptoms, loperamide or cholestyramine are recommended as the first step of treatment (Figure 5).

Budesonide is the best-documented treatment and significantly improves the clinical symptoms and the patient's quality of life. Three short-term, randomized controlled trials in CC have consistently shown that budesonide 9 mg daily for 6-8 wk is superior to placebo (Table 2)[91-93]. About 80% of patients responded to budesonide and had a decrease in the number of loose stools after 2-4 wk of therapy. In a Cochrane meta-analysis, the pooled odds ratio for clinical response with budesonide compared to placebo was 12.32 (95% CI 5.53-27.46), and the number needed to treat was two patients[94]. In a placebo-controlled trial including 41 patients, budesonide treatment was effective also in LC[95]. After 6 wk treatment, 18 of 21 patients (86%; 95% CI 65%-96%) in the budesonide group achieved a clinical response compared to eight of 20 patients (40%; 95% CI 22%-61%) in the placebo group, which yielded an odds ratio of 9.00 (95% CI 1.98-40.93; $P = 0.004$)[96]. The number needed to treat to achieve a clinical response with budesonide was three patients.

The relapse rate is high after cessation of successful short-term budesonide therapy in CC and 61%-80% of treated patients will have a recurrence of symptoms[91-93]. In clinical practice, tapering doses of budesonide to 3-6 mg/d have been used as maintenance therapy and may well control clinical symptoms. There is now evidence for such a strategy in CC, and two studies have proven maintenance therapy with budesonide 6 mg/d for 6 mo is well-tolerated and superior to placebo[97,98]. A total of 80 patients, who had responded to open-label budesonide, were randomized to budesonide 6 mg/d or placebo for 6 mo. Clinical response was maintained in 33/40 (83%) patients who received budesonide compared to 11/40 (28%) patients who

received placebo ($P = 0.0002$). Pooled odds ratio was 8.40 (95% CI, 2.73-25.81) with a number needed to treat of two patients for maintenance of clinical response with budesonide. Histological response was seen in 48% of patients who received budesonide compared to 15% of patients who received placebo ($P = 0.002$)[94]. However, 6 mo maintenance therapy did not alter the subsequent course, as the relapse risk after withdrawal of 24 wk maintenance treatment was similar to that observed after 6 wk induction therapy, and the median time to relapse was equal in the two groups (39 d *versus* 38 d)[97].

Other oral corticosteroids, such as prednisolone, are associated with more frequent side-effects, and the efficacy seems inferior to budesonide, although no formal comparative studies are available[99].

Bismuth subsalicylate has been shown to be effective in a small placebo-controlled study including nine patients with CC and five with LC[100]. This drug is not available in a number of countries because of concerns regarding drug toxicity.

Sulfasalazine or mesalazine have been extensively used in MC but not strictly evaluated in randomized placebo-controlled trials. In a recent trial, 64 patients with MC were randomized to mesalazine 2.4 g/d or mesalazine 2.4 g/d + cholestyramine 4 g/d for 6 mo. A high remission rate was seen in both treatment arms, and 85% of patients with LC and 91% of those with CC were in remission at study end. Combined therapy was superior in CC and induced an earlier clinical response in both diseases[101]. The benefit of mesalazine with or without cholestyramine needs to be confirmed in a placebo-controlled trial.

Antibiotics such as metronidazole or erythromycin have been used but not in a controlled fashion. Probiotic treatment shows uncertain results and need further evaluation[102]. *Boswelia serrata* extract has been tried in a placebo-controlled trial showing a non-significant trend in favor of active treatment[103].

In patients with unresponsive or steroid-resistant disease, immunosuppressive therapy may be considered, although the evidence is limited. An open study with azathioprine gave partial or complete remission in eight of nine patients with MC[104]. The efficacy of methotrexate has been assessed in a retrospective study[105]. Out of 19 patients with CC, a good response, generally seen within 2-3 wk of treatment, was seen in 16 and a partial response in two patients. The dose of methotrexate ranged from 5-25 mg/wk (median 7.5-10 mg/wk).

Surgical therapy may be considered for patients with severe unresponsive MC. Both split ileostomy and subtotal colectomy have been performed and reported as successful[54,106]. The indications for surgical therapy today are limited, considering the improvement of medical therapy.

The long-term prognosis of MC is generally good. In a follow-up study of CC, 63% of the patients had a lasting remission after 3.5 years, and in another cohort study, all 25 patients were improved 47 mo after diagnosis, and only 29% of them required ongoing medication[107,108]. A benign course was reported in 27 cases with LC, with resolution of diarrhea and normalization of histology in > 80% of patients within 38 mo[109]. Others have reported that 63% of patients with LC had a single attack, with a median duration from onset of symptoms to remission of 6 mo[20].

CONCLUSION

MC is a fairly common cause of chronic diarrhea, especially in elderly women, and may considerably impair the patient's quality of life. The correct diagnosis depends on the awareness of the condition by the clinician (referring the patient with chronic diarrhea to colonoscopy and not to barium enema), by the endoscopist (obtaining mucosal biopsies although the colonic mucosa is endoscopically normal) and by the pathologist (recognizing the histopathological features of MC). Treatment with budesonide is effective in the short term and improves the patient's symptoms and quality of life, but the optimal long-term therapy needs further study. The long-term prognosis is good and the risk of complications including colonic cancer is low.

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TOPIC HIGHLIGHT

Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, *Series Editor*

Recent advances in the management of radiation colitis

Jannis Kountouras, Christos Zavos

Jannis Kountouras, Christos Zavos, Department of Gastroenterology, Second Medical Clinic, Aristotle University of Thessaloniki, Ippokration Hospital, 54642 Thessaloniki, Greece
Author contributions: Kountouras J and Zavos C contributed equally to this work.
Correspondence to: Jannis Kountouras, MD, PhD, Professor of Medicine Gastroenterologist, Department of Gastroenterology, Second Medical Clinic, Aristotle University of Thessaloniki, Ippokration Hospital, 8 Fanariou St., Byzantio, 55133 Thessaloniki, Greece. jannis@med.auth.gr
Telephone: +30-2310-892238 Fax: +30-2310-892743
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Abstract

Radiation colitis, an insidious, progressive disease of increasing frequency, develops 6 mo to 5 years after regional radiotherapy for malignancy, owing to the deleterious effects of the latter on the colon and the small intestine. When dealing with radiation colitis and its complications, the most conservative modality should be employed because the areas of intestinal injury do not tend to heal. Acute radiation colitis is mostly self-limited, and usually, only supportive management is required. Chronic radiation colitis, a poorly predictable progressive disease, is considered as a precancerous lesion; radiation-associated malignancy has a tendency to be diagnosed at an advanced stage and to bear a dismal prognosis. Therefore, management of chronic radiation colitis remains a major challenge owing to the progressive evolution of the disease, including development of fibrosis, endarteritis, edema, fragility, perforation, partial obstruction, and cancer. Patients are commonly managed conservatively. Surgical intervention is difficult to perform because of the extension of fibrosis and alterations in the gut and mesentery, and should be reserved for intestinal obstruction, perforation, fistulas, and severe bleeding. Owing to the difficulty in managing the complications of acute and chronic radiation colitis, particular attention should be focused onto the prevention strategies. Uncovering the fibrosis mechanisms and the molecular events underlying radiation bowel disease could lead to the introduction of new therapeutic and/or preventive approaches. A variety of novel, mostly experimental, agents have been used mainly as a prophylaxis, and improvements have been made in radiotherapy delivery, including techniques to reduce the amount of exposed intestine in the radiation field, as a critical strategy for prevention.

Key words: Radiation colitis; Acute; Chronic; Prevention; Intestinal obstruction; Perforation; Fistula; Bleeding

Peer reviewer: NKH de Boer, PhD MD, Gastroenterology and Hepatology, VU University Medical Center, PO Box 7057, 1007 MB, Amsterdam, The Netherland

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INTRODUCTION

Radiation colitis is an insidious, progressive disease of increasing frequency. It is usually iatrogenic and unavoidable and frequently develops 6 mo to 5 years after regional radiotherapy for malignancy[1,2]. About half of all patients with malignancies undergo irradiation as part of their therapy. Considerable morbidity and mortality accompany radiation treatment because of the deleterious effects on adjacent normal tissues, mainly the colon and the small intestine. The type and extent of injury, depending on the dose of the radiation and the radiation sensitivity of the gut and the duration, is highly variable, ranging from 3 mo to 30 years[1,3]. Serious consequences may develop after years of gestation, and the disease, its treatment, and the disability produced are formidable. Apart from acute radiation colitis, manifestations of chronic radiation injury include proctitis, hemorrhages, fistulas, abscesses with signs of sepsis, perforations, strictures, and even cancer. Therefore, novel means to increase resistance of the intestine to radiation damage and effective therapeutic strategies are needed to prevent and manage this disease.

MANAGEMENT OF COLITIS CAUSED BY IRRADIATION

In general, prior to start, each treatment should be

individualized, and any predisposing factor should be identified during its course in order to early recognize and treat complications. Once complications have arisen, it is best to deal with the irradiated tissue by the most conservative modality, because the areas of intestinal injury do not tend to heal. This may require early diversion or resection as conservative therapy, because fistulas and bleeding will become recurrent and intractable. The effectiveness of non-surgical approaches remains far from desirable, and bleeding recurrence represents a major drawback that leads to a need for consecutive therapeutic sessions and combination of techniques[4]. If diversion fails to control bleeding, resection is necessary, even if it involves an abdominoperineal resection.

From another general viewpoint, there is a similarity in the activation of mucosal cytokines between inflammatory bowel disease (IBD) and radiation proctosigmoiditis. Indeed, as in the case of IBD patients, the mucosal levels of interleukin (IL)-2, -6, and -8 are significantly higher in both diseased and normal segments of colon in patients with radiation proctitis, compared with normal controls. In addition, IL-1β levels are significantly higher in diseased segments, compared with endoscopically normal-appearing segments in radiation proctitis. Tumor necrosis factor-alpha (TNF-α) levels are also significantly elevated in irradiated mice compared with non-irradiated controls[5]. These data may partially explain the beneficial effects of similar systemic and topical drugs including mesalamine compounds and steroids when used in radiation-induced proctosigmoiditis[6].

ACUTE RADIATION COLITIS (TABLE 1)

Empirical-experimental management

The majority of acute radiation colitis is self-limited, and only supportive management is required[7]. It must be emphasized, however, that acute radiation syndrome with a threshold dose of 8 Gy in man, represents a lethal clinical-pathological unit, enteritis and proctocolitis necro-hemorrhagica, with unknown causal therapy. In this respect, the detection of phospho-Elk-1, a protein acting as a transcription factor activating specific genes, might be considered as a suitable and very sensitive marker of acute radiation-induced injury of large and small intestine[8]. Whether Elk-1 inhibitors, such as the compound A (CpdA) or the protective agent U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene], the effect of which probably results from the IL-1β mRNA reduction *via* the inhibition of ERK pathway, can be used in the management of this syndrome remains to be investigated[9,10].

Inflammatory cell infiltration of the colon is observed at an early stage of radiation-induced colitis. The migration of inflammatory cells from the circulation requires interactions between cell adhesion molecules on the vascular endothelium and molecules on the surface of leukocytes. Specifically, circulating leukocytes are recruited to sites of inflammation by a well-regulated and coordinated process that largely occurs in postcapillary venules. Adhesion molecules are expressed on the surface of endothelial cells, and leukocytes are involved in an orderly sequence of cell-cell interactions that include leukocyte adherence to vascular endothelium and the subsequent transendothelial migration into the inflamed tissue. Finally, reactive oxygen metabolites produced by activated leukocytes can induce damage to various cellular components, including structural and regulatory proteins, carbohydrates, lipids, DNA and RNA. In this respect, upregulation of intercellular adhesion molecule (ICAM)-1 and the accumulation of inflammatory myeloperoxidase-positive cells have been observed during acute radiation colitis prior to an overt radiation-induced ulcer, thereby playing important roles in the development of radiation-induced colonic ulcer[11]. Moreover, there is direct *in vivo* evidence that antioxidant mechanisms of the intestinal mucosa are not mobilized during the acute tissue radiation response; four days after exposure, during the inflammatory phase, superoxide dismutases (SOD) and catalase are decreased and glutathione peroxidases and metallothioneins are induced. Dexamethasone treatment modulates only glutathione peroxidase expression and does not influence either metallothionein or SOD expression. These experimental data indicate that during the radiation-induced acute inflammatory response, an imbalance of the antioxidant network of intestinal mucosa occurs[12].

In view of the aforementioned data, modulation of the leukocyte recruitment and activation pathway seems to be a potential therapeutic strategy against acute radiation colitis. Further supporting this consideration, experimental studies have demonstrated that leukocyte rolling is mediated by P-selectin and that firm leukocyte adhesion is supported by lymphocyte function antigen-1 in radiation-induced colitis. P-selectin-dependent leukocyte rolling is a precondition for subsequent leukocyte adhesion in radiation-induced intestinal damage. Therefore, targeting P-selectin and/or lymphocyte function antigen-1 might protect against pathologic inflammation in the colon induced by radiotherapy[13]. Moreover, Cu/Zn-SOD1 supplementation in an

Table 1 Management of acute radiation colitis

Management of acute radiation colitis
Supportive management
Anti-diarrheal medications and by reducing fat and lactose intake; In intractable cases hospitalization is required for parenteral feeding and elementary diet
Elk-1 inhibitors
Compound A (CpdA)
U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene]
Modulation of leukocyte recruitment and activation pathway
Targeting P-selectin and/or lymphocyte function antigen-1
Cu/Zn-SOD1 supplementation
Synthetic somatostatin analog octreotide
Other measures
Antiemetics
Steroid-containing suppositories
Recombinant granulocyte colony-stimulating factor in neutropenia
Epidermal growth factor

experimental model of radiation-induced intestinal inflammation has also been shown to decrease oxidative stress and adhesion molecule upregulation in response to abdominal irradiation. Specifically, a significant increase in the flux of rolling leukocytes and number of firmly adherent leukocytes in intestinal venules is observed after irradiation. Although administration of SOD1 has no effect on leukocyte rolling, it decreases leukocyte adhesion to intestinal venules significantly and in a dose-dependent way. Treatment with SOD1, at doses that reduce leukocyte recruitment, abrogates the increase in hydroperoxides in intestinal tissue and ICAM-1 upregulation in intestinal endothelial cells. The inflammatory score, but not a combined histology damage score, is also significantly reduced by SOD1[14].

Diarrhea associated with acute radiation colitis frequently resolves with anti-diarrheal medications and by reducing fat and lactose intake. The diarrhea rarely requires discontinuation of treatment unless chemotherapy is given concurrently with radiation[15]. Intractable diarrhea during the combined treatment may require hospital admission for administration of parenteral feeding. Elementary diet may also be introduced as an alternative to parenteral nutrition[16].

Patients refractory to anti-diarrheal medications may benefit from administration of the synthetic somatostatin analog octreotide[7]. Specifically, it has been shown that subcutaneous octreotide administration (150 μg t.i.d.) for 5 d is apparently an effective, well-tolerated treatment modality for concurrent chemoradiotherapy-induced diarrhea refractory to loperamide[17]. Octreotide appears to be more effective than conventional therapy with diphenoxylate and atropine in controlling acute radiation-induced diarrhea and eliminating the need for radiotherapy interruptions[18].

Apart from anti-diarrheal medications, other measures of general management of acute radiation enteropathy include administration of antiemetics. Steroid-containing suppositories may be helpful in the treatment of patients with anorectal inflammation[7]. Severe neutropenia from chemotherapy might require growth factors, such as recombinant granulocyte colony-stimulating factor (G-CSF, filgrastim) or granulocyte-macrophage colony-stimulating factor (GM-CSF, sargramostim) to shorten the period of neutropenia, and avoid excessively delayed therapy from the bone marrow depression[19]. G-CSF is a cytokine known to activate neutrophils *in vivo* and GM-CSF mediates its effects on the neutrophil lineage through its effects on phagocytic accessory cells and its synergy with G-CSF[20,21].

Epidermal growth factor, an endogenous peptide, trophic to the gastrointestinal tract, significantly decreases the acute clinical manifestations of experimental radiation enteritis[22]. Therefore, it may be effective in human acute radiation colitis[23].

CHRONIC RADIATION COLITIS (TABLE 2)

Empirical-experimental management

Chronic radiation colitis is recognized as a frequent and clinically important sequel of abdominal and pelvic irradiation treatment for malignant disease. Since radiotherapy is now being used more than ever before in the therapy of solid organ neoplasms of the abdomen and the pelvis, the incidence of radiation colitis is likely to increase in the future[24-26]. Importantly, it is a precancerous lesion: Radiation-associated rectal cancer originates from dysplasia due to radiation colitis and has a tendency to be diagnosed at an advanced stage and to bear a dismal prognosis[27,28]. Therefore, management of chronic radiation colitis remains a major challenge owing to the progressive evolution of the disease that includes development of fibrosis, endarteritis, edema, fragility, perforation, partial obstruction, and even cancer. Patients with this condition are commonly managed conservatively. Because the obstruction is only partial, decompression is easily achieved by nasogastric suction and parenteral support. The patient is then often discharged on a liquid-to-soft diet. However, this therapeutic regimen does nothing for the underlying pathology. Although total parenteral nutrition corrects denutrition and facilitates deferred surgery in some patients, severe radiation enteritis remains a poorly predictable progressive disease with numerous relapses[29]. The problem, sooner or later, will return with the patient further depleted by the chronic radiation colitis. In a recent meta-analysis assessing the incidence and significance of malnutrition and examining the efficacy of therapeutic nutritional interventions used to manage gastrointestinal side effects in patients undergoing pelvic radiotherapy, it has been shown that there is no evidence favoring the use of nutritional interventions to prevent or manage bowel symptoms attributable to radiotherapy[30]. Regarding the underlying

Table 2 Management of chronic radiation colitis (IL; TNF-α)

Management of chronic radiation colitis
Empirical-experimental management
Total parenteral nutrition
Anti-IL-6R
Cyclooxygenase-2 inhibitors
Rho kinase inhibitors
Small molecular inhibitors of TNF-α
Targeting cadherin-catenin complex pathways
Recombinant human IL-11
Low-residue diet combined with bismuth subsalicylate or opiate drugs, such as loperamide or diphenoxylate (for mild diarrhea)
Aminosalicylates
Prostaglandin-inhibiting compounds
Oral steroids (for severe cases)
Probiotics (Lactobacillus bulgaricus)
Antioxidants
Colestyramine Balsalazide (in radiation-induced proctosigmoiditis)
Peroxisome proliferation-activated receptor activators
Sucralfate enemas
Short-chain fatty acids
Hyperbaric oxygen
Control of bleeding (by endoscopic cauterization using a heater, BICAP probe, Nd: YAG or argon laser)
Surgery (indicated in intestinal obstruction, perforation, fistulas, and severe bleeding)

pathology, vascular damage consisting of fibrin thrombi, fibrinoid necrosis and subintimal thickening of the arterioles leads to persistent local ischemia, which results in diffuse fibrosis of the lamina propria and submucosa. The diffuse fibrosis, in turn, accelerates vascular damage and further worsens local ischemia, forming a vicious cycle, finally leading to ulceration of the bowel wall and serious complications including massive gastrointestinal hemorrhages and perforations[31]. Therefore, surgical intervention appears to be appropriate when the diagnosis of chronic radiation colitis is confirmed[32].

Nevertheless, chronic changes in cytokine levels after abdominal irradiation in rodents have recently been documented[33]. Structural injury of the bowel wall and mesentery were scored and correlated with the levels of TNF-α, IL-6, transforming growth factor (TGF)-β1, -β2, -β3 and interferon (IFN)-γ mRNA in large and small bowel of mice 18-25 wk after whole abdominal irradiation with 12.5 and 13.5 Gy. Abdominal irradiation seems to induce considerable bowel damage associated with increased levels of all cytokines compared with sham-irradiated (0 Gy) mice. These experimental data demonstrate long-term cytokine expression changes in the bowel wall after irradiation that parallel the responses noticed in other tissues prone to radiation-induced fibrosis, such as cutaneous and pulmonary tissues, thereby having implications for the prediction, treatment and/or prevention of chronic radiation colitis. For instance, chronic IL-6 elevations, even prior to the start of irradiation, may predict patients at risk of radiation fibrotic bowel damage in the same way that IL-6 baseline elevations have been shown to identify patients with an increased risk of radiation pneumonitis and pulmonary fibrosis following thoracic irradiation[33]. Since studies in animal models of IBD have shown that various antibodies to pro-inflammatory cytokines and their receptors, such as IL-6 receptor (IL-6R) or TNF, appear to suppress chronic intestinal inflammation by inducing T-cell apoptosis[34], it is reasonable to assume that such antibodies (anti-IL-6R) might also be used to manage radiation colitis. In addition, reduction in cytokine expression with cyclooxygenase (COX)-2 inhibitors and small molecular inhibitors of TNF-α may reduce the frequency and severity of long-term bowel damage. There is some evidence that the COX-2 pathway is implicated in radiation-induced gut injury[31,35,36]. COX-2 and nuclear factor κB (NF-κB) expression have been associated with histopathological changes in the human colon and rectum following abdominal radiotherapy[31].

Besides, in radiation colitis involving aberrant glands, cellular proliferation increases and spotted oncogene p53 expression is noticed. Therefore, radiation colitis and aberrant glands with p53 overexpression might predict malignant potential of this condition[37].

Three typical phases of radiation proctitis are defined on histological grounds (acute damage, and early and late regenerative phases), essentially correlating with the time interval between radiotherapy and surgery. Such characteristics are mirrored by alterations in cadherin-catenin expression and localization in rectal crypts; morphology at both cellular and glandular levels in the large bowel is dependent to an extent on cell-cell adhesion mediated by cadherin-catenin complexes. In this regard, P-cadherin is highly expressed in the acute radiation damage and early regenerative phases, with a decreased level of expression during late regeneration. E-cadherin and associated catenins are translocated from the membrane to the cytoplasm in degenerating crypts, with return to normal membranous expression in regenerating crypts. Therefore, radiation-induced proctitis represents an *in vivo* model of mucosal damage and regeneration, thereby providing a valid model to study events during epithelial injury and repair: altered cadherin and associated catenins expressions appear to be predictive indicators closely associated with these processes[38]. On the other hand, because the E-cadherin-catenin complex plays a critical role in the maintenance of normal tissue architecture, mutation of any of its components is believed to result in loss of cell-cell adhesion, thereby contributing to neoplasia development. In this respect, adenomatous polyposis coli (APC) gene abnormalities, found to be the "gate-keeping" event for the initiation of colorectal neoplasia, may lead to a disruption of normal cell-cell adhesion through altered association with catenins and the cell adhesion molecule E-cadherin that binds catenins[39]. Translocation of the β-catenin protein, a key downstream effector of the Wnt signal transduction pathway, is frequently found in colorectal cancer. This protein is also observed in the cytoplasm and/or nucleus of non-neoplastic irradiated colonocytes. Nuclear translocation of β-catenin correlates with loss of APC and gain of cyclin D1 expression, suggesting the activation of the Wnt pathway during radiation-induced colorectal carcinogenesis. Because the translocation of β-catenin is found in irradiated-colonic mucosa as well as in colon cancer, the disruption of the β-catenin expression may be one of the early events in radiation-induced colonic oncogenesis[40]. Based on these data, interventions on cadherin-catenin complex pathways may also be used against chronic radiation colitis and radiation-induced colonic carcinogenesis. Finally, in a novel mouse model of radiation-induced colitis, a combination of high-dose γ-irradiation and lack of major histocompatibility complex (MHC) class Ⅱ expression on cells of hematopoietic origin results in the development of radiation colitis. Therefore, protection and/or inhibition from radiation-induced colitis seems to require MHC class Ⅱ antigen expression by cells of hematopoietic origin[41]. In this regard, administration of the recombinant pleiotropic human cytokine IL-11, which stimulates bone marrow stem cells to proliferate, has been shown to decrease intestinal mucosal injury produced by radiation in animals, thereby providing a potential therapeutic regimen for the treatment and/or prevention of chronic radiation colitis[42].

Diarrhea, with or without abdominal cramps, is the most common symptom of chronic radiation colitis[26]. The etiology of chronic radiation-induced diarrhea may be attributable to accelerated small and large

bowel transit, bacterial overgrowth, increased intestinal permeability, malabsorption of bile salts, lactose, fat and carbohydrate, and pancreatic insufficiency, all of which can exist with or without small bowel or large bowel strictures[43,44]. In case of colonic strictures, spurious diarrhea can occur. Moreover, the above mentioned microvascular changes in the bowel wall lead also to mucosal atrophy and a non-specific chronic inflammatory cell infiltrate, which has resulted in a mistaken diagnosis of celiac sprue[45]. However, in most cases, the pathophysiology of the diarrhea is uncertain. While changes in intestinal absorption and motility, unrelated to bacterial overgrowth, have been implicated in the etiology of diarrhea, there has been no comprehensive evaluation of gastrointestinal function in chronic radiation colitis. Perhaps partly as a result of this, present approaches to treatment have often been empirical. A low-residue diet (i.e. a low-fiber diet poor in foods that increase bowel activity) combined with bismuth subsalicylate or opiate drugs, such as loperamide or diphenoxylate, might be sufficient for mild diarrhea[1]; loperamide-N-oxide slows small intestinal transit, increases bile acid absorption, and is effective in the treatment of diarrhea associated with chronic radiation colitis[26]. Other antidiarrheal agents can be administered, including aminosalicylates and prostaglandin (PG)-inhibiting compounds[46,47]. In severe cases of radiation colitis, oral steroids have been tried with limited success[48]. Randomized controlled trials are not available, and all treatment regimens are based on evidence from small pilot studies, including the administration of sulfasalazine[49], glutathione (GSH)[50], and antioxidants[51]. Furthermore, antibiotics are indicated if there is small bowel bacterial overgrowth syndrome[52,53]. Preliminary results suggest that probiotics may also be useful for treatment of radiation bowel disease, although no robust data exist[54]. Other studies suggested that colestyramine, an agent that binds bile acids in the colonic lumen, might be effective in preventing radiation-induced diarrhea if administered in dosages of 4 g t.i.d. during radiation therapy[55]. In the presence of low serum magnesium levels, intravenous administration of magnesium sulfate, together with low residue diet and antidiarrheals, may also ameliorate the diarrhea[56].

Anti-diarrheal and bulk-forming agents have a role in the management of rectal urgency, frequency, and fecal incontinence, which might be induced by radiation damage of the myenteric plexus of the rectum and internal anal sphincter[57]. Sulfasalazine, 5-aminosalicylic acid (5-ASA) preparations and corticosteroid enemas have minimal or no effects on rectal tenesmus or bleeding[48].

However, recent pilot studies indicate that balsalazide, a new 5-ASA drug that yields a high concentration of active drug to the distal colon, is able to prevent or reduce symptoms of radiation-induced proctosigmoiditis[58]. In addition, irradiation-induced inflammatory response could be modulated pharmacologically based on the anti-inflammatory properties of 5-ASA, which is a peroxisome proliferation-activated receptor (PPAR) activator. PPAR agonists are now emerging as therapeutic drugs for various inflammatory diseases characterized by impaired PPAR expression: Irradiation drastically reduces mRNA and protein levels of PPAR-α and -γ. Specifically, 5-ASA treatment normalizes both PPAR-α and PPAR-γ during the post-irradiation period (after 7 and 3 d, respectively). By promoting PPAR expression and its nuclear translocation, 5-ASA interferes with the NF-κB pathway, both reducing irradiation-induced NF-κB p65 translocation/activation and increasing the expression of NF-κB inhibitor (IκB) mRNA and protein. Therefore, 5-ASA prevents irradiation-induced inflammatory processes as well as expression of TNF-α, monocyte chemotactic protein-1, inducible nitric-oxide synthase, and macrophage infiltration. In addition, 5-ASA restores the IFN-γ/signal transducer and activator of transcription (STAT)-1 and STAT-3 concentrations that were impaired at 3 and 7 d post-irradiation and are correlated with suppressor of cytokine signaling-3 repression. Collectively, these data suggest that PPAR agonists might be effective in the prevention of inflammatory processes and immune responses during and after pelvic radiotherapy[59].

Fecal incontinence appears to be a late complication that causes symptoms years after radiation treatment. The specific mechanisms that cause incontinence are changes in anal resting tone, squeeze pressure, and rectal volume or rectal compliance. Other aspects associated with incontinence include further disorders such as proctitis, colitis, and other disturbances involving the lower digestive tract. The therapeutic options mainly comprise management of associated aspects, such as proctitis or diarrhea; surgical intervention should be the absolute exception[60].

It has been reported that sucralfate treatment has a protective effect against experimental radiation colitis. Sucralfate enemas prior to radiation lead to reduction in: (a) the number of apoptotic colonic crypt cells; (b) the number of caspase-3 positive cells; (c) oncogene p53 accumulation and p21 expression; and (d) proapoptotic Bax/anti-apoptotic Bcl-2 ratio in rats. Therefore, the protective effects of sucralfate against radiation colitis might be partially due to the suppression of radiation-induced apoptosis in the colon and the protection of the colonic epithelial stem cell region[61]. Sucralfate administration may be also effective in human radiation proctocolitis[62]. In addition, when compared with oral sulfasalazine plus rectal prednisolone enemas, sucralfate enemas give a better clinical response in human proctosigmoiditis, are better tolerated, and, because of the lower cost, they might be the preferred short-term regimen[48]. Moreover, topical sucralfate induces a lasting remission in the majority of patients with moderate to severe rectal hemorrhage due to radiation proctosigmoiditis[63].

Clinically, short-chain fatty acids (SCFAs) have been proposed as possible therapeutic agents in several conditions including radiation proctitis. Although some promising effects have been observed in uncontrolled studies, a specific therapeutic role for SCFAs remains to be defined[64].

Hyperbaric oxygen

Hyperbaric oxygen application appears to be a very effective means of treatment of chronic radiation colitis and non-healing wounds in the involved anorectal region[65]. Hyperbaric oxygen therapy can be considered as a treatment option after failure of standard treatments in patients with severe radiation proctopathy[66]. The rationale for hyperbaric oxygen is the creation of an oxygen gradient in hypoxic tissue that stimulates the creation of new blood vessels. Neoangiogenesis improves the blood supply and reduces the ischemia and necrosis responsible for severe complications. In a retrospective study of patients with severe radiation colitis refractory to medical management, hyperbaric oxygen therapy provided clinical relief and can thus prove to be a useful alternative to conventional treatment in patients with chronic radiation-induced necrosis of the digestive tract[67]. Moreover, in a systematic review of the literature on the application of hyperbaric oxygen prevention and treatment of delayed radiation injuries, all but seven of the 74 publications analyzed reported positive results when hyperbaric oxygen was delivered as treatment for or prevention of delayed radiation injury. These results are particularly impressive in the context of alternative interventions[68]. Hyperbaric oxygen may also be helpful in management of bleeding due to chronic radiation colitis in patients not controlled with conservative measures such as formalin and laser therapy[69,70]. Hyperbaric oxygen treatment and infusion of PG E1 abolishes completely tarry stools and hematuria, and reverses the endoscopic findings of radiation colitis and cystitis[71].

Control of bleeding

Rectal bleeding due to radiation colitis usually results from telangiectasias. It is frequently minor, but blood transfusions may be required. Endoscopic cauterization using a heater, BICAP probe, Nd:YAG or argon laser can reduce bleeding.

Argon plasma coagulation therapy appears to be a simple, safe, and effective technique in the management of hemorrhagic radiation-induced proctosigmoiditis and is now generally accepted as the treatment of choice followed by local application of formalin if this fails[31,72,73]. Argon plasma coagulation, a non-contact thermal coagulation technique that reduces rectal bleeding in 80%-90% of cases, is applied endoscopically, with a probe passing through the endoscope that delivers a field of argon gas to the mucosal surface, where it is ionized by a high-voltage filament resulting in superficial mucosal heating and coagulation of friable blood vessels. Topical formalin therapy depends on direct application of a 4% concentration of the chemical soaked in gauze to the hemorrhagic areas under direct vision using a rigid sigmoidoscope. Thrombosis of the neovasculature and coagulation necrosis of the superficial mucosa ensues, with a complete response rate of 78%. While topical formalin appears to be slightly less effective than argon plasma coagulation therapy, formalin application alone or a combination of the two treatments has been advocated for severe cases of hemorrhagic radiation proctitis. Although formalin installation may be effective in controlling refractory bleeding due to radiation-induced proctitis, the procedure is not risk-free and may induce major complications such as acute colitis[74]. Preliminary results of a randomized study of the two therapeutic interventions, however, show equivalent efficacy but an absence of effect of either treatment on anorectal dysfunction.

Another approach to treat hemorrhagic radiation proctitis involves use of low-dose thalidomide, a potent inhibitor of (neo)angiogenesis, following a case report with successful outcome[75]. In addition, hormone therapy consisting of an estrogen-progesterone combination might provide a promising new additional symptomatic therapy for bleeding radiation colitis[76].

Rectal strictures should be managed initially non-operatively with a low-fiber diet, stool softeners, mineral oil enemas, and analgesics. Manual or endoscopic dilations of rectal strictures might be required. Short strictures with minimal angulation can be dilated by transendoscopic balloons or other dilators, albeit with considerable risk of perforation. Long, tortuous strictures should be managed operatively[77].

Surgery

About one third of patients with chronic radiation enteritis will need to be operated during follow-up. Surgical intervention is indicated in intestinal obstruction, perforation, fistulas, and severe bleeding. Surgery should be performed by an experienced team familiar with the treatment of radiation colitis. It is difficult to perform surgery for chronic radiation colitis because of the diffuse process of fibrosis and alterations in the gut and mesentery. The risk of anastomotic leak is high if the anastomosis is performed using irradiated tissue[78]. The risk can be lowered if at least one limb of the anastomosis did not receive prior radiotherapy[79]. It is difficult to distinguish between the normal tissue area and the irradiated area of the gut by gross evaluation during operation even when the fresh tissue is sent for frozen section. The accuracy in localizing injured intestine may be improved by intraoperative endoscopic evaluation, which can detect radiation-induced mucosal injury[80].

Resection of the affected intestine is significantly better than an enteric bypass procedure in overall outcome. However, extensive surgical resection of the diseased bowel may lead to short bowel syndrome and increase the need for total parenteral nutrition. Moreover, because of the progressive evolution of the fibrosis, the patient may require additional surgery. Surgical bypass of the damaged bowel is associated with a blind loop syndrome, and the patient may be still at risk of perforation, bleeding, abscess, and fistulae due to the persistence of the affected bowel. Bypass procedures should be performed when resection is not possible or as a temporary management before resection at a later date. Limited resection of the diseased intestine is the goal, but if the lesion is too diffuse, a bypass procedure

might be attempted.

Management of a pelvic fistula (e.g. vaginal or bladder fistula) is also complex and requires fecal diversion before the corrective surgery. Patients with fistulae frequently present with additional challenges such as electrolyte imbalance, malnutrition and infections. Many surgical techniques have been described to repair fistulae, but corrective surgery is best done when the patient is medically stable and enough time has elapsed after the surgical diversion. This permits the healing and decreased inflammation of the affected tissues[81,82].

In cases with severe fibrotic strictures, surgical intervention with establishment of a primary anastomosis may be required[83]. Strictureplasty may be an effective and safe tool to conserve intestinal length in certain highly selected patients with chronic radiation colitis and small-bowel strictures, namely those with limited intestinal reserve where strictures are located within long segments of diseased bowel which, if resected or bypassed, would have significant nutritional or metabolic consequences. Strictureplasty is not indicated for the treatment of perforation, hemorrhage, fistula, or short segments of disease in patients with adequate intestinal reserve[84].

Surgical complications of chronic radiation colitis such as intestinal obstruction, enterocutaneous fistula, intestinal stenosis, intestinal bleeding, severe proctocolitis and intestinal perforation should be managed operatively[85].

It is important to note that vigorous preoperative and postoperative nutritional support and evaluation are vital because of the poor healing qualities of the irradiated gut.

If conservative measures and local intervention to control bleeding prove unsuccessful, resection or ligation of the affected area(s) is preferred over a bypass procedure because the latter will allow the hemorrhage to continue and may lead to a higher mortality rate[86]. A promising surgical approach is small bowel transplantation, which may be considered in the pediatric population with radiation colitis.

PREVENTION OF RADIATION COLITIS

Based on the above data, it appears that the management of radiation bowel damage can be difficult and problematic; chronic radiation colitis is complex and rarely curable. Recent advances in the approaches to its prevention or amelioration are therefore particularly encouraging. Research to uncover the mechanisms of fibrosis and the molecular events underlying radiation bowel disease could lead to the development of new therapeutic and/or preventive approaches and provide the basis for predicting the risk of bowel damage and oncogenesis using levels and expressions of the mentioned cytokine IL-6, oncogene p53 or cadherin-catenin complexes, and for amelioration of bowel damage through inhibition, for example, of the COX-2 and Rho/Rho kinase pathways[33,87]. In this regard, the COX-2 inhibitor Rofecoxib® has been shown to suppress cytokine expression and to reduce acute bowel damage in rodents following abdominal irradiation[36]. In addition, piroxicam (a nonsteroidal anti-inflammatory agent) significantly decreases the incidence of colonic neoplasia in general and also delays the endoscopic appearance of colonic neoplasia in rats after pelvic irradiation[88]. Moreover, specific inhibition of Rho kinase is a promising approach for the amelioration of radiation fibrotic gut damage, as reported by a study investigating molecular pathways involved in the maintenance of fibrosis of the bowel wall of late radiation colitis patients[87]. Alterations in expressions of genes coding for Rho proteins was first established by molecular profile analysis of ileal biopsies. Primary cultures of gut smooth muscle cells derived from the ileal biopsies are associated with retention of fibrogenic differentiation *in vitro* and exhibit a typical cytoskeletal network, a high constitutive connective tissue growth factor level, increased collagen secretory capacity and altered expression of genes coding for the Rho family. Rho kinase blockade induces a simultaneous reduction in the number of actin stress fibers, α-smooth muscle actin and heat shock protein (Hsp) 27 levels. It also reduces connective tissue growth factor levels, the latter probably through NF-κB inhibition, leading to decreased expression of the type 1 collagen gene[87]. These observations show the involvement of the Rho/Rho kinase pathway in radiation fibrosis and intestinal smooth muscle cell fibrogenic differentiation, suggesting the potential role of Rho kinase inhibitors in ameliorating the radiation bowel damage.

Additional biomarkers potentially playing a role in the prediction, reduction or prevention of radiation colitis include genetic alterations of the cellular radiation response genes, such as the ataxia telangiectasia gene, and micronutrients, such as selenium and zinc. Genetic variants of the ataxia telangiectasia gene have been correlated with the risk of rectal hemorrhage associated with chronic radiation proctitis among prostate cancer patients who received the full brachytherapy prescription dose to defined volumes of the rectum[89]. The ataxia telangiectasia sequence alterations lead to an approximately sevenfold increase in mild to moderate (Radiation Therapy Oncology Group grades 1 and 2) radiation proctitis among patients who had received the full prescription dose to either low (< 0.7 mL) or moderate (0.7-1.4 mL) volumes of their rectum. Patients contemplating this increasingly popular radiation treatment modality for early prostate cancer should not only be better informed about the risks of bowel complications, but could also have their radiation dose prescriptions individualized based on genetic profiling.

Dietary supplementation of selenium and zinc may be useful in reducing anorectal sequelae after pelvic radiotherapy; an indirect relationship between baseline plasma levels of these micronutrients and abnormalities in anorectal function one year after radiotherapy for prostate cancer has been suggested. Notably, the heavy metal zinc induces Hsps, also known as stress proteins and molecular chaperones, which play a central role in protecting cellular homeostatic processes from environmental and physiologic insults by preserving the

structure of normal proteins and repairing or removing damaged ones. Lowering Hsps in cancer tissues can amplify the effectiveness of chemo- or radiotherapy[90].

Importantly, improvements in the delivery of radiotherapy, including techniques to reduce the amount of exposed intestine in the radiation field, also represent a critical strategy for prevention. The ideal radiation toxicity preventive therapy must have high efficacy, low toxicity, low cost, and not afford cancer protection. Unfortunately, the currently available therapy often does not fulfill all of these objectives and there is a need to identify patients who may truly benefit from preventive therapies. Specifically, the radiation therapy technique plays an essential role in reducing the rate of complications; particular attention should be paid to optimizing radiotherapy technique and dose prescriptions. The use of only anterior and posterior fields for pelvic radiation should be avoided, if possible, because of the high dose and large volume of intestine irradiated. A higher operative mortality was reported in trials using this technique preoperatively for rectal cancers[91,92]. The toxicity of radiation is directly related to the volume of small bowel being irradiated[93]. In many patients, therapy in the prone position with a special "belly" board allows the protrusion of the small intestine out of the radiation field[94,95]. Patients should be instructed to maintain a full bladder during the radiation session, which mechanically displaces the intestine out of the pelvis[96].

Modern radiation treatment techniques, such as three-dimensional treatment planning, also optimize the treatment technique by developing more accurate dose distributions. Notably, three-dimensional conformal radiotherapy techniques, including intensity-modulated radiotherapy, may not reduce late intestinal toxicity because margins around the cancer may not be able to be safely reduced and because of the prescription of higher radiation doses[97,98]. Brachytherapy, alone or as a supplement to external beam radiotherapy, is now increasingly being utilized to decrease normal tissue toxicity, without compromising treatment efficacy, in the management of prostate carcinoma[99,100]. Brachytherapy is a kind of radiotherapy whereby the source of radiation is located either within the malignant tissue (interstitial brachytherapy) or within a cavity in its immediate vicinity (intracavitary brachytherapy), rather than at a distance (typically 100 cm) from the center of the neoplasm target, as it is the case with external beam radiotherapy. Brachytherapy exploits the physical characteristics inherent with this modality of radiotherapy, whereby the high radiation dose is limited to the neoplasm target, while the surrounding normal tissues are spared from radiation by the rapid dose reduction (with the square of the distance). Brachytherapy alone, in the therapy of low-risk prostate carcinoma, is well tolerated, even in patients with a history of IBD[101].

Another related treatment, such as intensity-modulated radiotherapy (IMRT), uses sophisticated planning techniques to avoid critical structures. IMRT uses multiple segments of beams to shape the dose distribution to a desired result.

Operation, as a major risk factor, leads to the prolapse of the small intestine into the pelvis, exposing it to a full dose of radiation. Postoperative bowel adhesions also increase the volume of gut irradiated compared with normal intestine, usually mobile and able to move out of the radiation field. With gut adhesions, the intestine is trapped and is more likely to receive a high dose of radiation. If radiation therapy is anticipated after surgery, every attempt should be made at the time of surgery to displace the bowel outside of the radiation field[102]. One simple technique is the surgical placement of a polyglycolic, biodegradable mesh that moves the intestine out of the pelvis[103,104]. The procedure has negligible morbidity and it does not increase the operating time significantly. It also does not require a second operation to remove the mesh because it is absorbed 3 to 4 mo postoperatively. MRI can be used post-operatively to verify the position of the mesh, the small bowel, and its disappearance. Placement of a mesh during surgery allows a higher dose of radiation to be given postoperatively when indicated, thereby decreasing by 50% the volume of the small bowel exposed to the radiation[105,106]. Other techniques such as pelvic reconstruction, omentoplasty, and transposition of the large bowel also reduce the volume of gut at risk for radiotherapy up to 60%[106-109].

Amifostine (WR-2721) is an amino-thiol with well-established radioprotective effects. Recent studies have documented its effectiveness in protection of the salivary glands in patients receiving radiotherapy for head-and-neck cancer[110]. It has also been investigated for the prevention of chronic radiation colitis. According to preclinical studies, amifostine protects both the small and large intestine[111]. Specifically, it is converted intracellularly to an active metabolite, WR-1065, which in turn binds to free radicals and protects the cell from radiation damage[112]. In a randomized study, the late effects of radiation were significantly reduced in the group receiving parenterally administered amifostine. However, the median follow-up was quite short (24 mo), and longer follow-up is necessary to confirm the benefits of this medication because the incidence of late complications increases with time[113]. There is also evidence suggesting that intrarectal application of amifostine directly onto the rectum may reduce the risk of proctitis in patients undergoing radiotherapy for prostate cancer[114]; its intrarectal application is feasible and well tolerated. Systemic absorption of amifostine and its metabolites is negligible, and close monitoring of patients is not required after rectal administration[115]. Systemic administration of amifostine, used concurrently with radiotherapy in advanced rectal cancer, has been reported to reduce acute and late pelvic radiation toxicity[113]. Other investigators[116], however, were not able to demonstrate any protection afforded by amifostine.

Since cytotoxic effects of ionizing radiation on gastrointestinal epithelium may be related to oxidative stress, a number of agents have been used as a prophylaxis

treatment. Eicosanoids and free radicals release have been implicated in the pathogenesis. Selenium and/or vitamin E pretreatments are shown to improve post-irradiation disturbances in pro-oxidant-antioxidant balance, such as increased intestinal lipid peroxide and decreased GSH levels, increased intestinal SOD and GSH peroxidase activities and decreased glutathione transferase activity. This amelioriation has been confirmed by histopathological findings[117]. In another study, the early side effects of radiation were suggested to be prevented by vitamin A supplementation[118].

PGs have been investigated as potential radioprotectors. PGE2 and the PG analogs enprostil and misoprostol (Cytotec®) display radiation protection in animal studies[119-122]. Misoprostol suppositories also reduced symptoms of acute radiation colitis in patients undergoing radiation therapy for prostate cancer[123]. With respect to the mechanism of action, PGE2 has pro-proliferative and anti-apoptotic effects on epithelial cells in gastrointestinal injury. PGE2 decreases radiation-induced apoptosis and increases crypt survival[124].

Experimental data indicate that in control animals, glucagon-like peptide-2 (GLP-2) induces an increase in intestinal mucosal mass, along with an increase in villus height and crypt depth. GLP-2 administration before and after irradiation completely prevents the acute radiation-induced mucosal ulcerations and strikingly reduces the late radiation damage. Microscopic observations show an improved organization of the intestinal wall and an efficient wound healing process, especially in the smooth muscle layers. This therapeutic effect is mediated through an increased mucosal mass before tissue injury and the stimulation of still unknown mechanisms of tissue response to radiation damage. Although these preliminary results still need to be confirmed, GLP-2 might be a way to limit patient discomfort during radiotherapy and reduce the risk of consequential late effects[125].

Irradiated intestine consistently exhibits increased immunoreactivity of transforming growth factor (TGF)-β1. It has been demonstrated that mucosal barrier breakdown is closely associated with increased TGF-β immunoreactivity in subsequent radiation enteropathy. The highly significant correlation between TGF-β expression levels and alterations in late-responding tissue compartments also suggest a role for TGF-β in primary radiation colitis. A recent preclinical study showed a role for possible anti-TGF-β1 interventions to reduce delayed radiation fibrosis and enteropathy[126].

Preliminary studies also suggest that IFN-γ may be effective in the treatment of patients with radiation-induced cutaneous fibrosis. IFN-γ should be considered in Phase Ⅰ-Ⅱ studies to assess its toxicity and efficacy in the treatment of patients with radiation colitis[127].

Many special diets and nutrients, such as the mentioned fiber, elemental diets, SCFAs and amino acids like glutamine, may reduce small-bowel radiation toxicity. Specifically, probiotics (*Lactobacillus bulgaricus* strain isolated from yogurt) added as substrates can be given by an oral or enteral route to patients who undergo radiotherapy to prevent radiation-induced colitis and related malnutrition[128].

Glutamine and arginine support the mucosal barrier in several ways. In experimental studies, a 7-d glutamine- or arginine-enriched diet administered both pre- and post-irradiation showed that they have protective effects on gut mucosa in the post-irradiation state. However, pre- and post-irradiation administration together do not provide superior protection compared to post-irradiation administration alone[129].

Administration of insulin-like growth factor (IGF)-I immediately following abdominal irradiation increases small-intestinal mass and improves indicators of mucosal integrity, suggesting acceleration of small-intestinal mucosal recovery from radiation injury[130]. More recently, growth hormone and IGF-I have been demonstrated to protect intestinal cells from radiation-induced apoptosis both *in vitro*, by inhibiting apoptosis of the cells and preserving the mucosal integrity[131], and *in vivo*[132], utilizing IGF- I transgenic mice.

Pancreatic enzymes can exacerbate acute intestinal radiation toxicity, and suppressing pancreatic secretion with synthetic somatostatin receptor analogs, such as octreotide, can reduce both early and delayed radiation colitis[133]. Of note, in an experimental model, irradiation significantly increased intestinal and pancreatic myeloperoxidase activities and intestinal malondialdehyde levels of intestinal tissues, and octreotide treatment improved this elevation. The histopathologic evaluation of the mucosal structure was also preserved in the octreotide-treated group. Inflammation of pancreatic tissue was also confirmed with histopathological examinations. Moreover, irradiation seems to induce NF-κB overexpression, and octreotide treatment decreases the end organ damage and inflammation of the small intestine. Thus, octreotide appears to have beneficial effects on intestinal and pancreatic damage in abdominal irradiation through the inflammatory process[134].

Despite the aforementioned promising agents used in acute and chronic raditation colitis, further understanding of the pathophysiological mechanisms involved in the pathogenesis of acute and chronic irradiation colitis and the interaction of the molecular events controlling mainly apoptosis and fibrosis may assist in the development and establishment of new therapeutic approaches.

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TOPIC HIGHLIGHT

Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, *Series Editor*

Ischemic colitis: Clinical practice in diagnosis and treatment

Angeliki Theodoropoulou, Ioannis E Koutroubakis

Angeliki Theodoropoulou, Gastroenterology Unit Venizelion General Hospital of Heraklion, Heraklion 71110, Crete, Greece
Ioannis E Koutroubakis, Department of Gastroenterology, University Hospital Heraklion, Heraklion 71110, Crete, Greece
Author contributions: Theodoropoulou A reviewed the literature and wrote the first draft of the paper; Koutroubakis IE contributed to providing the idea and performing the review and editing of the manuscript.
Correspondence to: Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, Department of Gastroenterology, University Hospital Heraklion, PO Box 1352, Heraklion 71110, Crete, Greece. ikoutroub@med.uoc.gr
Telephone: +30-28-10392253 Fax: +30-28-10542085
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Abstract

Ischemic colitis is the most common form of ischemic injury of the gastrointestinal tract and can present either as an occlusive or a non-occlusive form. It accounts for 1 in 1000 hospitalizations but its incidence is underestimated because it often has a mild and transient nature. The etiology of ischemic colitis is multifactorial and the clinical presentation variable. The diagnosis is based on a combination of clinical suspicion, radiographic, endoscopic and histological findings. Therapy and outcome depends on the severity of the disease. Most cases of the non-gangrenous form are transient and resolve spontaneously without complications. On the other hand, high morbidity and mortality and urgent operative intervention are the hallmarks of gangrenous ischemic colitis.

Key words: Colon ischemia; Intestinal blood flow; Ischemic colitis; Thrombosis

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INTRODUCTION

Ischemic colitis (IC), first described by Boley *et al*, is the most common form of ischemic injury to the gastrointestinal tract representing more than half of the cases with gastrointestinal ischemia[1,2]. The incidence of IC is underestimated because it often has a mild and transient nature. Moreover, many cases are misdiagnosed as suffering from other diseases such as inflammatory bowel disease or infectious colitis.

An acute, self-limited compromise in intestinal blood flow which is inadequate for meeting the metabolic demands of a region of the colon is the underlying pathophysiology[3]. Colonic blood flow may be compromised by changes in the systemic circulation or by anatomic or functional changes in the local mesenteric vasculature. The original insult precipitating the ischemic event often cannot be established, but frequently occurs in the elderly patient with diffuse disease in small segmental vessels and various co-morbidities. Approximately 90% of cases of colonic ischemia occur in patients over 60 years of age although younger patients may also be affected[4].

IC presents either as an occlusive or a non-occlusive form. In most cases no specific occlusive lesion is recognized on angiography, and patients are referred to as suffering from non-occlusive colon ischemia.

The aim of this review is to transfer the current knowledge on diagnosis and management of ischemic colitis into daily clinical practice.

RISK FACTORS

A plethora of conditions may predispose to IC: Mesenteric artery emboli, thrombosis, or trauma may lead to occlusive vascular disease and impaired colonic perfusion[5]. Hypo-perfusion states due to congestive heart failure, transient hypotension in the perioperative period or strenuous physical activities and shock due to a variety of causes such as hypovolemia or sepsis can result in IC[3]. Mechanical colonic obstruction due to tumors, adhesions, volvuli, hernias, diverticulitis or prolapse may also infrequently cause IC[3]. There is a long list of medications that predispose to colon ischemia. Major classes of pharmacologic agents known to be associated with IC include the following[6]: antibiotics, appetite suppressants (phentermine), chemotherapeutic agents (vinca alkaloids and taxanes), constipation inducing medications, decongestants (pseudoephedrine), cardiac glucosides, diuretics, ergot alkaloids, hormonal therapies, statins, illicit drugs,

immunosuppressive agents, laxatives, nonsteroidal anti-inflammatory drugs, psychotropic medications, serotonin agonists/antagonists and vasopressors. Iatrogenic causes may result in IC. Ischemic colitis follows aortic reconstruction with an incidence of 2% to 3% and is higher after abdominal aortic aneurysm repair[7,8]. IC may be a complication of coronary artery bypass surgery or a rare complication of colonic surgery or colonoscopy[3].

A state of increased coagulability, although not extensively investigated, has been raised as a significant factor in the pathogenesis of IC. Some cases of IC have been reported to be associated with genetic defects such as deficiencies of protein C, protein S, and antithrombin Ⅲ[9-11], factor V Leiden (FVL) mutation[12,13], and prothrombin 20210G/A mutation[14], as well as acquired factors such as antiphospholipid antibodies[15]. Protein Z deficiency has also been reported in IC patients[16]. A thrombophilic tendency in the majority of patients was shown in a study of comprehensive thrombophilic screening in colon ischemia[17]. The most significant associations were found with the antiphospholipid antibodies and the FVL mutation[17]. These results were confirmed by another recent study in which thrombophilic disorders were found in 28% of patients studied[18].

IC might also spontaneously appear in apparently healthy individuals. In these cases no clear cause for the ischemia is identified. This idiopathic or "spontaneous" form is generally thought to be related to localized non-occlusive ischemia of the bowel[5]. In younger patients a predisposing cause is more easily recognized. Vasculitides, estrogens, cocaine and methamphetamine use, psychotropic drugs, sickle cell disease, long-distance running and heritable disorders of coagulation should be considered[19-24]. In a recent study[25], the frequency of the 506 Q allele of the factor V (FV) 506 RQ (Leiden) mutation and the mutant 4G allele of plasminogen activator inhibitor (PAI) polymorphism were found to be significantly higher in young patients with IC compared with healthy controls.

PATHOPHYSIOLOGY

The colon is predisposed to ischemia by its relatively low blood flow and its less developed microvasculature plexus compared with the small bowel. Two major arteries supply most of the blood to the colon: the superior mesenteric artery (which supplies the ascending and transverse colon) and the inferior mesenteric artery (IMA) (which supplies the descending and sigmoid colon). The internal iliac arteries supply the rectum.

The colon is protected from ischemia by a collateral blood supply *via* a system of arcades connecting the two major arteries. The anatomy is highly variable, however, and certain areas are more vulnerable in some people[26]. The splenic flexure and sigmoid colon are regions where two circulations meet each other (so-called watershed areas), have more limited collateral networks and therefore ischemic damage is more common in these areas. The marginal artery of Drummond is one of the collateral vessels supplying the splenic flexure; 5% of the population has a diminished or absent marginal artery of Drummond[27]. These patients are at particular risk of ischemia. The right colon may be vulnerable in systemic low-flow states, as the marginal artery of Drummond is poorly developed here in 50% of the population[28]. The vasa recta are smaller and less developed in the right colon compared to the left colon. Collateral flow between the IMA and the internal iliac arteries occurs *via* the superior and middle/inferior rectal (hemorrhoidal) vessels. Ischemic damage of the rectum is rare because of its dual blood supply from the mesenteric and iliac arteries.

Classification

Clinically, ischemic colitis may be classified into gangrenous and non-gangrenous forms. The latter can also be subdivided into transient and chronic forms.

According to the classification of Brandt and Boley the following types are suggested[29]: (1) Reversible ischemic colonopathy; (2) Transient IC; (3) Chronic ulcerative IC; (4) Ischemic colonic stricture; (5) Colonic gangrene; and (6) Fulminant universal.

The non-gangrenous form accounts for 80%-85% of cases[26]. The disease is transient, and reversible in about 50% of cases. Chronic forms, presenting either as chronic segmental colitis or strictures, occur in 20%-25% and 10%-15% of cases, respectively[26,29]. Predictive factors of the chronic form are older age, longer elapsed time from the onset of illness to the termination of subjective symptoms, and a prolonged period until normalization of the white blood cell count or the erythrocyte sedimentation rate[5]. Gangrene occurs in about 15% of patients and requires laparotomy as soon as possible[26]. Fulminant pancolitis is rare, occurring in only 1% of cases[26].

A worse prognosis has been reported in elderly patients. There are conflicting results with regard to the relationship between the medical history of patients and the severity of IC. High blood pressure, history of cancer, diabetes mellitus, aortic surgery, peripheral vascular disease and involvement of the right side of the colon have been suggested by some authors to be predisposing factors for a worse evolution of the disease[30-32]. In the study by Anon *et al*[33], factors predicting poor prognosis in ischemic colitis were the absence of hematochezia, tachycardia and peritonism, anemia, hyponatremia and colonic stenosis.

Any part of the colon may be affected but the left colon is the predominant location in approximately 75% of patients[5]. Splenic flexure is involved in approximately one-quarter of patients[5] and isolated right colon ischemia (IRCI) in about 10% of cases[29]. In a recent biopsy-proven study, IRCI accounted for 26% of cases[34]. Its clinical presentation was found to be different in patients who presented more commonly with abdominal pain without bloody diarrhea. IRCI has been reported to be associated with hemodialysis and chronic renal failure and in patients with shock. It is associated with severe colitis and patients have a worse outcome than those with colon ischemia involving other regions, including

a five-fold need for surgery and a two-fold increase in mortality[34]. Patients on hemodialysis who develop IRCI have a particularly unfavorable outcome[34]. Insufficient collateralization and blood flow to the right side of the colon is believed to be the reason for the poor prognosis in these patients. Alternatively, it is possible that the presence of an acute superior mesenteric artery occlusion and thus its outcome reflects that of acute mesenteric ischemia.

Clinical presentation

The clinical presentation varies, depending on the severity and extent of the disease. None of the symptoms and signs is specific. Most patients present with a sudden onset of crampy abdominal pain, diarrhea and an urge to defecate. The pain is mild, located over the affected bowel, usually to the left side of the lower abdomen and hypogastrium, followed by mild rectal bleeding within 24 h. The blood may be bright red or maroon, frequently mixed with the stools. Rectal bleeding is usually minimal. Significant hematochezia accompanied with hemodynamic instability or the need for blood transfusion suggests a different diagnosis. The presence of an associated ileus may be manifested by anorexia, nausea and vomiting.

Clinical examination of the abdomen reveals mild to moderate tenderness over the affected area of the colon. Rectal examination shows heme-positive stools. Fever is unusual while the white cell count is generally raised. In cases of severe ischemia with transmural infarction and necrosis, marked tenderness with peritoneal signs may be present on physical examination accompanied by metabolic acidosis and septic shock.

DIAGNOSIS

Given that the presentation of colon ischemia is not specific and is highly variable, diagnosis and management is clinically challenging. Diagnosis requires a high index of clinical suspicion. The chronology of symptoms and the clinical situations upon which these symptoms appear must be taken into account.

Special attention must be paid to the presence of conditions that predispose to the disease, such as strenuous physical activity, dehydration, illicit drugs, thrombophilic tendency, aortic surgery or cardiac bypass, vasculitis, major cardiovascular episode accompanied by hypotension or an obstructing lesion of the colon.

The presence of diarrhea, abdominal pain and tenderness as well as mild lower gastrointestinal bleeding, even in the absence of any risk factor, should prompt consideration of IC as a cause. Early and repeated clinical evaluation in addition to radiological and endoscopic assessment is necessary to avoid complications. Common clinical conditions should be excluded. The differential diagnosis includes infectious colitis, inflammatory bowel disease, pseudomembranous colitis, diverticulitis and colon carcinoma. Severe forms may be difficult to distinguish from acute mesenteric ischemia.

All patients with clinical suspicion of IC should have stool cultures for *Salmonella, Shigella, Campylobacter* and *Escherichia coli* O157:H7[35]. The latter organism has been implicated in causing colonic ischemia. Infection with parasites or viruses such as cytomegalovirus should also be excluded.

Laboratory tests

Various laboratory markers of ischemia have been investigated such as: lactate, LDH, CPK, amylase levels, leucocytes, alkaline phosphatase, inorganic phosphate, intestinal fatty acid binding protein and alfa-glutathione S-transferase[36]. These markers have been studied mainly in acute bowel ischemia, and none has been found to be sufficiently specific to diagnose IC. They are uncommon in mild ischemia and only increase with advanced and severe ischemic damage, late in the course of the disease.

Imaging techniques

Plain abdominal radiography can reveal nonspecific findings such as thumbprinting, air-filled loops, colonic aperistalsis, mural thickening and exhausted bowel in up to 21% of patients[3]. It is a useful examination for excluding colon infarction[37]. When intra-abdominal air secondary to perforation, air within the bowel wall, or air in the portal vein, is demonstrated by plain radiography, an emergency exploratory laparotomy is indicated.

Barium enema may suggest colon ischemia in up to 75% of patients with thumbprinting being the most common finding. Ulcers, ridges, edema, eccentric mural deformity, succulation and strictures may also be seen. Findings are non specific[38]. Barium enema should be avoided in cases where there is a suspicion of gangrene or perforation. Barium enema also makes the later use of angiography or endoscopy more difficult because of residual contrast agent.

Computed tomography (CT) is often used as the initial diagnostic test when assessing patients with nonspecific abdominal pain. It may suggest the diagnosis and location, exclude other serious medical conditions, narrow the differential diagnosis possibilities and illustrate the complications. Although intrinsic colonic abnormalities cannot be used to diagnose or predict the development of infarction[39].

In non-transmural IC, the initial bowel wall thickening, thumbprinting, and pericolonic stranding, with or without peritoneal fluid, can be seen on CT images. In these cases, CT usually demonstrates the double halo or target sign. After reperfusion of the ischemic bowel wall, the sign may be produced by edema in the submucosa and appear as low attenuation or by hemorrhage and appear as high attenuation. If there is total vascular occlusion without reperfusion (infarction), the colonic wall remains thin and unenhancing, associated with dilatation of the lumen. In these cases, CT may demonstrate a thrombus in the corresponding mesenteric vessel. If ischemia is transmural, strictures may form. Occasionally, a toxic megacolon develops. Pneumatosis and/or gas in the mesenteric veins are ominous signs when associated with bowel wall thickening and are due to bowel infarction.

Pneumatosis coli or pneumatosis intestinalis can be diagnosed by demonstrating air bubbles in the colonic or intestinal wall. The gas bubbles are arranged in a linear fashion and are best visualized with the window settings for bone or lung[40].

Mesenteric angiography usually has no role in the evaluation and management of IC because at the time of symptom onset, colon blood flow has returned to normal. Damage from hypoperfusion is often at the arteriolar level, whereas mesenteric vessels and arcades are patent. There are two exceptions where angiography may have some utility: when acute mesenteric ischemia is considered and cannot be clearly distinguished from IC by clinical presentation, or when there is isolated involvement of the right side of the colon, suggesting superior mesenteric artery occlusion.

Sonography is a sensitive technique for the early detection of changes in the colon wall resulting from ischemia, and it can suggest this cause in the appropriate clinical setting. Location and length of the involved colonic segment, colon wall thickening, bowel wall stratification and abnormal echogenicity of the pericolic fat and peritoneal fluid are some of the findings on sonography[41].

Color Doppler sonography may be useful in the differentiation between inflammatory and ischemic bowel wall thickening[42]. Sonography may provide data for identifying patients who will develop necrosis. In one study, altered pericolic fat or the absence of improvement in sonographic follow-up studies were factors associated with transmural necrosis[41]. Nevertheless, overlying bowel gas, operator-dependent quality and poor sensitivity for low flow vessel disease limit its use.

Scintigraphy has recently been used in the diagnosis of ischemic colitis. In-111 or Tc-99m-labeled leukocyte scintigraphy has been studied and has demonstrated successful imaging of bowel infarction while Tc-99m(V) DMSA was recently found to have no role in the detection and diagnosis of IC[43-45].

Colonoscopy

In recent years, colonoscopy has replaced barium enema as the most common diagnostic method and the gold standard for confirmation of IC. It is more sensitive and allows visualization of colonic mucosa and histological analysis of biopsies. However, with the exception of colonic gangrene, neither endoscopic nor histological findings are specific[29] and highly depend on the duration and severity of ischemic injury. Diagnosis requires early colonoscopy (< 48 h). Serial studies in connection with the clinical setting are necessary to establish the diagnosis.

Ischemic tissue damage to the colon is thought to be a result of both local hypoperfusion during the ischemic period and reperfusion injury when blood flow returns. When the ischemic period is brief, reperfusion may be significant and accounts for most of the histologic and endoscopic damage present in IC[2]. Reperfusion injury may be associated with the release of oxygen free radicals which cause lipid peroxidation within cell membranes, resulting in cell lysis and tissue damage.

When the ischemic period is of long duration, hypoperfusion deprives the involved bowel of oxygen and nutrients, leading to hypoxia and direct cell death[2]; damage progresses from the lumen outwards to the serosa (from the mucosa and submucosa to deeper layers).

In the early stages only the mucosa and the submucosa are involved. Hemorrhagic nodules may be seen at colonoscopy and represent bleeding into the submucosa. These findings parallel the "thumbprints" or "pseudotumors" found on barium studies[5]. The purple submucosal hemorrhages usually dissipate within 48 h or are followed by ulceration. Hence, the initial diagnostic study should be performed soon after the onset of symptoms. Focal areas of pale and edematous mucosa interspersed with areas of petechial hemorrhage or superficial ulceration may also be seen in mild cases[2,46]. Later, segmental erythema with or without ulcerations and bleeding may be observed. A single longitudinal ulcerated or inflamed colon strip represents the characteristic single-stripe sign[47]. In more severe ischemia when transmural infarction of the bowel wall occurs, the mucosa appears gray-green or black over a significant area. Pseudopolyps and pseudomembranes may also co-exist[5]. In chronic stages, weeks or months later, stricture, mucosal atrophy and granularity or a mucosal pattern suggestive of "segmental ulcerative colitis" may occur[2].

Histologic changes in IC include edema, distorted crypts, mucosal and submucosal hemorrhage, inflammatory infiltration in the lamina propria, granulation tissue, intravascular platelet thrombi and necrosis. In the phase of stricture, inflammation is minimal and fibrosis predominates[5].

Endoscopic findings which distinguish between IC and inflammatory bowel disease are the segmental distribution, rectum sparing and rapid resolution on serial examinations[3]. Special care should be taken during colonoscopy to avoid overinflation which can lead to the risk of perforation. Distention of the bowel with room air may cause a further reduction in intestinal perfusion. Using carbon dioxide as the insufflating agent which is rapidly absorbed, and has the benefit of vasodilation and direct improvement in colonic perfusion, may minimize these risks[26]. When signs of peritonitis are present, endoscopy should be avoided. When endoscopy reveals findings of gangrene, colonoscopy should be stopped and laparotomy performed as soon as possible.

Total colonoscopy when it is considered safe, is preferred because 30% of IC cases occur proximal to the left flexure[3]. Given the high morbidity and mortality of IRCI and the vague presenting symptoms, early diagnosis and aggressive management is critical.

TREATMENT

Treatment depends on acuteness and severity of presentation. Most cases of IC are transient and resolve

spontaneously. Such patients do not require specific therapy. Very mild cases can be managed on an outpatient basis with liquid diet, close observation and antibiotics. Patients with more severe symptoms must be hospitalized. In the absence of colonic gangrene or perforation, general measures of supportive care are recommended. Patients should be placed on bowel rest and given intravenous fluids to resuscitate extracellular volume and reduce intestinal oxygen requirements. Parenteral nutrition should be considered for patients who need prolonged bowel rest and have major medical contraindications to surgery[26]. Cardiac function and oxygenation should be optimized. Swan-Ganz catheterization may assist in guiding fluid status and cardiac function in hemodynamically unstable patients. Vasopressors or any medications which are associated with colon ischemia should be withdrawn if possible. Oral cathartics and bowel preparations should not be given because they can, in some cases, precipitate colonic perforation or toxic dilatation of the colon. Likewise, the use of systemic corticosteroids may potentiate ischemic damage and predispose to colonic perforation. Local corticosteroids may have a role in the treatment of patients with chronic IC although no published experience supports their use. A nasogastric tube should be placed if ileus is present. Decompression of a distended colon by use of a rectal tube may be useful. Empiric broad-spectrum antibiotics are given to cover aerobic and anaerobic bacteria and minimize bacterial translocation and sepsis which has been shown to occur with the loss of mucosal integrity[48]. The use of antibiotics is based on several experimental studies which showed a reduction in severity and extent of bowel damage when antibiotics were given before or during an ischemic event[2,49]. Antibiotics have resulted in prolonged survival after intestinal ischemia in rats[49]. Although there is a lack of substantial evidence in humans, this practice is justified because of the difficulty in predicting who will progress to gangrenous colitis. In experimental studies[5], substances such as papaverine, isoproterenol, bradykinin, histamine, serotonin, adenosine, vasoactive intestinal polypeptide and glucagon have been found to dilate colonic vasculature and improve local colonic blood flow and tissue oxygenation.

Frequent clinical follow up of the abdomen, careful monitoring of vital signs and serial radiographic and colonoscopic examinations are needed. Clinical suspicion of colonic infarction justifying an emergency laparotomy may arise if there are signs of clinical deterioration despite conservative therapy, such as sepsis, persistent fever and leukocytosis, peritoneal irritation, protracted pain, diarrhea or bleeding, protein-losing colopathy for more than 14 d, free intra-abdominal air, or endoscopically-proved extensive gangrene[26].

About 20% of patients with acute IC will require surgery with an associated mortality rate of up to 60%[31]. At laparotomy, the diagnosis is confirmed and all affected bowel resected. It is important to ensure normal surgical margins. The external appearance of the bowel may be normal during laparotomy since the serosa may be unaffected, despite extensive mucosal damage. The extent of resection should be guided by the distribution of disease seen on preoperative studies. Some authors have reported on intraoperative techniques such as Doppler ultrasonography, intraoperative colonoscopy, evaluation of the antimesenteric serosal surface by hand-held photoplethysmography, pulse oximetry or transcolonic oxygen saturation and intravenous fluorescein for assessment of colonic viability[49,50]. In general, the resected segment should be examined in the operating room for mucosal injury. If needed, additional colon should be removed. Questionably viable areas of colon are generally resected. A colectomy is followed by colostomy or ileostomy. Patients with left-sided IC undergo resection with a proximal stoma and a distal mucous fistula or Hartman pouch. Primary anastomosis is unusual. Rarely, an ileocolostomy may be performed in patients with right-sided IC and viable ileum and tranverse colon. In a series by Longo *et al*[51], the stoma was closed in 75% of patients with IC who underwent segmental resection *vs* only a third of those with total colonic involvement.

Fortunately, in the majority of patients, signs and symptoms of the disease resolve within 24 to 48 h and complete clinical, radiographic and endoscopic resolution occurs within 2 wk. In these circumstances no further therapy is indicated. In severe but reversible injury, when segmental ulcerative colitis exists, the colon may take 1 to 6 mo to heal[29]. Asymptomatic patients should have frequent follow-up examinations to document healing or the development of strictures or persistent colitis. In such cases, the patient may have persistent diarrhea, rectal bleeding or repeated episodes of sepsis, which may lead to perforation. Chronic ischemia may respond to topical steroid preparations in addition to general conservative measures. Resection of the affected segment is curative and subsequent development of further ischemic disease is rare. Asymptomatic strictures should be observed, since some may return to normal within 12 to 24 mo with no specific therapy[31]. When a stricture produces symptoms of obstruction, segmental resection is adequate while endoscopic dilation has been proposed as an alternative to surgery[48].

CONCLUSION

The etiology of ischemic colitis is multifactorial and the clinical presentation variable. The diagnosis is based on a combination of clinical suspicion, endoscopic and histological findings. Therapy and outcome depend on the severity of the disease. Most cases of the non-gangrenous form are transient and resolve spontaneously without complications. High morbidity and mortality and urgent operative intervention are the hallmarks of gangrenous ischemic colitis.

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TOPIC HIGHLIGHT

Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, *Series Editor*

Diagnosis and management of splanchnic ischemia

Jeroen J Kolkman, Marloes Bargeman, Ad B Huisman, Robert H Geelkerken

Jeroen J Kolkman, Marloes Bargeman, Department of Gastroenterology, Medisch Spectrum Twente, 7500 KA Enschede, The Netherlands
Ad B Huisman, Department of Interventional Radiology, Medisch Spectrum Twente, 7500 KA Enschede, The Netherlands
Robert H Geelkerken, Department of Vascular Surgery, Medisch Spectrum Twente, 7500 KA Enschede, The Netherlands
Author contributions: All authors contributed to this review paper.
Correspondence to: Dr. Jeroen J Kolkman, Department of Gastroenterology, Medisch Spectrum Twente, PO Box 50.000, 7500 KA Enschede, The Netherlands. j.kolkman@mst.nl
Telephone: +31-53-4872412 Fax: +31-53-4872979
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Abstract

Splanchnic or gastrointestinal ischemia is rare and randomized studies are absent. This review focuses on new developments in clinical presentation, diagnostic approaches, and treatments. Splanchnic ischemia can be caused by occlusions of arteries or veins and by physiological vasoconstriction during low-flow states. The prevalence of significant splanchnic arterial stenoses is high, but it remains mostly asymptomatic due to abundant collateral circulation. This is known as chronic splanchnic disease (CSD). Chronic splanchnic syndrome (CSS) occurs when ischemic symptoms develop. Ischemic symptoms are characterized by postprandial pain, fear of eating and weight loss. CSS is diagnosed by a test for actual ischemia. Recently, gastro-intestinal tonometry has been validated as a diagnostic test to detect splanchnic ischemia and to guide treatment. In single-vessel CSD, the complication rate is very low, but some patients have ischemic complaints, and can be treated successfully. In multi-vessel stenoses, the complication rate is considerable, while most have CSS and treatment should be strongly considered. CT and MR-based angiographic reconstruction techniques have emerged as alternatives for digital subtraction angiography for imaging of splanchnic vessels. Duplex ultrasound is still the first choice for screening purposes. The strengths and weaknesses of each modality will be discussed. CSS may be treated by minimally invasive endoscopic treatment of the celiac axis compression syndrome, endovascular antegrade stenting, or laparotomy-assisted retrograde endovascular recanalization and stenting. The treatment plan is highly individualized and is mainly based on precise vessel anatomy, body weight, co-morbidity and severity of ischemia.

Key words: Splanchnic ischemia; Mesenteric ischemia; Tonometry; Blood flow; Chronic splanchnic syndrome; Chronic splanchnic disease; Chronic mesenteric ischemia; Celiac artery compression syndrome; Ischemic colitis

Peer reviewer: Dr. Daniel R Gaya, Gastrointestinal Unit, Molecular Medicine Centre, School of Molecular and Clinical Medicine, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, United Kingdom

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INTRODUCTION

In this review we will cover the current insights in splanchnic or gastrointestinal ischemia. This disorder is still rarely seen in daily practice, and randomized controlled trials are absent, therefore the view of this paper is highly personal and partly authority-based in its conclusions. The spectrum of ischemic bowel disease is broad, ranging from transient left-sided ischemic colitis (with a good prognosis) to full blown intestinal infarction, with a high death rate. We will focus on new developments in clinical presentation, diagnostic approaches, and treatment options. Splanchnic ischemia can develop during low-flow states in patients with patent vessels, and in subjects with varying degree of splanchnic artery stenoses or splanchnic venous thrombosis. The prevalence of significant splanchnic arterial stenoses, or chronic splanchnic disease (CSD) is high, ranging from 30% to 50%[1,2]. Chronic splanchnic syndrome (CSS) occurs when ischemic symptoms develop. The most characteristic ischemic symptoms consist of postprandial pain, with resultant fear of eating and weight loss. When epigastric bruit is included, these are the so-called classical triad of CSS. In most patients

with CSS, this triad is incomplete. The true incidence of CSS is currently unclear, but is rare compared to CSD due to abundant collateral circulation.

Two important developments occurred in the last decade. Firstly, validation of the gastric exercise tonometry, which is currently the only clinically available and validated diagnostic test to ascertain the presence of splanchnic ischemia[3,4]. Using an ischemia-specific test it should be possible (1) to identify patients with symptomatic vessel stenoses, or CSS, which can be treated, and (2) to make this diagnosis in time and thus prevent the disaster of acute intestinal infarction. Secondly, the increasing evidence that one vessel CSD may cause splanchnic ischemia resulting in one vessel CSS, and can be successfully treated with appropriate selection procedures[5]. An important difference in presentation, treatment and outcome has been shown to exist between single and multi-vessel disease[6]. In the latter group, the clinical presentation is often less typical, with diarrhea, unexplained gastric ulcers, or dyspepsia-like symptoms. These insights stem mainly from our work with tonometry.

An entirely different entity consists of patients suffering from splanchnic ischemia without splanchnic stenoses; the so-called non-occlusive mesenteric ischemia (NOMI). It can be seen as a consequence of physiological adaptation mechanisms during low-flow states were blood is dispersed from the gastrointestinal region to more vital organs[7]. This situation is very common in intensive care and operative units, but can also be seen in outpatients. Treatment consists of aggressive fluid resuscitation and medication. However, bowel infarction can still occur.

In the last decade a change in imaging of the splanchnic vessels occurred. Duplex ultrasound, although operator dependent and suitable for 80% of patients, is still the first choice. Visceral angiography has increasingly been replaced by CT and MR-based angiographic reconstruction techniques. The clinically important advantages and disadvantages of these techniques will be discussed. Whichever technique is used, it leaves the clinician with only anatomical information. To decide whether a given stenosis has caused the symptoms, information on actual ischemia is required. This information can be obtained using tonometry, which has a proven accuracy of 80%-90%. Other tests including, serological iFABP, endothelial progenitor cell measurement, or MR angiography (MRA)-based saturation measurements, may serve that purpose in the near future.

Treatment options have changed considerably over the last decade. Apart from the classical transabdominal vascular reconstructive surgery techniques, minimally invasive endoscopic treatment of the celiac axis compression syndrome, endovascular antegrade stenting, or laparotomy-assisted retrograde endovascular recanalization and stenting have broadened our therapeutic "armory" considerably. The main patient characteristics to guide therapy choice, which include anatomical considerations, as well as body weight, co-morbidity and severity of ischemia, will be discussed.

EPIDEMIOLOGY

The prevalence of CSD is not insignificant, and rises with increasing age. In a 30-year-old angiographic study of 713 patients, 5% of the splanchnic arteries were occluded and in 70% of these occlusions the IMA was involved[8]. In a retrospective study including 980 patients with a mean age of 68 years who underwent angiography for various indications, 8% had significant stenoses of at least one splanchnic artery[9]. In a screening study with duplex ultrasonography in 553 healthy elderly subjects with a mean age of 84 years, stenoses in the celiac artery (CA) or superior mesenteric artery (SMA) were found in 18%[10]. In patients with atherosclerotic disorders of aorta, iliac and femoral vessels the incidence ranged from 25% to 40%[11,12].

A minority of patients with CSD will develop CSS or acute splanchnic syndrome (ASS). A follow-up of the study in elderly subjects in whom duplex had shown CSD, revealed no CSD-related mortality after 6 years of follow-up[13]. This risk is increased in subjects with 2 and 3 vessel CSD. In the study by Thomas *et al* 4.5% of patients with three-vessel CSD developed CSS and another 1.5% died of ASS after a follow-up of an average of 2.6 years[9]. In the Detroit experience, of the 23 patients with severe acute intestinal ischemia studied between 1963 and 2000, 12 (52%) patients had undetected CSS symptoms well before presentation[14].

ANATOMY AND (PATHO)-PHYSIOLOGY

Anatomy

Three major arteries supply blood to the stomach, small intestine and colon. The first branch, the celiac artery (CA) supplies the stomach, proximal duodenum, liver and spleen. The second, the superior mesenteric artery (SMA) supplies the distal duodenum, small intestine and proximal colon. The third branch supplies the distal colon and the rectum. There is an abundance of collateral vessels to protect the gastrointestinal tract from ischemia. Branches of these arteries enter the serosa of the gut on the mesenteric side and form a vascular plexus around the gut. After penetration of the bowel wall, a dense submucosal plexus is formed. From this plexus, arterioles penetrate the muscularis mucosa to the superficial mucosal layers. At the mucosal tip they branch into an intense capillary network of capillaries and venules. Each villus has a single, central arteriole. This arteriole travels to the tip of the villus, then splits into a network of capillaries, which form a central venule at the base of the villus. This is why countercurrent exchange can take place[15]. The tip of the villus is quite susceptible to ischemia[16].

Blood flow

Under normal conditions, approximately 20% of the cardiac output goes through the splanchnic vessels. This splanchnic blood flow doubles after a meal to approximately 2000 mL/min. Blood draining from the bowel enters the splanchnic veins and finally drains into

the portal vein. The liver, therefore, receives its blood supply from two sources: venous blood from the portal vein and arterial blood from the hepatic artery, which branches from the CA in 75% and from the SMA in remaining 25%. This dual blood supply renders the liver relatively protected against ischemia. When the blood flow to the bowel decreases below a certain level (the critical O2 delivery level), the cells will switch to anaerobic glycolysis, resulting in lactate production[17]. In the gastrointestinal system this occurs when blood flow is reduced below 50% of the basal rate[18,19]. In most cases of splanchnic ischemia, the arterial lactate levels will remain normal despite increased lactate production by the gut. The reason for this discrepancy is the large lactate metabolizing capacity of the liver. Thus, systemic lactic acidosis is a late phenomenon in these patients, indicating severe transmural ischemia and probably liver involvement as well.

The regulation of the splanchnic blood flow involves both vasoconstrictive and vasodilating substances. The main vasoconstricting substances are the catecholamines and endothelin, especially endothelin-1[20,21]. The main splanchnic vasodilators are nitric oxide (NO) and prostaglandins. It is assumed that part of the gastrointestinal toxicity of NSAIDs can be attributed to vasoconstriction because of reduced mucosal prosta-glandin concentration[22].

During low-flow states, splanchnic vasoconstriction is an early and profound phenomenon[23], which may lead to blood flow reduction below the 50% threshold. Splanchnic ischemia develops well before systemic hemodynamic instability arises[24]. This splanchnic vasoconstriction may be triggered by shock states, including hemorrhage, sepsis, dehydration or cardiac failure, from vasoactive medications, nicotine and cocaine abuse, or even in strenuous exercise[19,25-27] or severe psychological stress[28]. This combination of ischemia despite normal vessel anatomy, has given rise to the term non-occlusive mesenteric ischemia (NOMI).

Ischemic and reperfusion damage

After the onset of ischemia, an ischemic and reperfusion phase can be distinguished. When this ischemia lasts for less than 6-8 h all processes are reversible; thereafter, transmural gangrene may be the result of completely interrupted blood flow. The ischemic phase starts when the energy-containing ATP is depleted due to lack of oxygen, leading to disruptions of the tight junctions. Also, membrane-bound enzymatic pumps stop functioning, leading to inflow of luminal electrolytes and water into epithelial cells resulting in cell death. Both effects lead to reduced intestinal epithelial barrier function, with bacteria and other luminal contents entering the blood stream[29]. At the same time, the mucosal enzyme xanthine dehydrogenase is converted to xanthine oxidase (XO), which at this stage is harmless. These early effects of the ischemic phase alone are localized and can remain clinically undetected for many hours. The reperfusion phase starts when oxygen-enriched blood re-enters the ischemic tissue. This reperfusion may begin after partial dissolution of an embolus or thrombus or after revascularization. This oxygen is transformed into reactive oxygen species (ROS) by the abundantly present XO. ROS are toxic to proteins and DNA[30], and diffuse into tissues, leading to intensification and spreading of the damaged area. This so-called ischemia-reperfusion cascade initiates an inflammatory and thrombotic response in the submucosal layer of the villus. In the future, antagonists of leukocyte vessel wall adhesions, an early event in the ischemia-reperfusion cascade, could attenuate these inflammatory and thrombotic responses[31]. Successful restoration of splanchnic blood flow, containing toxic products, from the ischemic area into the systemic circulation might trigger multiple organ failure.

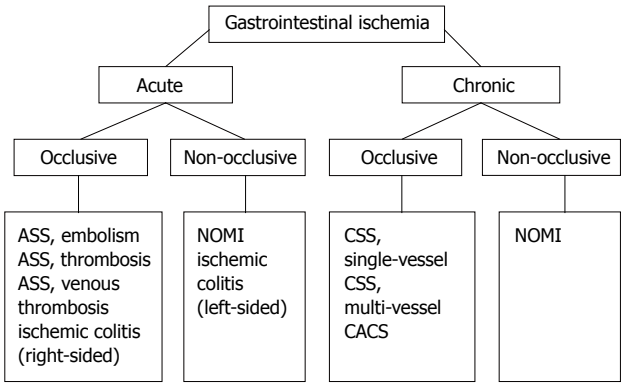

Figure 1 Classification of splanchnic, or gastrointestinal ischemia. ASS: Acute splanchnic syndrome; CSS: Chronic splanchnic syndrome; CACS, Celiac artery compression syndrome; NOMI: Non-occlusive mesenteric ischemia.

CLINICAL PRESENTATION

The naming of gastrointestinal ischemic syndromes is often confusing and requires a brief introduction. We will divide the different syndromes based on duration of complaints and vessel abnormalities (Figure 1). Acute splanchnic syndrome (ASS; synonym: acute mesenteric ischemia, acute bowel infarction) is characterized by a sudden onset of abdominal pain due to interrupted splanchnic circulation. It consists of occlusive disorders: acute splanchnic emboli, venous thromboses and end-stage arterial thrombotic occlusions, and the non-occlusive disorder, NOMI. Chronic splanchnic syndrome (CSS) is defined by a combination of chronic splanchnic disease (CSD) with ischemic symptoms. Celiac artery compression syndrome (CACS) is defined by the combination of eccentric celiac artery compression by the arcuate ligament of the diaphragm and chronic abdominal symptoms caused by ischemia. NOMI may be diagnosed with chronic or remittent splanchnic hypoperfusion, for example in heart failure[32] or in dialysis patients[33]. Finally, ischemic colitis is a separate entity and will be discussed separately.

Acute splanchnic syndrome

Acute splanchnic ischemia can result from arterial thrombosis, acute embolism, venous thrombosis or non-occlusive ischemia[34-39]. Most often the superior mesenteric

artery is involved. Clinically, it is recognized by an acute onset of abdominal pain, which might be accompanied by nausea, vomiting and hypotension. On physical examination and laboratory testing there are usually minimal abnormalities at first[40]. If left untreated, the pain often disappears. Without restoration of blood flow and depending on the collateral circulation, a full blown peritonitis follows within hours or days, with translocation of bacteria and SIRS, or systemic inflammatory response syndrome, and multiple organ failure. Mortality is high, ranging from 32%-80% depending on the etiology. In the last four decades a reduced mortality rate was observed for venous thrombosis and arterial embolism (now 32% and 51%), while the mortality of NOMI and arterial thrombosis remained unchanged at 73% and 77%[39]. Therefore, the most important factors for improvement of survival should be a high index suspicion, a proper diagnosis of CSS before ASS develops and an immediate restoration of blood flow.

Chronic splanchnic syndrome

Chronic splanchnic syndrome (CSS), a synonym for chronic mesenteric ischemia, gastrointestinal ischemia or intestinal angina, is a relatively rare disorder and may be under-diagnosed. After institution of a multidisciplinary approach team for the evaluation of insufficiently explained abdominal pain in the Medisch Spectrum Twente Hospital, the recognition of CSS increased from seven to 23 persons per million per year[41]. The major symptoms of CSS are outlined in Table 1. The most characteristic is postprandial pain, starting 15-30 min after a meal, and persisting for 1-3 h. Patients often report fear of eating, and take smaller meals, with less fat and proteins. Weight loss, the second characteristic finding in CSS, is almost always caused by reduced intake due to this fear of eating and not to malabsorption. Diarrhea, unexplained gastric ulcers or even gastroparesis can also be presenting symptoms.

This multidisciplinary team, with nationwide referrals, also found a differentiation between single- and multi-vessel disease[6]. Although the clinical presentation is quite similar, the course and outcome justifies separate discussion of both groups[42].

Single-vessel disease

The etiology of isolated stenoses of the splanchnic arteries, most often the celiac artery, is caused in most cases by splanchnic arteriosclerosis or external compression by the crux of the diaphragm[5]. Due to the presence of abundant collateral vessels, it was generally assumed that a single stenotic lesion rarely, if ever, causes complaints[43]. In 1972, Szilagyi *et al*[44] reviewed the entire literature on CA compression syndrome and found no proof of any abnormality of intestinal structure or function that could be attributed to this compression, nor proof that treatment had more than a placebo effect. However, several papers were published with good results for CA decompression operations[45-47]. These opinions were challenged recently by our group. We have shown that by using tonometry as a functional test, we could successfully distinguish patients that benefited from surgery from those who did not, and that the disappearance of symptoms after successful revascularization was associated with normalization of this functional test[5]. The prognosis of single-vessel CSS seems rather benign. In 50 patients with an isolated stenosis, no mortality and low post-operative morbidity were seen on follow-up, which contrasts sharply with multi-vessel CSS[6]. No difference in short-term outcome between patients with CACS or atherosclerotic lesions was observed. In our view, single-vessel CSS can be diagnosed when (1) there is a significant stenosis on duplex ultrasound or angiography (> 70%), (2) the clinical history fits CSS and (3) the functional test (gastric exercise tonometry) indicates splanchnic ischemia.

Table 1 Clinical picture in 107 CSS patients[6]

Patient characteristics in 107 CSS patients	
Age	Mean 55 years, range 18-85
Male:Female	26%:74%
Duration of symptoms	Mean 18 mo, range 3-192
Reported weight loss	78%
BMI	Mean 20.8 kg/m^2, range 12.0-33.2
BMI < 20 kg/m^2	35%
Weight loss (kg/mo)	Mean 1.3 kg/mo, range 0-8
Pain after meal	86%
Pain after exercise	43%
Abdominal buit	24%
Classical abdominal angina	22%
Cardiovascular history	40.20%
Nicotine use	45.80%

Multi-vessel disease

When 2 or 3 of the main splanchnic arteries have significant stenoses the ratio of CSS versus CSD increases to almost 90% in patients referred to our unit (unpublished data). Although the clinical presentation is in essence not very different from patients with single vessel disease, (postprandial pain and weight loss as the main symptoms) sometimes quite atypical presentations can be seen. Even experienced clinicians can miss an adapted lifestyle that masks a case of slowly progressive CSS. In multi-vessel CSS the clinical presentation can mimic simple dyspepsia with bloating and fullness, gastroparesis, unexplained diarrhea or simply lack of energy. When the disease progresses, the pain may be provoked by small triggers such as a simple drink, or even during rest. Abdominal vascular resting pain, persisting abdominal pain not related to a meal, are important prognostic indicators, and often indicate imminent or ongoing bowel infarction, or ASS. It should be remembered that the time to develop irreversible ischemic changes is about 6-8 h in end-stage 2- or 3-vessel CSS.

Ischemic colitis

There are two types of ischemic colitis: left-sided and right-sided ischemic colitis. Left-sided ischemic colitis usually presents with abrupt onset abdominal pain, followed by bloody diarrhea, which may persist for days

to weeks. It is often associated with low-flow states[48], coagulation disorders[49], cardiac abnormalities or after abdominal aortal surgery. Low-flow states may be induced by arrhythmias or cardiac dysfunction, drugs, dehydration or (aortic) surgery. Isolated right-sided ischemic colitis usually presents with abdominal pain, but rarely with bloody diarrhea[50]. Right-sided ischemic colitis is usually associated with SMA stenosis or occlusion. It should therefore be considered as part of CSS or ASS.

The late stages of ischemic colitis can be a clinical challenge, with clinical presentation and endoscopic findings mimicking both Crohn's disease and ulcerative colitis. In most cases isolated ischemic colitis will be transient, although persistent colitis, stricture formation and even gangrenous colitis have been seen to develop[48].

NOMI

NOMI has already been mentioned as cause of ASS in 20%-30% of patients. Most NOMI patients however, never develop ASS, and this condition is quite common due to the early splanchnic vasoconstriction with reduced circulating blood volume of any cause. Moreover, the bowel has limited capacity to preserve aerobic metabolism. NOMI is very common and widely recognized in intensive care and peri-operative medicine, where it is referred to as intramucosal acidosis or mucosal ischemia[7]. Here, the clinical signs of NOMI may range from abdominal tenderness, nausea, diarrhea, ulceration, bleeding and full thickness ischemia, and may lead to bowel wall necrosis and even death. In critically ill patients, this process can easily lead to translocation of bacteria and endotoxins, causing SIRS and multi organ failure[51].

We also distinguished a group of patients with 'abdominal migraine' characterized by symptoms compatible with splanchnic ischemia, abnormal functional tests (tonometry) indicating ischemia, splanchnic angiography without relevant pathology, and good response to vasodilators[4,52].

DIAGNOSTIC METHODS

Duplex ultrasound

Duplex-ultrasound is widely used as screening tool for detection of splanchnic stenosis. In experienced hands the CA and the SMA can be visualized in 80%-90% of patients. Proper visualization can be difficult because of the location behind the, often air-filled, stomach. First, vessel anatomy is established using the B-mode. This is followed by assessment of blood-flow pattern and velocity. The arterial blood-flow in the splanchnic vessels varies during the cardiac cycle. The normal CA has a biphasic signal. Retrograde flow in the common hepatic artery may indicate proximal CA stenosis or occlusion. The SMA normally has a triphasic signal. A biphasic signal in the SMA is normal after a meal or when the right, or rarely the common hepatic artery, comes from the SMA as an anatomic variant; it may indicate a proximal stenosis if it occurs in the fasting state. Blood-flow measurement is performed using a measurement angle of less than 60 degrees. A significant stenosis is characterized by areas of high velocity jets (small streaks of very high, sometimes turbulent flow) within the artery, and overall increased flow velocity. The widely accepted cut-off values, published by Moneta in 1993, are: for the CA a Peak Systolic Velocity (PSV) and End Diastolic Velocity (EDV) of 200 m/s and 55 m/s; and for the SMA, PSV and EDV of 275 and 45 m/s, respectively[53]. Using these threshold values, the sensitivity and specificity for stenoses > 70% were 89% and 44% for the inspiration CA, 89% and 62% for the expiration CA, 100% and 61% for the inspiration SMA, and 80% and 42% for the expiration SMA. One criticism of the "Moneta criteria" has been that he used a cohort from the general population with atherosclerotic disease, who did not necessarily suffer from chronic abdominal symptoms. In daily practice, duplex ultrasound is performed under fasting conditions. Some studies suggested measurement after test meals, because patients with ischemia had suppressed augmentation of blood-flow following a meal compared to subjects with normal vessels[54-58]. Until recently, post-test meal splanchnic duplex made no headway in daily practice. Duplex-derived flow velocities after splanchnic artery bypass grafting may be affected by graft diameter or type of reconstruction and are not equal to the preoperative criteria[59].

MRA

By using contrast, so-called contrast enhanced (ce)-MRA it is possible to identify splanchnic artery stenoses and even collaterals, for example Riolan's artery[60-62]. Surprisingly, we could identify only two studies that compared digital subtraction angiography (DSA) with MRA in 19[63] and 23 patients[61] with good correlation. In our own experience, with 25 patients in whom DSA and MRA were performed within 2 mo, the MRA had a 95% sensitivity and 90% specificity, compared to DSA as the "gold standard". We observed a significant inter and intra observer variation in grading of stenoses in 45% of the cases[64]. Although widely used, it can therefore not be considered a gold standard investigation for assessment of vessel anatomy. A potential advantage of MRA is its ability to measure actual flow through the splanchnic and portal circulation. The arterial flow is harder to measure than in veins, because of the smaller caliber and the pulsatile character of arterial flow[56]. However, a consistent relationship between flow in the arteries and veins was observed[65,66]. Several studies with healthy volunteers and patients have shown augmentation of flow after a meal and significant differences between patients with vessel stenoses and healthy volunteers[54,58]. These results may be promising for future use in diagnosing CSS.

CT angiography

With the introduction of the multi-slice CT scan, CT angiography of the abdominal arteries has become possible. There are several studies showing that CT angiography is an accurate way to image the splanchnic arteries, veins, and collaterals[67-70]. Surprisingly, studies focusing on CSD and comparing this technique to the gold

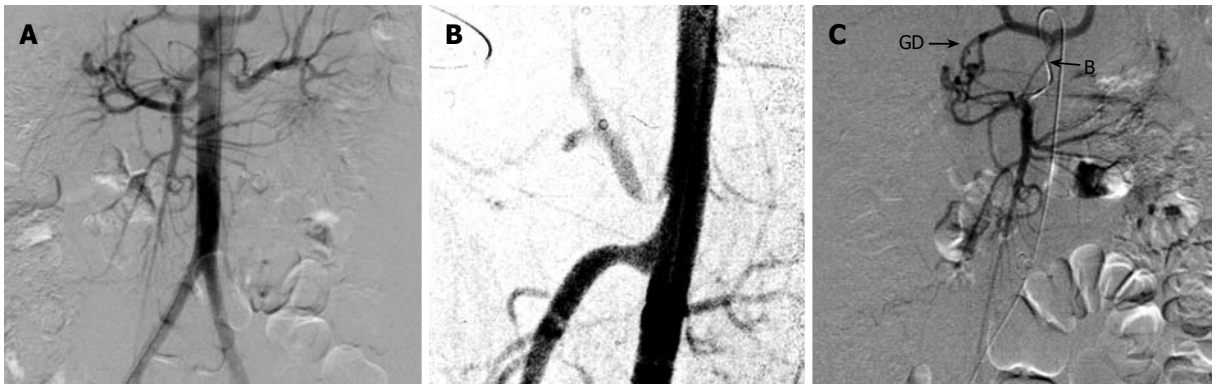

Figure 2 Collateral vessels: gastroduodenal (GD) artery and Buehlers artery (B). A: The gastroduodenal (GD) artery and Buehlers artery (B) are visible on non-selective aortography indicating stenosis of the origin of either the CA or SMA. The late filling of the CA points to a stenosis in its origin; B: Lateral aortography showing an asymmetrical stenosis of the CA; C: On selective canulation of the SMA, both collaterals are more clearly visible.

standard (DSA) in a representative group of patients, are lacking to our knowledge. It is essential to use multi-slice scanners with slice thicknesses of 2 mm at most (preferably 1 mm) to allow accurate visualization of the arteries. With the state-of-the-art technology, CT-scanning in inspiration and expiration is possible and is a prerequisite when there is suspicion of celiac artery compression syndrome. The advantages of CT angiography are clear: in a patient with acute abdominal complaints it can show or exclude arterial and venous obstruction, bowel involvement (wall distention and thickening, presence or absence of contrast enhancement, pneumatics), as well as alternative diagnoses. Among these are perforations, pancreatitis, and abscesses. Similarly, in the setting of chronic unexplained postprandial pain, CT angiography may also show alternative diagnoses. In our experience, these include pancreatitis, pancreatic cancer, and retroperitoneal tumors. Adding the advantages of minimal invasiveness and lower costs, CT angiography can be a serious competition for conventional angiography[67,69,71,72].

Angiography

Intra-arterial digital subtraction angiography (DSA) of the splanchnic vessels can be used to perform endovascular therapeutic procedures in the same session, including infusion of papaverine and angioplasty or stenting of stenoses. The combination of high diagnostic accuracy and the possibility for intervention makes angiography the procedure of choice in patients suspected of symptomatic splanchnic stenoses, especially with imminent or ongoing infarction. In acute splanchnic infarction, angiography serves as guideline for endovascular or operative revascularization.

A state-of-the art visceral angiography involves three steps: (1) a non-selective anterior-posterior abdominal aortic angiography. When collaterals show in this stage, they indicate significant splanchnic artery stenosis and are considered pathological (Figure 2A and B); (2) a lateral aortography during maximal inspiration and expiration, for detection of external compression of the CA and/or, rarely, the SMA; and (3) selective angiography of all three splanchnic vessels to obtain a detailed view of the vascular anatomy, stenoses and anatomical variations[42]. Although CT and MR angiography are gaining ground as the principal investigative tools for splanchnic vessel anatomy, detailed angiographic information of anatomy, stenosis, collaterals and anatomical variations is, in our view, essential in the preparation of an optimal revascularization strategy.

Endoscopy

Although almost all patients with CSS underwent upper GI endoscopy during the work-up of their complaints, abnormalities were rarely noted. Reports on gastroparesis and gastric gangrene have been published, but are also rare. In our experience, 7% of patients with CSS presented with unexplained gastroduodenal ulcers (Helicobacter negative, no NSAID-use). In colonic ischemia endoscopy is the mainstay of diagnosis. The typical picture consists of superficial ulceration, mucosal friability, edema, and patchy areas of either bleached or cyanotic mucosa[73,74]. Colonic ischemia of longer duration may mimic ulcerative colitis or even Crohn's disease. Some papers have focused on endoluminal light spectroscopy oximetry. With this technique, mucosal hemoglobin oxygen saturation can be measured[75-80]. In a recent study it was shown that mucosal saturations are low in patients with chronic splanchnic ischemia, compared to healthy subjects. After successful treatment in these patients, mucosal oxygen saturations increased substantially[76]. There are a several potential limitations to this technique. Firstly, ischemia is patchy in nature, and can therefore be missed. Secondly, ischemia might be present in parts of the small bowel that are difficult to reach endoscopically. Thirdly, ischemia might only be present in a situation of increased oxygen demand (after a meal, or during exercise), especially earlier in the course of the disease.

Tonometry

Tonometry of the gastrointestinal tract has the unique potential to the detect ischemia, irrespective of flow or metabolism. Tonometry is based on a general physiological principle that during ischemia, anaerobic metabolism leads to increased production of acids, which are buffered locally by bicarbonate ions, leading to increased carbon dioxide tension (PCO_2) in the tissue. This relation between

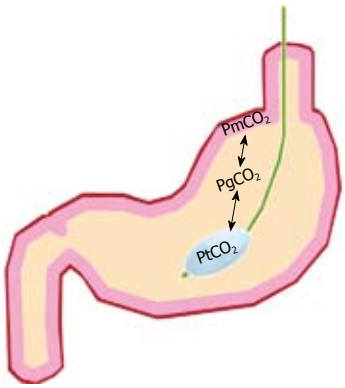

Figure 3 Tonometer balloon placed in the stomach nasogastrically. CO_2 diffuses rapidly over different membranes, therefore the tonometer PCO_2 ($PtCO_2$) will be in equilibrium with gastric luminal PCO_2 ($PgCO_2$) and mucosal PCO_2 ($PmCO_2$). The PCO_2 can be measured from the catheter either from injected saline using blood gas analyzers or by connection to a semi-automated Tonocap device. The underlying physiological principle is that ischemia is always associated with PCO_2 increase. Therefore, focal measurement of ischemia is possible for long periods *via* a minimally invasive technique.

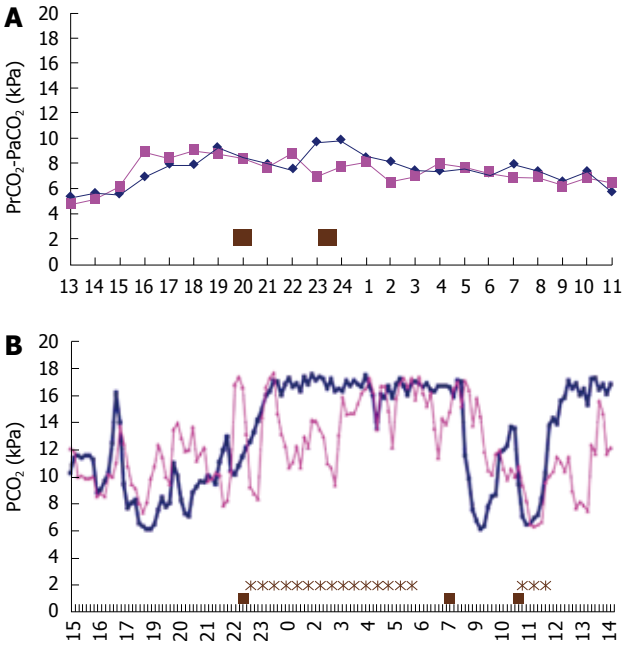

Figure 4 Imminent ASS and normal gastric and jejunal PCO_2 pattern. A: Normal 24 h PCO_2 pattern in the stomach (squares) and jejunum (diamonds) with variation in PCO_2, but no peaks above 11 kPa following meals; B: Imminent bowel infarction in a patient with severe 3 vessel CSS. After her evening meal she had pain for almost 6 h, and extreme ischemia with PCO_2 > 16 kPa for 7 h. She was treated with endovascular stent placement the day after this measurement, with immediate relief of complaints. She is still doing well, over 3 years later.

ischemia and increased PCO_2 has been observed in all ischemic models and animals studied. The most specific marker of ischemia is an increased difference between luminal and arterial CO2, the PCO_2 gradient, which is barely influenced by other systemic factors, including hyper- or hypoventilation. The luminal PCO2 can be measured conveniently using a nasogastric tonometry catheter and air tonometry (Figure 3). The unique property of tonometry in measuring ischemia *per se*[3] sets it apart from all other diagnostic methods. Indeed, when blood flow is gradually reduced, the PCO2 gradient remains normal until the blood flow decreases to < 50% of the basal flow and then increases sharply[18,19].

In patients with suspected chronic ischemia, gastric tonometry has been used initially using a test meal with variable, but overall disappointing, results[81-83]. The main methodological problems involved buffering effects by gastric acid and dilution effects of the ingested test meals[84]. We therefore developed a tonometric test involving 10 min of submaximal exercise, in order to provoke GI ischemia[19]. The diagnostic accuracy of the gastric exercise tonometry test (GET) was evaluated in a cohort of patients referred for suspected CSS. GET had a 78% sensitivity and 92% specificity for ischemia detection[4]. We have used GET to guide treatment in patients with single vessel stenoses. The main finding in this study was the tight relationship between normalization of GET and disappearance of symptoms after anatomically successful revascularization[5]. We also re-examined the potential use of a 24-h tonometry test, including test meals, with standardization of the test circumstances, including potent acid suppression and standard test meals[85]. In a pilot study in 33 patients referred for suspected CSS, the 24 h tonometry showed promising results, with a sensitivity of 76% and a specificity of 94%, comparable to those of exercise testing[86]. We are now analyzing a study comparing GET and 24-h tonometry. The preliminary data suggest that 24-h tonometry permits accurate measurement of postprandial and fasting PCO_2 levels; following meals, gastric and small bowel PCO_2 gradients may physiologically increase up to 10 kPa. During ischemia, gradients exceed 11 kPa (60 mmHg)[87]. Also, prolonged PCO2 increases for several hours (up to 7 h in one subject, Figure 4), especially in combination with abdominal pain during fasting, indicate imminent infarction, and thereby provide invaluable extra information.

Serological markers

Currently, there is no reliable marker to diagnose gastrointestinal ischemia. Studies have been performed using several markers, including (L and D)-lactate, LDH, D-dimer; however, none of these was proven to be sensitive or specific. In contrast, various animal models have successfully used markers like D-lactate and i-FABP (intestinal Fatty Acid Binding Protein) as an early marker of intestinal ischemia. This enzyme is present in the mature enterocytes of the small intestine, in the highest concentration at the villi[88,89], the region most susceptible to ischemia, and is released early after an ischemic insult. Therefore it seems a good candidate marker for early ischemia detection[90]. There are a few patient studies indicating its potential as marker for ASS[89,91], but also during pancreatitis[92] or inflammatory bowel disease[93]. We performed a pilot study comparing tonometric responses to a test meal and indeed found increased i-FABP in these subjects[94]. More studies are needed before the role of this and other promising plasma markers can be established.

WORK-UP AND TREATMENT

In the work-up of patients in whom splanchnic ischemia is suspected four questions should be addressed: (1) is the history compatible with splanchnic ischemia, (2) which of the three vessels are narrowed, to what degree and are pathological collaterals present, (3) is there (functional) evidence of actual ischemia, and (4) is the impairment of the splanchnic blood flow in the short-term threatening for the bowel.

Endovascular techniques have emerged allowing for stent placement in CA and SMA in most patients. The choice is between dilation or stent placement. Dilation alone has a low short-term success rate, and we currently use it only as diagnostic tool to distinguish between CSS and CSD. Although some vessels can be treated *via* the femoral artery; the sharp downward angulation (60 degrees) of the AMS and CA often necessitates brachial artery canulation in many cases. In our center, both techniques are often combined, using the femoral catheter as a guide for the stent placed *via* the brachial catheter. Compared to operative revascularization, the main disadvantage of stent placement is the shorter long-term patency. The latter was shown in three recent studies, of which two compared endovascular and open repair. Atkins *et al* reported in a cohort of 80 patients with CSS primary patency at 1 year of 58% after endovascular and 90% after open repair and a primary assisted patency of 65% *vs* 96% respectively[95]. Bieble *et al* reported that in a cohort of 49 patients with CSS, 75% after endovascular and 89% after open repair were symptom free after 2 years[96]. The main difference in this study was the restenosis rate of 8% after open versus 25% after endovascular treated patients, with lower complication rates in the latter. Sarac *et al*[97] reported a primary, primary assisted and secondary patency of 65%, 97% and 99% in a cohort of 87 endovascular treated splanchnic arteries. The low short-term morbidity makes it an excellent choice in patients with limited life expectancy or those too weak or underweight for operative revascularization.

A variety of surgical techniques have been advocated for open repair of the splanchnic arteries, including reimplantation, transarterial and transaortic endarterectomy, antegrade and retrograde aortovisceral bypass using vein or arterial autograft bypasses and prosthetic bypass, with early success rates between 91% and 96% and late success rates between 80% and 90%[98]. The choice of technique is usually based on the preference and experience of the surgeon. However, the majority of centers with wide experience believe that antegrade autogeneous revascularization techniques of both the CA and the SMA in selected cases offers the best long-term results. The disadvantage of major aortic surgery is the not inconsiderable burden for the patient. In general, we prefer antegrade two-vessel reconstruction for young patients with CSS and a body mass index above 19.5 kg/m^2, and endovascular or minimal invasive retrograde single or multi-vessel reconstructions for patients with relevant comorbidity or reduced life expectancy. Also endovascular repair could act as bridge to open repair after full recovery and weight gain in selected young patients with end stage CSS.

Acute splanchnic syndrome

The diagnosis of ASS begins with a high index of suspicion. Any patient with acute onset of abdominal pain that remains unexplained after proper investigation for two hours should be suspected to have ASS. An urgent investigation of vessel patency should be ordered. The choice is between an acute angiography and a CT scan. Simultaneously with the CT scan or DSA, the volume status should be aggressively restored to counterbalance the splanchnic vasoconstriction, which is almost always present in these patients. All necrotic bowel should be removed and blood flow restored as soon as possible. The latter will involve intravenous heparin in venous ASS, revascularization or embolectomy in arterial ASS, and in selected cases, intra-arterial vasodilation in ASS-NOMI[73]. In arterial ASS, the choice of revascularization, and whether it should be done before or after bowel resection, depends on local expertise. Bowel vitality can be hard to assess initially, therefore second- and third look operations to ascertain bowel vitality are often advised and seem prudent. It has been shown that aggressive treatment might be responsible for the modest improvement in outcome of ASS[39]. In cases of limited extent of severe bowel ischemia we advise immediate retrograde endovascular revascularization[99,100] and resection of the ischemic bowel to diminish the detrimental cascade of ischemia-reperfusion resulting in multi organ failure and high mortality.

Acute ischemic colitis

Isolated acute left-sided ischemia can usually be treated conservatively, as it is almost always non-occlusive in nature. Right-sided ischemic colitis should be considered as ASS and treated accordingly. Patients with left-sided ischemic colitis should be treated with intravenous fluid and bowel rest. Broad spectrum antibiotics are advised, which might reduce bowel damage[48,101], but there is no evidence to back this up. Left-sided ischemic colitis subsides in 2 wk[48] in most patients. About 20% of patients with acute ischemic colitis ultimately need surgery, either because the ischemic colitis persists or complications occur. A non-responsive left-sided ischemic colitis can manifest as ongoing sepsis refractory to medical treatment, persistent diarrhea, bleeding, or protein-losing enteropathy for more than 14 d. In some patients progressive peritonitis or gangrene of the colon develops, with a mortality rate of 30%-60%[48,102].

CHRONIC SPLANCHNIC SYNDROME

Single-vessel CSS and CACS

Patient selection is crucial in these patients. When single-vessel CSS is diagnosed by history, vessel anatomy and functional tests, treatment may help to relieve the symptoms. The prognosis quod vitam is good; therefore

treatment is aimed at relief of symptoms only. Some patients prefer conservative measures including small meals and proton pump inhibitors. When a revascularization is indicated, the choice of technique depends on vessel abnormalities as well as local experience.

In CACS patients primary stent placement is not an option because the repeated force by the diaphragm with each respiration will fracture the stent in the short-term. Different techniques are currently used to release the CA from compression by the diaphragm's crux. One potential complication is the development of reflux disease[5], which is related to the damage to the crux, which also plays an important role in the physiological anti-reflux barrier. Recently, we have performed the release by an endoscopic retroperitoneal technique with equally good results compared to the open approach in the short-term[103]. The problem of reflux seems to be reduced, although more studies with longer follow-up times are needed.

Multi-vessel CSS

Most patients with abdominal symptoms and multi-vessel stenoses have, in our experience, CSS. The risk of developing ASS is considerable[6], therefore the treatment goal is aimed at symptom relief as well as prevention of ASS. There are no prospective studies on the conservative treatment of CSS, but advice on diet and lifestyle have been described. Most patients have already changed their eating pattern, with smaller and more frequent meals that contain less fat and protein. It should be strongly advised to stop smoking because it causes strong splanchnic vasoconstriction. The use of proton pump inhibition is not evidence based, however, it makes sense as these drugs reduce the secretion of gastric acid, and thus gastric metabolic demand, while increasing the gastric blood flow[104]. Atherosclerosis is a generalized disorder; therefore the usual measures should be initiated including treatment of hypertension, hyperlipidemia, and diabetes.

Most multi-vessel CSS patients have an indication for revascularization. Whether patients with multi-vessel (asymptomatic) CSD should be treated to prevent ASS is uncertain. It may be considered in young patients in good health, but firm evidence is lacking. The choice of revascularization in multi-vessel CSS again depends on anatomy, experience, and comorbidity as discussed before.

NOMI

Chronic splanchnic ischemia due to NOMI comes in two different patient groups. The first group has severe underlying medical conditions, with reduced effective circulating volume and splanchnic vasoconstriction and ischemia. These include dialysis patients[33] and chronic heart failure[32] patients. Treatment is difficult because the treatment of their underlying disease requires reduction of intravascular volume, which may worsen their abdominal complaints. In our experience the use of nitrates, ketanserin and alpha-inhibitors may have a positive effect on the abdominal symptoms, whereas calcium channel blockers seemed to worsen these. A second group we encountered are patients with a clinical presentation similar to CSS, with normal microvasculature but functional tests indicating ischemia. As discussed earlier, in some of these we have observed vascular spasms during angiography, with onset of pain within minutes thereafter[4]. In a pilot study, we treated them with vasodilators, nitrates, ketanserin or nicorandil. Over 50% of patients show a reduction of abdominal pain of at least 50% on a visual analog scale, which is sustained for years in most cases[52]. Further studies are needed to assess the prevalence, precise mechanism and the best treatment options of this disorder.

Post-intervention care

After revascularization, severe reperfusion injury can occur. The exact risk factors for its development are unknown. In our experience it is more common in patients with serious and long-standing multi-vessel disease. The following pattern in reperfusion damage after revascularization is typical. The first 1-2 d after revascularization the patient has good clinical recovery. Most could start eating again without pain or special discomfort. After 2-3 d symptoms develop, in hours to days, consisting of nausea, abdominal pain, similar or even worse than before treatment, diarrhea and in extreme cases, protein-losing enteropathy with very low serum albumin levels. Massive ascites may develop during reperfusion.

It is crucial to distinguish reperfusion injury from vessel occlusion; therefore vessel patency has to be ascertained with CTA or DSA. This bowel reperfusion syndrome can persist for days to weeks. We therefore treat these patients with parenteral nutrition, intravenous fluids, and proton pump inhibitors. The end of the reperfusion syndrome is usually heralded by increased appetite, reduced pain and reduced diarrhea. Patients can restart oral intake, be taken off parenteral nutrition and generally have uneventful recovery within weeks thereafter. No long-term complications have been observed in these patients.

CONCLUSION

Splanchnic ischemia has developed into a broad spectrum of diseases. These are characterized by onset, vessel anatomy, and presence of ischemia. Each syndrome has different characteristics, outcome, and treatment options, therefore a state-of-the art vessel anatomy assessment and accurate functional test are crucial. Tonometry is the only validated test assessing the adequacy of the splanchnic blood-flow and consequently is crucial in proper patient selection. Treatment options, including noninvasive, minimal invasive and classical open vascular reconstructive techniques, are wide and require a multi-disciplinary team-approach for proper selection and follow-up.

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Scutellaria barbate extract induces apoptosis of hepatoma H22 cells *via* the mitochondrial pathway involving caspase-3

Zhi-Jun Dai, Xi-Jing Wang, Zong-Fang Li, Zong-Zheng Ji, Hong-Tao Ren, Wei Tang, Xiao-Xu Liu, Hua-Feng Kang, Hai-Tao Guan, Ling-Qin Song

Zhi-Jun Dai, Xi-Jing Wang, Hong-Tao Ren, Xiao-Xu Liu, Hua-Feng Kang, Hai-Tao Guan, Ling-Qin Song, Department of Oncology, the Second Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China
Zong-Fang Li, Zong-Zheng Ji, Department of General Surgery, the Second Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China
Wei Tang, Department of life Science, Shaanxi Normal University, Xi'an 710061, Shaanxi Province, China
Author contributions: Dai ZJ and Wang XJ designed the research; Dai ZJ, Li ZF, Ren HT, Tang W and Song LQ performed the research; Li ZF, Ji ZZ, Liu XX and Kang HF contributed to the reagents, materials, analysis; Dai ZJ and Guan HT analyzed the data; Dai ZJ and Ren HT wrote the paper.
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Correspondence to: Dr. Zhi-Jun Dai, Department of Oncology, the Second Affiliated Hospital, Medical School of Xi'an Jiaotong University, No. 157, West 5th Road, Xi'an 710004, Shaanxi Province, China. dzj0911@126.com
Telephone: +86-29-87679526 Fax: +86-29-87679282
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Abstract

AIM: To study the growth inhibitory and apoptotic effects of *Scutellaria barbata* D.Don (*S. barbata*) and to determine the underlying mechanism of its antitumor activity in mouse liver cancer cell line H22.

METHODS: Proliferation of H22 cells was examined by MTT assay. Cellular morphology of PC-2 cells was observed under fluorescence microscope and transmission electron microscope (EM). Mitochondrial transmembrane potential was determined under laser scanning confocal microscope (LSCM) with rhodamine 123 staining. Flow cytometry was performed to analyze the cell cycle of H22 cells with propidium iodide staining. Protein level of cytochrome C and caspase-3 was measured by semi-quantitive RT-PCR and Western blot analysis. Activity of caspase-3 enzyme was measured by spectrofluorometry.

RESULTS: MTT assay showed that extracts from *S. barbata* (ESB) could inhibit the proliferation of H22 cells in a time-dependent manner. Among the various phases of cell cycle, the percentage of cells in S phase was significantly decreased, while the percentage of cells in G_1 phase was increased. Flow cytometry assay also showed that ESB had a positive effect on apoptosis. Typical apoptotic morphologies such as condensation and fragmentation of nuclei and blebbing membrane of apoptotic cells could be observed under transmission electron microscope and fluorescence microscope. To further investige the molecular mechanism behind ESB-induced apoptosis, ESB-treated cells rapidly lost their mitochondrial transmembrane potential, released mitochondrial cytochrome C into cytosol, and induced caspase-3 activity in a dose-dependent manner.

CONCLUSION: ESB can effectively inhibit the proliferation and induce apoptosis of H22 cells involving loss of mitochondrial transmembrane potential, release of cytochrome C, and activation of caspase-3.

Key words: *Scutellaria barbate*; Hepatoma; Apoptosis; Mitochondrial transmembrane potential; Serum pharmacology

Peer reviewers: Debbie Trinder, PhD, School of Medicine and Pharmacology, University of Western Australia, Fremantle Hospital, PO Box 480, Fremantle 6959, Western Australia, Australia; Dr. Katja Breitkopf, Department of Medicine II, University Hospital Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

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INTRODUCTION

Scutellaria barbata D.Don (*S. barbata*) is a perennial herb, also known as Ban-Zhi-Lian (barbat sskullcap) in traditional Chinese medicine. It is mainly distributed in southern China and has been used as an antitumor agent for lung cancer, digestive system cancer, hepatoma,

breast cancer, and chorioepithelioma as well as an anti-inflammatory agent and a diuretic in China and Korea[1-9]. Extracts from *S. barbata* (ESB) have *in vitro* growth inhibitory effects on a number of human cancers including leukemia, colon cancer, hepatoma and skin cancer[4-10]. However, its antitumor mechanism still remains unclear.

It was reported that many Chinese herbs have anticancer properties and induce apoptosis[11]. Three apoptotic pathways have been addressed, including the mitochondrial pathway[12,13], death receptor pathway[14], and endoplasmic reticulum stress-mediated apoptosis pathway[15]. The mitochondrial pathway initiates apoptosis in most physiological and pathological situations. Permeabilization outside mitochondrial membrane plays the most important role in mitochondrial apoptosis. In the mitochondria-initiated pathway, mitochondria undergoing permeability transition release apoptogenic proteins such as cytochrome C or apoptosis-inducing factor from the mitochondrial intermembrane space into the cytosol[16]. Released cytochrome C can activate caspase-9, and activated caspase-9 in turn cleaves and activates executioner caspase-3. After caspase-3 activation, some specific substrates for caspase-3 such as poly (ADP-ribose) and polymerase (PARP) are cleaved, and eventually lead to apoptosis[17].

In this study, *S. barbata* extract showed anti-tumor activity *in vitro* and could inhibit the growth of mouse H22 hepatoma cells by inhibiting cell apoptosis and cytotoxic effects, demonstrating that the extract from *S. barbata* can strongly inhibit cell proliferation and induce apoptosis of H22 cells through the mitochondrial dysfunction pathway.

MATERIALS AND METHODS

Reagents and animals

New bovine serum (Gibco, USA), RPMI-1640 medium(Gibco, USA), propidium iodide (PI) (Sigma, USA), dimethyl sulfoxide (DMSO), ribonuclease (RNase A), rhodanmin123 (Rh123), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). Mouse monoclonal antibodies against caspase-3 and cytochrome C were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Apoptotic cell Hoechst 33258 detection kit was purchased from Nanjing Kai-ji Biotechnology Development Ltd (China), and fluorescence probes Rhodamine 123 was purchased from Sigma (USA). Male SD rats weighing 220-250 g were purchased from the Experiment Animal Center, Medical School of Xi'an Jiaotong University (China).

Preparation of S. barbata extract and drug containing serum

S. barbata crude extract (ESB) was purchased from Xi'an Zhongxin Biotechnology Development Ltd (China). One kilogram of *S. barbata* was extracted three times with water as previously described[18]. Final qualification was 10:1. More specifically, stems of SB were cut into small pieces, boiled in water for 2 h, put into a filtrate, and concentrated by spray drying until the specific density reached 1.15-1.18.

"Serum pharmacology" was used to study the *in vitro* pharmacological activity of herb medicine as previously described[19]. ESB-containing serum was prepared as previously described[18,20]. Twenty male SD rats were randomly divided into control group, high ESB dose group, medium ESB dose group, and low ESB dose group (n = 5). Rats in the high, medium and low ESB dose groups received intragastric ESB of 6, 3 and 1.5 g/d per kg of body weight. Rats in the control group received normal saline, twice a day for 3 d. Two hours after the last administration, blood was immediately obtained from the heart and kept at room temperature for 4 h. The serum was separated by centrifugation at 2400 r/min for 10 min, collected following twice of filtration with a 0.22 μm cellulose acetate membrane, calefied in 56℃ water for 30 min, and stored at -20℃ for use.

Cell lines and culture

Mouse H22 hepatoma cells, purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1×10^5 U/L penicillin and 100 mg/L streptomycin in an incubator containing a humidified atmosphere with 50 mL CO_2 at 37℃. The cells were subcultured until reaching logarithmic growth phase. The viability of H22 cells, stained with trypan blue, was above 97%.

Cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Sigma, USA). H22 cells were seeded at a concentration of 5×10^3 cells/well in a 96-well plate, and grown at 37℃ until adherence. At end of the treatment, 50 μg/10 μL of MTT was added and the cells were incubated for another 4 h. Two hundred μL of DMSO was added to each well after the supernatant was removed. After the plate was shaken for 10 min, cell viability was detected by measuring the absorbance at 490 nm wavelength using an enzyme-labeling instrument (EX-800 type) in quintuplicate.

Cell viability (%) = the absorbance of experimental group/the absorbance of blank control group × 100%.

Detection of morphological apoptosis

Staining of cells with uranyl acetate and lead citrate was performed to detect morphological changes. Briefly, adherent H22 cells were treated with ESB at a high dose for 48 h. The treated cells were digested with pancreatin and fixed in 3% glutaraldehyde precooled at 4℃ for 2 h. To make ultra-thin sections of copper, cells were washed with PBS, fixed in 1% osmic acid for an additional hour, dehydrated in acetone and embedded in epoxide resin. After stained with uranyl acetate and lead citrate, the

sections were examined under a Hitachi-800 transmission electron microscope as previously described[21].

Nuclear staining

H22 cells were harvested by centrifugation, washed with PBS and fixed in 1% glutaraldehyde for 1 h at room temperature. The fixed cells were washed with PBS, stained with 200 μmol/L Hoechst 33258 for 10 min. Changes in nuclei after stained with Hoechst 33258 were observed under a fluorescence microscope (Olympus, BX-60, Japan).

Cell cycle analysis

H22 cells were incubated at 5×10^5 cells/well in 6-well plates, treated with a homologous drug for 48 h. The detached and attached cells were harvested and fixed in 70% ice-cold ethanol at -20℃ overnight. After fixation, cells were washed with PBS, resuspended in 1 mL PBS containing 1 mg/mL RNase (Sigma) and 50 μg/mL PI (Sigma), and incubated at 37℃ for 30 min in the dark. Samples of 10 000 cells were then analyzed for DNA content by FACScan flow cytometry (Beckman, USA), and cell cycle phase distributions were analyzed with the CellQuest acquisition software(BD Biosciences).

Detection of mitochondrial membrane potential

Mitochondria transmembrane potential ($\Delta\psi m$) was detected under laser scanning confocal microscope(LSCM) with Rhodamine 123 (Rh123) staining as previously described[22]. About 1×10^6 cells were harvested by trypsinization, washed twice with PBS, and incubated with Rh123 at the final concentration of 1 μL/mL for 20 min at 37℃ in the dark, centrifuged at 1000 r/min for 5 min, washed twice with a medium, resuspended in the medium, cultured at 37℃ in an incubator containing 50 mL CO_2 for 60 min. Fluorescence intensity was determined at an excitation wavelength of 488 nm, emission wavelength of 530 nm under a laser scanning confocal microscope (Olympus, FluoView™ FV300, Japan). The fluorescence intensity of Rhodamine 123 in cells represents the mitochondrial membrane potential[23].

Western blot analysis

H22 cells (2.5×10^7) were collected by centrifugation at 2000 r/min for 10 min at 4℃, washed twice with cold PBS (pH 7.2), centrifuged at 2000 r/min for 10 min. Protein content was determined using a Bio-Rad protein assay reagent with bovine serum albumin as the standard. Total proteins (30 μg/lane) were separated by 15% SDS-PAGE gel electrophoresis, and transferred to a 0.45 μm PVDF membrane (Amersham Pharmacia Biotech). The blots were incubated with the desired primary antibody overnight at the following dilutions: caspase 3 (1:1000), cytochrome C (1:1500), and β-actin (1:1500). Primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Subsequently, the membrane was incubated with appropriate secondary antibodies for 1 h at room temperature. The immunoblots were analyzed by densitometry on a GelDoc 2000 system (Bio-Rad Laboratories Inc. USA) as previously described[17,24].

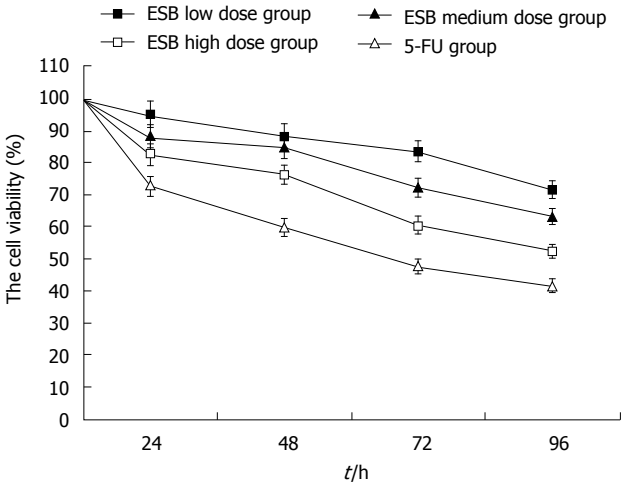

Figure 1 Inhibition of H22 cell proliferation by ESB. H22 cells were treated with different doses of ESB. The number of cells was determined at 0, 24, 48, 72, and 96 h, respectively. The viability of cells was detected by MTT assay. ANOVA analysis showed that the growth of H22 cells was inhibited by ESB in a dose- and time- dependent manner ($P < 0.05$).

Assay for caspase-3 activity

Caspase-3 activity was assayed using the caspase-3 activity assay kit (Nanjing Kai-ji, China) as previously described[25,26]. In brief, standard curve was plotted by detecting the absorbance of standard samples with terminal concentrations at each well, respectively. After 1 h incubation at room temperature, H22 cells were collected and lysed completely in a caspase assay buffer. The activity of caspase-3 was assayed in triplicate using a plate-reading iuminometer (Turner Designs, Sunnyvale, CA) at the wavelength of 405 nm. Nkat was used to represent the activity measured[25].

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed with analysis of variance (ANOVA) using the statistical software SPSS 11.0. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of ESB on proliferation of H22 cells

H22 cells were treated with different doses of ESB. The growth rate of H22 cells was evaluated after 0, 24, 48, 72, and 96 h, respectively. The cell viability of H22 cells in different ESB treatment groups was significantly higher than that in 5-FU treatment group (Figure 1). High and medium dose ESB inhibited the proliferation of H22 cells ($P < 0.05$), while low dose ESB could not obviously inhibit the proliferation of H22 cells ($P > 0.05$). MTT assay showed that high and medium dose ESB inhibited the proliferation of H22 cells *in vitro* in a time-dependent manner.

Morphological observation of apoptosis of H22 cells induced by ESB

High resolution transmission electron microscopy showed that normal H22 cells were round and regular in shape

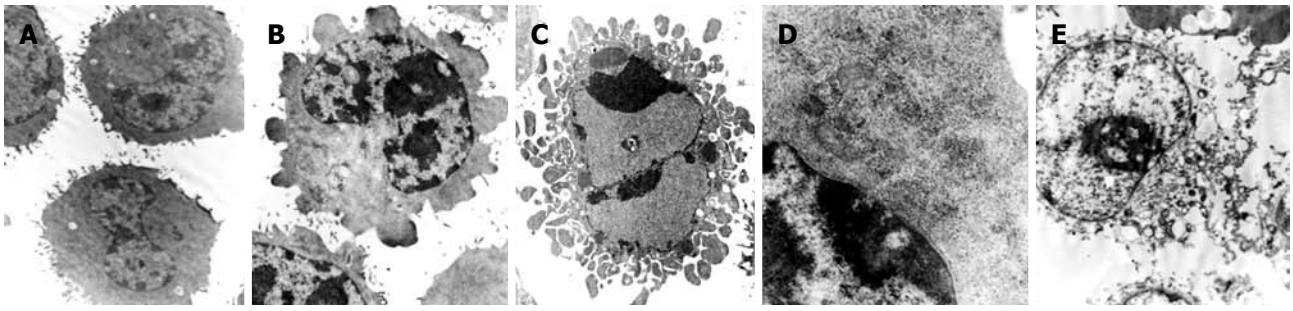

Figure 2 Morphological observation of H22 cells by EM after treatment. A: normal hepatoma H22 cells (5000 ×); B: karyopyknosis and chromatic agglutination in high ESB dose group (5000 ×); C: apoptotic body in high ESB dose group (5000 ×); D: chondriosome swelling in high ESB dose group (6000 ×); E: cellular swelling and necrosis in 5-FU group (5000 ×).

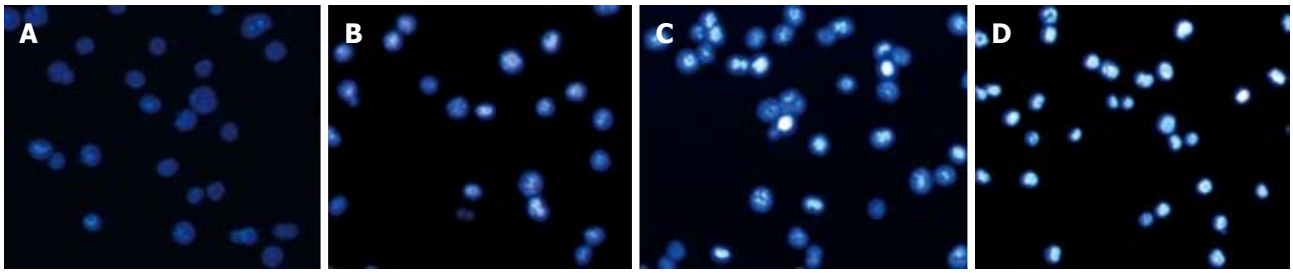

Figure 3 Cell apoptosis observed with Hoechst 33258 staining under a fluorescence microscope (× 200). After cells were treated with different doses of ESB for 48 h, Hoechst 33258 staining was used to observe apoptotic cells as described in MATERIALS AND METHODS. The number of apoptotic cells gradually increased in a dose-dependent manner with marked morphological changes found in cell apoptosis including condensation of chromatin and nuclear fragmentation. A: Control group; B: Low dose treated group; C: Medium dose treated group; D: High dose treated group.

with chromatin margination in few tumor cells (Figure 2A). After treatment with a high ESB dose for 48 h, a part of nuclear membrane domed outward with a sharp angle. The typical morphologies of apoptotic H22 cells such as chromatic agglutination and fragmentation of nuclei, chondriosome swelling, formation of apoptotic body, could be observed in high ESB dose group (Figure 2B-D), while in 5-FU group, cellular swelling and necrosis could be observed in many fields of vision.

Detection of apoptosis of H22 cells by Hoechst 33258 staining

After treatment with different doses of ESB for 48 h, H22 cells were stained with Hoechst 33258 and observed under a fluorescence microscope. The condensely stained chromatin of apoptotic cells was more bright than that of normal cells. The characteristics of apoptosis, such as nuclear shrinkage, DNA condensation and fragmentation, were found in ESB treatment group (Figure 3B-D), while no apoptosis occurred in blank control group (Figure 3A). The percentage of apoptotic cells in control group and low and high ESB dose groups was 3.36% ± 2.14%, 14.57% ± 4.28%, 43.15% ± 5.33%, 72.65% ± 6.52%, respectively. Furthermore, the number of apoptotic cells gradually increased in a dose-dependent manner.

Effect of ESB on cell cycle distribution by flow cytometry

The effects of ESB on cell cycles were analyzed by flow cytometry. The percentage of cells was significantly decreased at S phase and increased at G_1 phase in high ESB dose group.

Table 1 Effect of ESB on cell cycle and apoptosis of H22 cells by flow cytometry (mean ± SD)

Groups	*n*	G_0/G_1	S	G_2/ M	Apoptosis rate
Control	5	37.63 ± 2.12	32.73 ± 2.24	21.75 ± 1.52	0.51 ± 0.12
ESB low dose	5	39.35 ± 2.25	33.56 ± 3.12	21.20 ± 1.27	1.07 ± 0.15
ESB medium dose	5	45.91 ± 2.56[a]	30.65 ± 2.64	17.15 ± 1.34[a]	3.15 ± 0.27[a]
ESB high dose	5	56.05 ± 2.37[b]	21.33 ± 3.42[b]	12.30 ± 1.25[b]	7.83 ± 0.43[b]

Cell cycle distributions in control and ESB-treated cells were determined by PI staining and flow cytometric analysis. Results presented were representative of three independent experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

The sub-G1 population indicated apoptotic-associated chromatin degradation. The ratio of cell apoptosis in blank control group, and low, medium, high ESB dose groups was 0.51%, 1.07%, 3.15%, 7.83%, respectively. There was significantly difference between the 4 groups ($P < 0.05$). These results suggest that high ESB dose can induce cell cycle arrest at G_0/G_1 phase and apoptosis in H22 cells (Table 1).

Effect of ESB on mitochondrial membrane potential

Mitochondria play an essential role in apoptosis. To assess whether ESB affects the function of mitochondria, mitochondrial membrane potential was detected under a laser scanning confocal microscope with Rh123 staining. The fluorescence intensity of Rhodamine123 in H22 cells of blank control group was the strongest (Figure 4).

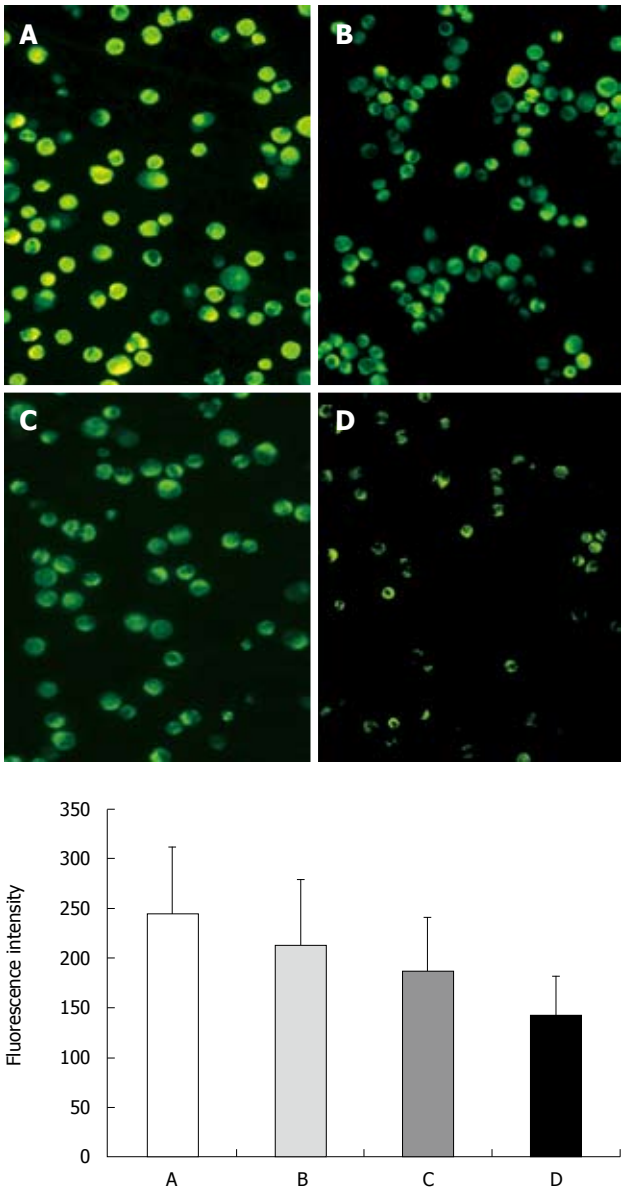

Figure 4 Effect of ESB on Δψm measured with laser scanning confocal microscope by staining with Rhodamine 123 (200 ×). A: Control group; B: Low ESB dose group; C: Medium ESB dose group; D: High ESB dose group. Fluorescence intensity (FI) indicates membrane potential of mitochondria in the cells. FI was decreased in a dose-dependent manner ($P < 0.05$, ANOVA analysis).

After treatment with different doses of ESB for 48 h, ANOVA analysis showed that the fluorescence intensity was decreased in a dose-dependent manner ($P < 0.05$).

Caspase-3 activity in ESB-induced apoptosis of H22 cells

Caspase-3, acting on downstream of the mitochondrial signaling pathway, is a major mediator of apoptosis. Dysfunction of mitochondria provoked us to detect the changes of caspase-3 activity in H22 cells following ESB treatment. The expression intensities of caspase-3 protein in the control and low-high ESB dose groups were 0.21 ± 0.02, 0.33 ± 0.04, 0.59 ± 0.03, and 0.85 ± 0.05, respectively (Figure 5). Western blot analysis revealed that there was a gradual increase in caspase-3 protein in low-high ESB dose groups ($P < 0.05$), indicating that

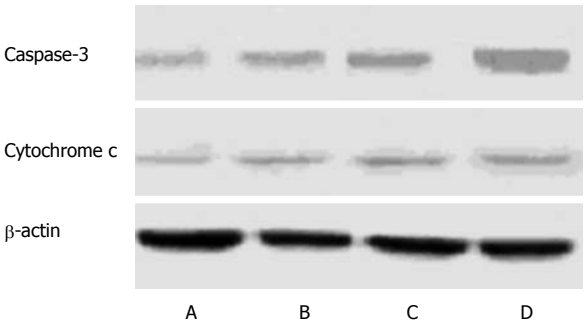

Figure 5 Protein level of caspase-3 and cytochrome C in H22 cells. A: Control group; B: Low ESB dose group; C: Medium ESB dose group; D: High ESB dose group. After treatment with different doses of ESB for 48 h, cellular proteins were detected by Western blot analysis.

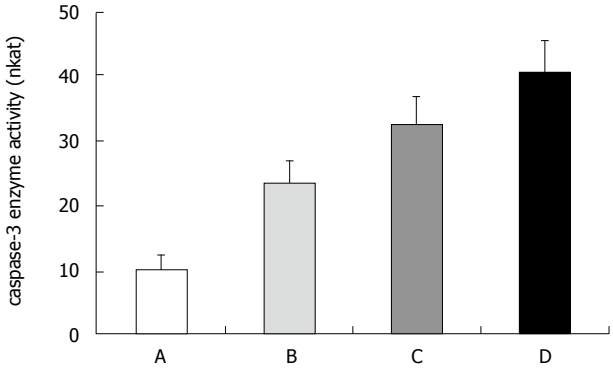

Figure 6 Effect of ESB on caspase-3 enzyme activity in H22 cells. A: Control group; B: Low ESB dose group; C: Medium ESB dose group; D: High ESB dose group. There was a dose dependent increase in activity of caspase-3 enzyme in ESB treated cells ($P < 0.05$). This assay was done triplicate, independently. Nkat was used to represent activity measured.

caspase-3 can be activated by ESB.

Caspase-3 activities were detected after treatment with different doses of ESB for 48 h, showing that caspase-3 activity was induced by ESB in a dose-dependent manner (Figure 6).

Release of cytochrome C from mitochondria in ESB-induced apoptosis

Cytochrome C release from mitochondria into cytosol is a critical step in the apoptotic cascade. The reduction of mitochondrial membrane potential may facilitate the release of cytochrome C, which will then activate the apoptotic pathway to trigger cell death. The protein level of cytochrome C in cytosol was measured in H22 cells treated with different doses of ESB by Western blot analysis with mouse monoclonal cytochrome C antibodies. As shown in Figure 5, the amount of cytosolic cytochrome C in the cytosolic fraction after ESB treatment was significantly increased in a dose-dependent manner ($P < 0.05$).

DISCUSSION

S. barbata, which has been traditionally used in treatment of inflammation, hepatitis, tumor and gynecological

diseases in China and Korea[1-9]. Studies have shown that *S. barbata* contains a large number of alkaloids and flavones, alkaloid, sterides, and polysaccharides[27,28]. However, the active site of chemical structure for antitumor activity has not been fully determined[29]. Recent studies indicate that *S. barbata* extract (ESB) is effective against hepatoma, lung and digestive system cancers, *et al*[4-10], and can be used in combination with other traditional Chinese medicines in treatment of other tumors.

In pharmacology study, crude Chinese drugs or their compounds are often added directly into the culture system of cells or organs *in vitro*[30]. However,, experimental results *in vitro* are often different from those *in vivo*. Serum pharmacology has been extensively used to study the effects and mechanisms of Chinese drugs *in vitro*[30]. It is believed that serum pharmacology is more scientific and beter for Chinese drugs than traditional pharmacology in which crude drugs are directly added into the culture system of cells or organs *in vitro*[19-20,30,31]. In this study, we investigated the effects of ESB on inducing apoptosis of H22 cells with serum pharmacology.

H22 cells were treated with different doses of ESB containing serum, and the growth rate of H22 cells was evaluated by MTT assay after 0, 24, 48, 72, and 96 h, respectively. High and medium ESB dose inhibited the proliferation of H22 cells, while low ESB dose could not obviously inhibit the proliferation of H22 cells. MTT assay showed high and medium ESB dose inhibited the proliferation of H22 cells *in vitro* in a time-dependent manner, which may provide useful information for development of anti-tumor drugs.

Morphological changes of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies[32]. These morphological changes were also observed in our study under transmission electron microscopy and fluorescence microscope after treatment with a high ESB dose for 48 h. Typical morphologies of apoptotic H22 cells, such as chromatic agglutination and fragmentation of nuclei, chondriosome swelling, formation of apoptotic body, were observed in ESB high ESB dose group (Figure 2B-D), but no apoptosis occurred in blank control group. Furthermore, fluorescence microscopy showed that the number of apoptotic cells gradually increased in a dose-dependent manner.

Blocking of cell cycle is one of the mechanisms of ESB by which the growth and proliferation of tumor cells are inhibited[33]. Flow cytometry showed that cell apoptosis was significantly decreased at S-phase, increased at G_1-phase, and reached its peak at subG_1-phase. The blocking of cell cycle may be one of the mechanisms of ESB by which the growth of H22 cells is inhibited and cell apoptosis is induced.

Mitochondria play a critical role in apoptosis induced by chemotherapeutic agents[12-14]. Many agents can induce, directly or indirectly, apoptosis by insult to the mitochondria[34,35]. Apoptosis could cause loss of $\Delta\psi m$ and release of cytochrome C into cytosol, and induce caspase-9-dependent activation of caspase-3[13]. In this study, the effect of ESB on $\Delta\psi m$ was examined using Rhodamine 123, a mitochondrial potential probe, showing that H22 cells lost $\Delta\psi m$ following ESB treatment. Forty-eight hours after ESB treatment, the cells exhibited significant alterations in $\Delta\psi m$, and the fluorescence intensity of disruption of $\Delta\psi m$ gradually decreased in a dose-dependent manner

One of the major apoptotic pathways is activated by the release of cytochrome C from mitochondria into cytosol[36], which is the hallmark of cells undergoing apoptosis. In this study, Western blotting analysis was performed to measure the protein level of cytochrome C in H22 cells after treatment with different doses of ESB. The amount of cytosolic cytochrome C in the cytosolic fraction after ESB treatment was increased in a dose-dependent manner (Figure 6).

Caspases are cystein proteases that play a key role in the execution phase of apoptosis[37]. Caspase-3, a member of the family of caspases, extensively studied as "the executor of apoptosis", plays a crucial role in cell death[38]. Apoptosis mediated by caspase-3 occurs in many cancer cells. In this study, Western blot analysis revealed that caspase-3 protein was gradually increased in the low-high ESB dose groups. At the same time, caspase-3 enzyme activity was increased in a dose-dependent manner. These results indicate that caspase-3 can be activated by ESB. ESB treatment resulted in loss of mitochondrial membrane potential, release of cytochrome C and caspase-3, demonstrating that ESB induces apoptosis and mitochondria are involved in apoptosis mediated by ESB.

In conclusion, ESB has antiproliferative activities against H22 cells by inducing apoptosis involving loss of $\Delta\psi m$, release of cytochrome C, and activation of caspase-3.

COMMENTS

Background

Medicinal plants have been used as traditional remedies for hundreds of years. Among them, *Scutellaria barbata D.Don* (*S. barbata*) has been traditionally used in treatment of hepatitis, inflammation, osteomyelitis and gynecological diseases in China. Studies indicate that extracts from *S. barbata* have growth inhibitory effects on a number of human cancers. Reports are available on the treatment of lung, breast and digestive system cancer, hepatoma, and chorioepithelioma with *S. barbata* extracts. However, the underlying mechanism of the antitumor activity of *S. barbata* extracts remains unclear.

Research frontiers

Studies have confirmed that many Chinese herbs have antitumor properties and induce apoptosis. In the process of signal transduction of cell apoptosis induced by drugs, mitochondria play a great role in promoting apoptosis signal and releasing caspase. Permeabilization of the outside mitochondrial membrane plays the most important role in mitochondrial apoptosis, during which loss of $\Delta\psi m$ and release of cytochrome C into cytosol, and caspase-9- dependent activation of caspase-3 occur sequentially.

Innovations and breakthroughs

There is no evidence that the mitochondrial pathway is involved in apoptosis induced by S. barbata. The present study was undertaken by culturing mouse liver cancer H22 cells treated with serum containing different concentrations of ESB. ESB containing serum induced apoptosis of H22 cells, and apoptosis was

involved in loss of mitochondrial transmembrane potential, release of cytochrome C, and activation of caspase-3.

Applications

This experimental study on the mechanism of the antitumor activity of S. barbata, may offer new evidence for S. barbata in the treatment of hepatoma in clinical practice.

Terminology

ESB is an extract from *Scutellaria barbata*; Δψm indicates mitochondrial transmembrane potential;1 nanokatal defined as the amount of enzyme required to increase the rate of reaction by 1 nmol/s under defined assay conditions.

Peer review

This study examined the anti-tumour effects of Scutellaria barbate. The authors used serum containing extract from *S. Barbata* (ESB) to determine its effect on proliferation of H22 hepatoma cells *in vitro*. ESB inhibited cell proliferation by inducing cell cycle arrest at Go/G1 phase and by increasing apoptosis with a reduction in mitochondrial membrane potential, release of cytochrome C and capase-3 activation. This work is novel and improves our understanding of the mechanisms of action of ESB.

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MLH1 promoter germline-methylation in selected probands of Chinese hereditary non-polyposis colorectal cancer families

Heng-Hua Zhou, Shi-Yan Yan, Xiao-Yan Zhou, Xiang Du, Tai-Ming Zhang, Xu Cai, Yong-Ming Lu, San-Jun Cai, Da-Ren Shi

Heng-Hua Zhou, Xiao-Yan Zhou, Xiang Du, Tai-Ming Zhang, Xu Cai, Yong-Ming Lu, Da-Ren Shi, Department of Pathology, Cancer Hospital/Institute, Fudan University; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China
Shi-Yan Yan, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China
San-Jun Cai, Department of Abdominal Surgery, Cancer Hospital/Institute, Fudan University, Shanghai 200032, China
Author contributions: Zhou HH and Yan SY participated in the treatment of patients, analyzed the data, performed the research and wrote the manuscript; Zhou XY, Du X and Shi DR participated in the treatment of patients, collected the data and designed the research; Cai SJ participated in the treatment of patients, collected the data; Other authors contributed to the new reagents/analytic tools.
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Correspondence to: Da-Ren Shi, Department of Pathology, Cancer Hospital, Fudan University, 270 Dong An Road, Shanghai 200032, China. shidaren2000@yahoo.com
Telephone: +86-21-64046008 Fax: +86-21-64046008
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Abstract

AIM: To detect the *MLH1* gene promoter germline-methylation in probands of Chinese hereditary non-polyposis colorectal cancer (HNPCC), and to evaluate the role of methylation in *MLH1* gene promoter and molecular genetics in screening for HNPCC.

METHODS: The promoter germline methylation of *MLH1* gene was detected by methylation-specific PCR (MSP) in 18 probands from unrelated HNPCC families with high microsatellite-instability (MSI-H) phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. At the same time, 6 kindreds were collected with microsatellite-stability (MSS) phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes as controls. The results of MSP were confirmed by clone sequencing. To ensure the reliability of the results, family H65 with nonsense germline mutation at c.2228C > A in *MSH2* gene was used as the negative control and the cell line sw48 was used as the known positive control along with water as the blank control. Immunochemical staining of *MLH1* protein was performed with Envision two-step method in those patients with aberrant methylation to judge whether the status of *MLH1* gene methylation affects the expression of MLH1 protein.

RESULTS: Five probands with *MLH1* gene promoter methylation were detected in 18 Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. Two of the five probands from families H10 and H29 displayed exhaustive-methylation, fulfilling the Japanese criteria (JC) and the Amsterdam criteria (AC), respectively. The other 3 probands presented part-methylation fulfilling the AC. Of the 13 probands with unmethylation phenotype, 8 fulfilled the JC and the Bethesda guidelines (BG), 5 fulfilled the AC. The rate of aberrant methylation in *MLH1* gene in the AC group (22.2%, 4/18) was higher than that in the JC/BG groups (5.6%, 1/18) in all HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. However, no proband with methylation in *MLH1* gene was found in the families with MSS phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. No expression of MLH1 protein was found in tumor tissues from two patients with exhaustive-methylation phenotype, whereas positive expression of MLH1 protein was observed in tumor tissues from patients with partial methylation phenotype (excluding family H42 without tumor tissue), indicating that exhaustive-methylation of *MLH1* gene can cause defective expression of MLH1 protein.

CONCLUSION: Methylation phenotype of *MLH1* gene is correlated with microsatellite phenotype of *MMR* genes, especially with MSI-H. Exhaustive-methylation of *MLH1* gene can silence the expression of MLH1 protein. *MLH1* promoter methylation analysis is a promising tool for molecular genetics screening for HNPCC.

Key words: Hereditary non-polyposis colorectal cancer; *MLH1*; Methylation; Germline; Methylation-specific PCR; Microsatellite phenotype

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INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is characterized by an autosomal dominant inheritance of early-onset microsatellite instability (MSI)-positive colorectal cancer and an increased risk of other cancers, including cancers of the endometrium, stomach, ovary, urinary tract, pancreas, and small bowel. HNPCC accounts for 5%-10% of all colorectal cancers and is caused by a mutation in one of the following DNA mismatch repair (MMR) genes: *MLH1, MSH2, MSH6, PMS1* and *PMS2*[1-3]. Germline mutations in *MLH1* and *MSH2* account for > 90% of all known MMR mutations in HNPCC[4], and germline mutations in *MSH6* account for 5%-10%, whereas mutations in other genes are rare[3,5]. MSI has been observed in approximately 13% of sporadic colorectal cancers (CRC) and in virtually all CRC arising in patients with HNPCC. Germline mutations in *MMR* genes, high-frequency microsatellite instability (MSI-H) and loss of MMR protein expression are the hallmarks of HNPCC. Epigenetic silencing is usually considered a kind of somatic phenomenon and somatic *MLH1* promoter hypermethylation is generally accepted in the tumorigenesis of sporadic tumours. However, little is known about the maintenance of epigenetic state in the germline[6] and abnormal *MLH1* gene promoter methylation in normal body cells is controversially discussed as a mechanism predisposing patients to HNPCC. Recently, aberrant methylation in MMR genes, *MLH1* or *MSH2*, has been supposed as a basic factor for cancer[7]. Promoter hypermethylation in *MLH1* gene of colorectal tumors correlates well with loss of MLH1 protein in sporadic MSI-positive cases[8,9]. This study was to investigate the *MLH1* gene germline epimutation by methylation-specific PCR (MSP) in 18 Chinese HNPCC kindreds with MSI-H but without germline mutations in *MSH2, MLH1,* or *MSH* gene, in order to identify HNPCC families and provide experimental information for HNPCC database.

MATERIALS AND METHODS

Materials

From January 1998 to October 2005, 24 Chinese HNPCC families fulfilling different clinical criteria were registered at the Department of Abdominal Surgery in Shanghai Cancer Hospital/Institution. Germline mutations in *MLH1*, *MSH2* and *MSH6* genes were excluded by DNA-PCR-based sequencing in the probands of all Chinese HNPCC families[10-12]. Of them, 18 unrelated HNPCC probands were selected for the study objects with the phenotype of MSI-H, and the remaining 6 were for the control group with the phenotype of microsatellite stability (MSS). Each participant was asked to give 10 microliters of peripheral blood and consented for access to archival tumor tissue. The characteristics of the selected cases are listed in Tables 1 and 2. To ensure the reliability of the results, family H65 with nonsense germline mutation at c.2228C > A in *MSH2* gene was used as the negative control and the cell line sw48 was used as the known positive control for the methylation in *MLH1* gene as well as water as the blank control. This study was proved by the Medical Ethical Committee of Cancer Hospital, Fudan University. The procedures of the study were in accordance with the international rules and regulations.

Table 1 Characteristics of 18 probands with MSI-H

Case	Gender	Age (yr)	Criteria	MSI	*MLH1/MSH2/MSH6* mutation study
H21	M	38	AC	MSI-H	NM
H22	M	46	AC	MSI-H	NM
H28	F	30	AC	MSI-H	NM
H29	F	37	AC	MSI-H	NM
H32	M	51	AC	MSI-H	NM
H42	M	65	AC	MSI-H	NM
H46	M	48	AC	MSI-H	NM
H57	F	47	AC Ⅱ	MSI-H	NM
H63	F	47	AC	MSI-H	NM
H10	M	41	JC	MSI-H	NM
H12	F	50	JC	MSI-H	NM
H41	M	46	JC	MSI-H	NM
H55	M	49	JC	MSI-H	NM
H7	M	38	BG	MSI-H	NM
H8	M	43	BG	MSI-H	NM
H30	M	48	BG	MSI-H	NM
H35	F	38	BG	MSI-H	NM
H51	F	27	BG	MSI-H	NM

AC: Amsterdam criteria; JC: Japanese criteria; BG: Bethesda guidelines; MSI-H: High microsatellite instability; NM: No mutation.

Table 2 Characteristis of 6 probands with MSS

Case	Gender	Age (yr)	Criteria	MSI	*MLH1/MSH2/MSH6* mutation study
H16	F	44	JC	MSS	NM
H20	F	54	BG	MSS	NM
H44	F	39	BG	MSS	NM
H48	M	28	BG	MSS	NM
H50	M	55	BG	MSS	NM
H54	M	43	BG	MSS	NM

JC: Japanese criteria; BG: Bethesda guidelines; MSS: Microsatellite stability; NM: No mutation.

DNA extraction

Genomic DNA from peripheral blood and the cell line sw48 was extracted with the QIAGEN (Hilden, Germany) DNA extraction kit following its manufacturer's introductions. Concentration of the genomic DNA was determined with an ultraviolet spectrophotometer (Beckman, DU640 type).

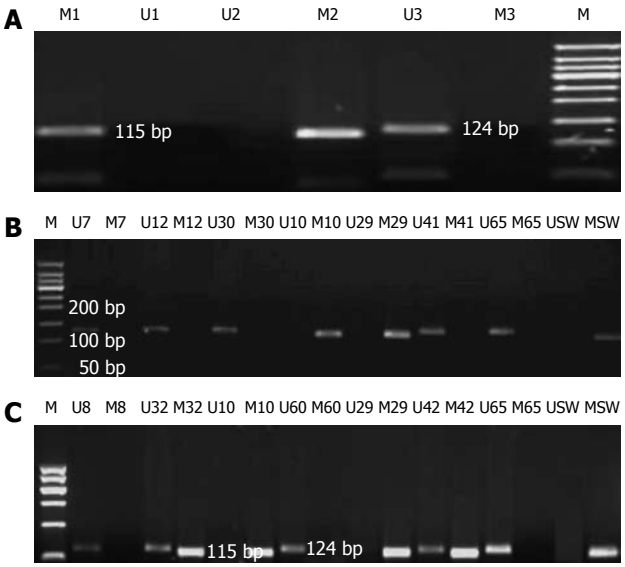

Figure 1 Results of *MLH1* MSP assay using primers that amplify methylated (M) or unmethylated (U) alleles (Lane M represents 100-bp DNA marker). A: MLH1 MSP assay in families H10, H65 and cell line SW48. M1, M2, U1and U2 indicate the methylated and unmethylated products of familiy H10, cell line SW48, and the methylaed band (115 bp); M3 and U3 indicate the methylated and unmethylated products in family H65; B: MLH1 MSP assay in families H7, H12, H30, H10, H29, H41, H65 and cell line SW48. U7, U12, U30, U41 and U65 indicate the unmethylated products of families H7, H12, H30, H41, and H65,respectively; M10, M29 and MSW indicate the methylated products of families H10, H29, and positive control SW48, respectively; C: MLH1 MSP assay in families H8, H32, H10, H60, H29, H42 H65 and cell line SW48. U8, U60 and U65 indicate the unmethylated products of families H8, H60 and H6, respectively; M10, M29 and MSW indicate the methylated products of families H10, H29, and positive control SW48, respectively; U32, M32 and U42, M42 are products of families H32 and H42, respectively.

PCR for methylation in MLH1

MSP exploits the effect of sodium bisulfite on DNA, which efficiently converts unmethylated cytosine to uracil with methylated cytosine unchanged. Consequently, after treatment, methylated and unmethylated alleles have different sequences that can be used to design allele-specific primers.

Genomic DNA was modified with sodium bisulfite as described previously[13,14]. The modified DNA was then subjected to MSP using primer pairs engineered to amplify either methylated or unmethylated DNA. Methylated and unmethylated primer pair sequences were also designed as previously described[15] and synthesized (Sangon, shanghai). PCR was carried out with HotstarTaq DNA polymerase (Cat. No. 203203): preheating at 94℃ for 10 min, followed by 40 cycles of denaturation at 94℃ for 45 s, annealing at 58℃ for 45 s and extension at 72℃ for 45 s, and a final elongation at 72℃ for 7 min. PCR products were subjected to 2% agarose gel electrophoresis. The products of exhaustive-methylation only indicated a methylated band of 124 bp and the unmethylated products only indicated an unmethylated band of 115 bp, while the partially methylated products indicated both of them. After observation of clear and expected bands, the products were purified using the QIAquick gel extraction kit (Qiagen) and sequenced on a 3700 DNA sequence system (ABI, USA) in order to check the correct bisulfite modification. Appropriate positive and negative reference samples were included. Each result of sequencing was analyzed by DNA Star 5.08 bioanalysis software.

Immunochemical staining for MLH1

A monoclonal antibody against MLH1 (Pharmingen, San Diego, CA, USA) was prepared at a 1:40 dilution and detected by the Envision two-step method to judge whether the status of methylation in *MLH1* gene would affect the expression of MLH1 protein. The expression of MLH1 was diminished in cancer tissues in the absence of detectable nuclear staining of neoplastic cells. Infiltrating lymphocytes and normal colonic crypt epithelium next to the tumor area served as internal positive controls.

RESULTS

Five probands with *MLH1* gene methylation were found in 18 unrelated Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. The rate of abnormal methylation in *MLH1* gene was approximately 27.8% (5/18). Among the 18 patients, 2 displayed exhaustively methylated phenotype and the other 3 presented partially-methylated phenotype. The exhaustive methylation accounted for 11.1% (2/18) in the HNPCC families with MSI-H but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. Perhaps, the changes might be much lower in all unselected HNPCC families. Among the 13 probands with unmethylation phenotype, 8 fulfilled the Japanese criteria (JC)/Bethesda guidelines (BG), 5 fulfilled the Amsterdam criteria (AC). All probands with partially-methylated phenotype fulfilled the AC, whereas probands of families H10 and H29 displaying exhaustively-methylated phenotype fulfilled the JC and AC, respectively. The rate of aberrant methylation in *MLH1* gene in the AC group (22.2%, 4/18) was higher than that in the JC/BG groups (5.6%, 1/18) in all HNPCC families with MSI-H phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. However, no proband with methylation in *MLH1* gene was found in HNPCC families with MSS phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. In our study, the expected size of bands was clear and specific. The study was repeated in triplicate to make sure all results credible (Figure 1A-C). Moreover, all exhaustively and partially methylated PCR products were purified and clone-sequenced in order to further substantiate the results of MSP (Figure 2). We believed that the methylation in *MLH1* gene might be related with microsatellite phenotype. No expression of MLH1 protein was observed in tumor tissues from two patients with exhaustively methylated phenotype, while positive expression of MLH1 protein was found in tumor tissues from patients with partially methylated phenotype (excluding family H42 without tumor tissue), suggesting that exhaustive-methylation in *MLH1* gene can cause defective expression of MLH1 protein and influence its function while the partial methylation in *MLH1* gene may have no impact on the expression of MLH1 protein.

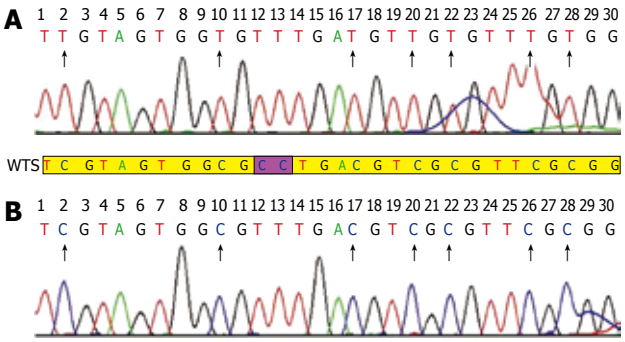

Figure 2 Methylation analysis of the promoter *MLH1* gene by clone sequencing. Arrow indicates CpG dinucleotide, WTS indicates the wild-type sequence of transcription start site. A: Unmethylated band presenting all unmethylated cytosines was converted to uracil after bisulfite modification; B: Methylated band presenting all unmethylated cytosines was unchanged after bisulfite modification.

DISCUSSION

HNPCC syndrome is the most common form of hereditary colorectal cancer. Predisposed individuals have a higher risk of developing cancer. The syndrome is primarily due to heterozygous germline mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2* genes. The resulting mismatch repair deficiency leads to MSI which is the hallmark of tumors arising within this syndrome, as well as a variable proportion of sporadic tumors. Diagnostic guidelines and criteria for molecular testing of suspected families have been proposed and continuously updated. However, not all families fulfilling these criteria show mutations in MMR genes and/or MSI implicating other unknown carcinogenic mechanisms and predisposition genes. This subset of tumors is the focus of current clinical and molecular research.

Germline mutations in the coding regions of *MSH2* and *MLH1* genes are known to be responsible for up to 45%-64% of all HNPCC families[16], and those of *MSH6* account for 10% of HNPCC kindreds[17]. We have previously detected germline mutations in the entire coding regions of *MSH2, MLH1* and *MSH6* genes in 24 probands meeting the AC, 15 probands fulfilling the JC and 19 probands meeting the BG by PCR-gene-sequencing with 20 germline mutations detected including two mutations occurring in a same patient and three novel mutations[10,11]. Subsequently, 3 new mutations are found by mRNA-based PCR sequencing[12]. It was speculated that the remaining probands without mutations in *MSH2*, *MLH1*, and *MSH6* genes might be associated with other aberrant types of genes. It was reported that DNA methylation associated with transcriptional silencing of *MLH1* is the underlying cause of MMR defects in most sporadic colorectal cancers with a MSI+ phenotype[9,18]. Moreover, reversal of methylation with 5-aza-deoxycytidine not only results in reexpression of MLH1 protein, but also restoration of the MMR capacity in MMR-deficient cell lines[9]. These findings further substantiate that the promoter methylation in *MLH1* gene is another deficient mechanism of *MLH1* gene.

Hypermethylation of CpG island in the promoter sequence has been proved to be an important mechanism of gene silencing and is particularly associated with transcriptional silencing of tumor suppressor genes in sporadic cancers[19,20]. Germline mutations might occur in individuals with a well-characterized genetic disease but lack an identifiable mutation in known disease genes[21]. It was recently reported that monoallelic promoter hypermethylation in *MLH1* gene is observed in peripheral blood from a number of patients with early-onset colorectal cancer[7,22-24]. The above results indicate that *MLH1* promoter-germline mutation might be related to HNPCC.

Our study demonstrated 5 probands with *MLH1* gene methylation (including 2 exhaustive-methylations which fulfill the JC and the AC, respectively, and 3 part-methylations fulfilling the AC) in 18 unrelated Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. The rate of aberrant methylation in *MLH1* gene (22.2%, 4/18) was higher in probands fulfilling the AC than that (5.6%, 1/18) in those meeting the JC and BG. Of the 13 probands with unmethylated phenotype, 8 fulfilled the JC and BG (61.5%, 8/13), 5 fulfilled the AC (38.5%, 5/13). However, no proband was detected with the aberrant methylation in *MLH1* gene in the 6 suspected HNPCC families with MSS phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. These findings illuminate that the promoter methylation in *MLH1* gene is likely another underlying cause of MMR defect in HNPCC fulfilling the AC. In order to ravel whether the aberrant methylation in *MLH1* gene influences the expression of MLH1 protein, immunostaining of MLH1 protein was carried out in 5 probands with *MLH1* aberrant methylation in our study. No expression of MLH1 protein was found in 2 probands with exhaustively methylated phenotype, whereas positive expression of MLH1 protein was observed in 2 probands with partially methylated phenotype (excluding family H42 without tumor tissue) suggesting that exhaustive methylation in *MLH1* gene can cause defective expression of MLH1 protein and influence its function while partial methylation of *MLH1* gene may have no impact on the expression of *MLH1* gene, revealing that methylation in *MLH1* gene may be related with the microsatellite phenotype and influence the expression of MLH1 protein and its function, which is consistent with the reported findings in other studies[8,9].

In neoplastic cells, stable allele-specific loss of transcription due to aberrant methylation in an unmutated promoter region can identify hypermethylation as a direct mechanism of tumor suppressor gene inactivation[25]. Moreover, the promoter methylation can be passed in somatic mitosis, which is reversible. Persons with hypermethylation in *MLH1* alleles of somatic cells can predispose to the development of cancer in patterns with hereditary nonpolyposis colorectal cancer. It was reported that epimutation can be transmitted from a mother to her son[26], which is consistent with transgenerational epigenetic inheritance.

In the present study, the rate of aberrant methyla-

tion in *MLH1* gene was only 27.8% (5/18) in selected HNPCC with MSI-H phenotype but without germline mutations in *MLH1*, *MSH2* and *MSH6* genes. Among the probands with aberrant methylation, the rate of methylation in those fulfilling the AC accounted for 80% (4/5), which was significantly higher than that [20% (1/5)] in those meeting the JC and BG. Methylation analysis of the *MLH1* promoter should be performed for all early-onset or multiple colorectal cancer patients with MSI-H tumors or loss of MLH1 protein expression due to unknown causes in HNPCC probands fulfilling the AC.

There is evidence that aberrant methylation in the promoter region of *MLH1* alleles is functionally equivalent to a pathogenic *MLH1* germline mutation and mimics the clinical phenotype of Lynch syndrome. 'Sporadic' HNPCC-patients need to be treated Lynch syndrome patients. Individuals carrying *MLH1* germline epimutations are at a high risk of developing colorectal and other tumors and should be considered carriers of germline mutations. Inheritance should be discarded in each case, until more conclusive data are obtained. *MLH1* promoter methylation analysis should be performed at least for the first degree relatives with positive methylation to exclude the inheritance of a familial epimutation[27]. Identification of hypermethylation as an epigenetic defect has important implications for surveillance recommendations, since these patients should be treated like Lynch syndrome patients.The heritability of methylation needs to be further investiged.

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COMMENTS

Background

Germline mutations in mismatched repair genes, such as MLH1, MSH2 and MSH6, lead to hereditary nonpolyposis colorectal cancer (HNPCC) and not all families fulfilling these criteria show mutations in mismatched repair genes. It is well known that MLH1 promoter methylation is related with sporadic colorectal cancer. However, MLH1 promoter germline-methylation in Chinese HNPCC patients has not yet been reported.

Research frontiers

Germline mutations in MMR genes, such as MSH2, MLH1 and MSH6 contribute to the early diagnosis of HNPCC and screening of HNPCC families. Few studies on MLH1 promoter germline-methylation are available.

Innovation and breakthroughs

Five patients with *MLH1* gene methylation were found in this study by methylation-specific PCR in 18 unrelated Chinese HNPCC probands with high microsatellite-instability phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* gene. The rate of abnormal methylation in *MLH1* gene was approximately 27.8% (5/18) and the rate (22.2%, 4/18) in probrands fulfilling the Amsterdam criteria, which was higher than that (5.6%, 1/18) in those meeting the Japanese criteria/Bethesda guidelines.

Applications

MLH1 promoter methylation analysis can be used for the microsatellite phenotype of mismatched repair genes and is a promising tool for molecular genetics screening of HNPCC.

Terminology

HNPCC is an abbreviation of hereditary nonpolyposis colorectal cancer; MSP is an abbreviation of methylation-specific PCR.

Peer review

In this study, MLH1 promoter germline-methylation was detected in 18 unrelated Chinese HNPCC probands with high microsatellite-instability phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* gene. The rate of aberrant methylation in probands meeting theAmsterdam criteria was higher than that in those fulfilling the Japanese criteria/Bethesda guidelines. However, the function of MLH1 promoter germline-methylation should be further studied with a large of samples.

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Metrically measuring liver biopsy: A chronic hepatitis B and C computer-aided morphologic description

Nicola Dioguardi, Fabio Grizzi, Barbara Fiamengo, Carlo Russo

Nicola Dioguardi, Fabio Grizzi, Carlo Russo, Laboratories of Quantitative Medicine, Istituto Clinico Humanitas IRCCS, Rozzano 20089, Milan, Italy
Barbara Fiamengo, Department of Pathology, Istituto Clinico Humanitas IRCCS, Rozzano 20089, Milan, Italy
Author contributions: Dioguardi N designed and conducted the study, analyzed the data and prepared the manuscript; Grizzi F was responsible for the implementation of the fractal dimension software and for the metric and the immunology of inflammation; Fiamengo B did the anatomical interpretation of the liver biopsy sections; Russo C developed and implemented the computer metrical method.
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Correspondence to: Nicola Dioguardi, MD, Scientific Superintendence, Istituto Clinico Humanitas IRCCS, Via Manzoni 56, Rozzano 20089, Milan, Italy. nicola.dioguardi@humanitas.it
Telephone: +39-2-82244501 Fax: +39-2-82244590
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Abstract

AIM: To describe a quantitative analysis method for liver biopsy sections with a machine that we have named "Dioguardi Histological Metriser" which automatically measures the residual hepatocyte mass (including hepatocytes vacuolization), inflammation, fibrosis and the loss of liver tissue tectonics.

METHODS: We analysed digitised images of liver biopsy sections taken from 398 patients. The analysis with Dioguardi Histological Metriser was validated by comparison with semi-quantitative scoring system.

RESULTS: The method provides: (1) the metrical extension in two-dimensions (the plane) of the residual hepatocellular set, including the area of vacuoles pertinent to abnormal lipid accumulation; (2) the geometric measure of the inflammation basin, which distinguishes intra-basin space and extra-basin dispersed parenchymal leukocytes; (3) the magnitude of collagen islets, (which were considered truncated fractals and classified into three degrees of magnitude); and (4) the tectonic index that quantifies alterations (disorders) in the organization of liver tissue. Dioguardi Histological Metriser machine allows to work at a speed of 0.1 mm^2/s, scanning a whole section in 6-8 min.

CONCLUSION: The results are the first standardized metrical evaluation of the geometric properties of the parenchyma, inflammation, fibrosis, and alterations in liver tissue tectonics of the biopsy sections. The present study confirms that biopsies are still valuable, not only for diagnosing chronic hepatitis, but also for quantifying changes in the organization and order of liver tissue structure.

Key words: Liver measurement; Image analysis; Liver lesion; Liver tectonics

Peer reviewer: Mark D Gorrell, PhD, Professor, Centenary Institute of Cancer Medicine and Cell Biology, Locked bag No. 6, Newtown, NSW 2042, Australia

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INTRODUCTION

The main purpose of this paper is to describe a rigorous method based on the fundamentals of measurement theory[1], which metrically defines the changes in magnitude of liver tissue prime basic structural elements that occurring during the course of chronic hepatitis B and C.

Each available score to evaluate hepatic lesions is characterized by some methodological inaccuracy[2-4]. In fact, transient elastography (Fibro-Scan)[5,6] is limited by the skill of the operator and because liver stiffness is not only dependent from fibrosis, and serological assays not directly involved in tissue evolution, but in patient diagnosis[7-11]. In addition to the inherent risks of excising a liver specimen[12], current morphometric analyses[13-17] are time-consuming, depend on subjective choices of the regions of interest, involve the interactive elimination of Glisson's capsule and staining artefacts, and use the International System (IS), which is unsuitable for measuring the irregular shapes found in histology[18-21].

The study concerning the status of the liver tissue affected by chronic viral hepatitis was suggested by three

main needs. The first was ethical because methodological accuracy and repeatability are essential. The second was clinical because many problems remain unsolved in hepatology, such as non-responders to therapy[3], and regression of cirrhosis[2]. The third need was economic, as the price of the metrical data supplied by the "Dioguardi Histological Metriser" analysis is relatively low, and the repeatable biopsy interpretation is obtained with a few minutes.

The lack of an appropriate geometry had prevented the real measurement of irregular liver structures, until Mandelbrot's fractal geometry[22] (also called the geometry of irregularity) offered a correct approach for obtaining reproducible and closer to reality metrical measurements of hepatocellular mass, inflammation, and fibrosis, and also provided a quantitative index for evaluating the organization of liver tissue tectonics. In order to apply these new measurements, we constructed a practical and fully-automated machine that we called the "Dioguardi Histological Metriser", which is capable of measuring 10 parameters to describe the status of the residual hepatocyte mass (including hepatocyte vacuolization), inflammation, fibrosis, and the loss of liver tissue tectonics in liver biopsy sections, at a speed of 0.1 mm^2/s. Hepatitis B and C virus infections do not usually affect the biliary system.

The study concerning the status of the liver tissue affected by chronic viral hepatitis B and C, was suggested by three main needs: (1) Ethical: methodological accuracy and reproducibility are essential; (2) Clinical: because many questions remain unsolved in hepatology, such as non-responders patients to therapy[3], and regression of cirrhosis[2]; (3) Economical: metrical data analysis supplied by the "Dioguardi Histological Metriser" is not expensive reproducible and is obtained within a few minutes.

MATERIALS AND METHODS

Case list

We studied 398 patients randomly collected (250 male) aged 52 ± 12 years with chronic hepatitis B or C, who were admitted to the hepatology departments of the Istituto Clinico Humanitas (ICH) IRCCS, Rozzano, and the University of Milan Department of Gastroenterology, Ospedale Maggiore IRCCS, Milan, Italy. The biopsies were performed in accordance with the guidelines of the Ethics Committees of ICH and Ospedale Maggiore IRCCS. All of the liver specimens were approximately 17 ± 12 mm^2.

The logarithmic curve of the ordered set according to the fibrosis data magnitude obtained from the 398 patients, can be interpreted as the trajectory (from α to ω)of the ideal dynamics of collagen deposition during the course of chronic hepatitis (Figure 1).

Histological methods

Three consecutive 2 μm thick sections were cut from formalin-fixed, paraffin-embedded biopsy specimens: the first was stained with hematoxylin and eosin for diagnostic purposes; the second was treated to identify inflammatory cells by using monoclonal antibodies raised against leukocyte common antigen (LCA: Dako, Milan, Italy) and a standardized immunoperoxidase method[23], and hepatocellular lipids vacuoles; and the third was stained with Sirius red to visualize fibrosis.

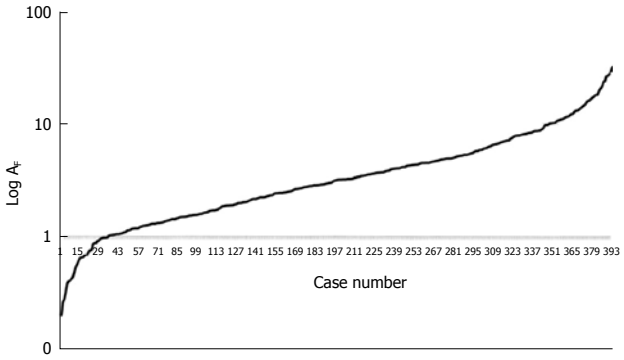

Figure 1 Markovian logarithmic curve obtained with the fibrosis content in each single biopsy section from 398 patients, ordered by increasing severity. A_F = area of fibrosis.

Semi-quantitative analyses

Expert hepatopathologists graded and staged the biopsy sections using the Knodell[24], Sheuer[25], Ishak[26], and METAVIR[27] semi-quantitative scoring systems.

Example of liver tissue geometric analysis

A specific example of the set of metrical parameters obtained by quantitatively evaluating liver residual parenchyma, inflammation, fibrosis, and disordered liver tissue tectonics is shown in Figure 2.

Methodology validations

Variations in the water bath temperatures used to distend the histological sections were tested at 41, 43, 45 and 47℃, (which accounted for 12% of the variations in fibrosis). Variations in paraffin section thickness were tested using five sequential thicknesses from 2-6 μm, (which accounted for 20% of the variations in fibrosis). Variations in staining times (tested using nine sequential sections stained with a freshly-made Sirius red solution for 15-135 min), (which accounted for 13% of the variations in fibrosis). Intra-sample variability in the tissue area covered with Sirius-red-stained collagen was assessed using three series of thirty 2 μm-thick sections obtained from three biopsies, two series of fifteen 4 μm-thick sections obtained from two further biopsies, and one series of ten 6 μm-thick sections obtained from a sixth biopsy. The results showed wide intra-sample variability, because of the highly irregular distribution of the collagen matrix. Also the loss of the thinner matrix components because of histological section processing might have play played a role in this result.

Statistical analysis

The results were analyzed using Statistica software (StatSoft Inc., Tulsa, OK, USA). Variability was evaluated using the coefficient of variation (CV) given by the formula CV = (SD/mean) × 100%. *P* values of less than 0.05 were considered statistically significant.

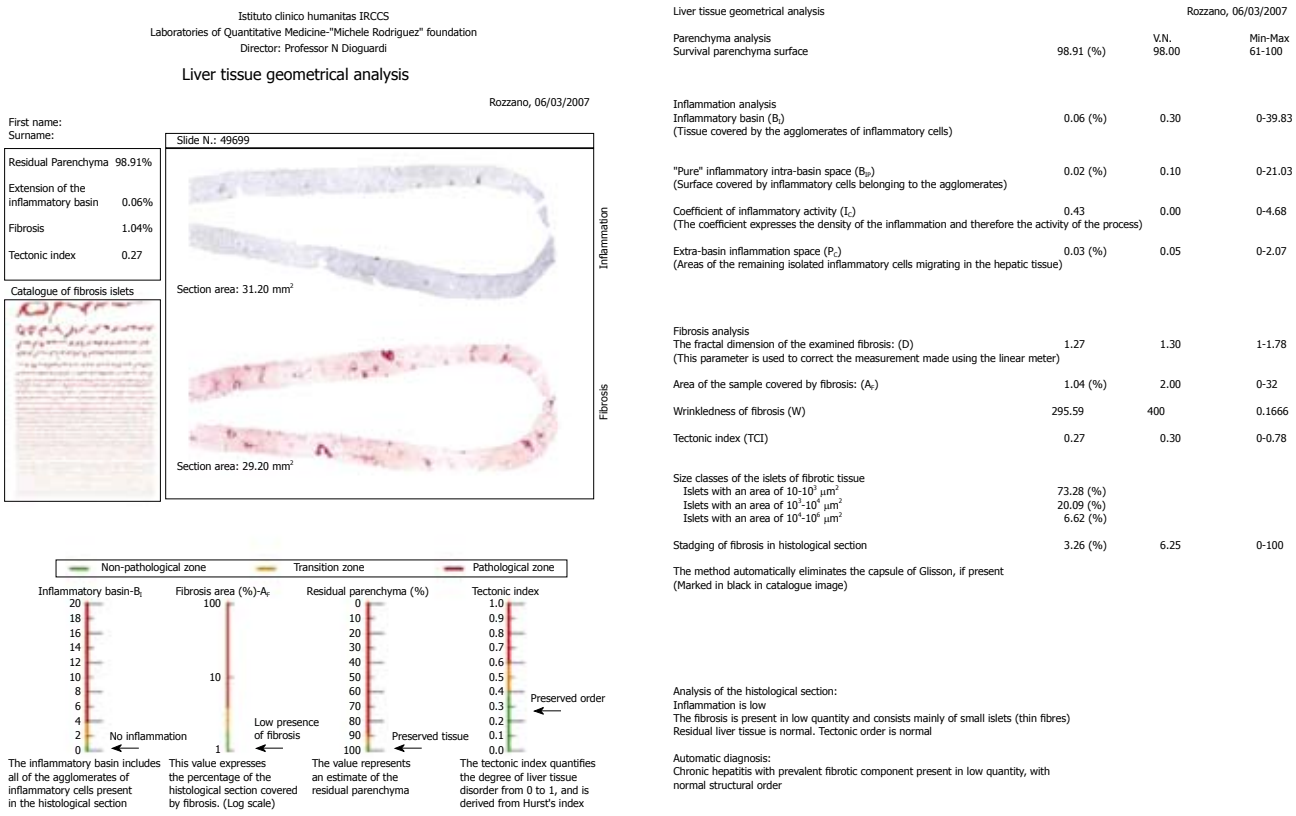

Figure 2 An example of LTGA. The machine completes the analysis by providing a common language description of the histological pattern and diagnosis.

The model and its pathological transformation

Our model for measuring the state of liver tissue is based on two canonical points: the choice of the prime structural elements of the tissue, and the most general kind of tissue organization[1].

Prime structural elements (determine property of the system) and are the most representative structural elements of an organ tissue insofar as they have the property of changing their shape and size over time, without losing their individuality.

Prime liver structural elements were considered: (1) the parenchyma (i.e. the *substantia jecuris*), which consists of the hepatocytic mass that, in this phase of the research, includes regenerative nodular hepatocytes; (2) the dispersed set of topical immunological cells; (3) the collagen scaffold that consists collagen fibers and included the portal spaces; and (4) the tectonic, defined by the Malpighi-Kiernan lobular organization of the liver. All of these elements were taken in their strictly structural form.

The most general kind of organization[1] of an organ tissue is the state of its prime structural elements defined by quantitative relationships. It determines tissue tectonics and the reference for every structural change in organ architecture which, in the case of the liver, is the lobular structure.

Pathological events occurring during the course of chronic viral hepatitis transform the shape and size of these prime physical structures, and consequently alter the most general organization of the liver system.

The main events altering the natural physical state of the prime structural elements and tectonics of liver tissue, are enlargement, reduction and "vacuolization". The pathological transformations determined by these events during the course of chronic viral hepatitis, can be interpreted as follows: (1) necrosis reduces lipid hepatocyte determines vacuolization increases the size of the parenchyma; (2) Increase in the number of dispersed cells generated by the topical general immune system determine inflammatory cell clusters; (3) Growth of septa that evolve into porto-portal or porto-central fibrotic bridges results in expansion of the collagen support network (which appears as Sirius-red-stained islets in a histological section). Taken together these individual trasnformations (in shape and size) generate a loss of the natural harmonu in the inter-relationship of the prime elements. This loss of order is also measurable.

The Dioguardi Histological Metriser

We designed and built our own user-friendly Liver Tissue Geometric Analyser (LTGA or Dioguardi Histological Metriser; patent pending), which automatically ensures correct microscope focusing, metrically evaluates the image of an entire digitalized histological slide, and defines the areas covered by the residual parenchymal mass (including lipid vacuolization), inflammation and fibrosis; it also disregards any unfilled spaces (vascular and biliary cavities or sinusoidal spaces) and artefactual tissue-free spaces.

The Dioguardi Histological Metriser consists of two parts: a "client" (or dedicated microscope system) that captures and digitizes the images of the specifically stained histological section, and a central "server" that receives the images, automatically measures the parameters

Figure 3 Liver fibrosis. A: Prototypical examples of multifarious Sirius-red-stained collagen islets making-up the liver collagen network; B: the Metriser automatically selected and excluded Glisson's capsule (black islets) from the computation of fibrosis by means of an appropriate algorithm.

listed in Table 1, and sends the results back to the client.

In this study, the microscope system consisted of a Leica DMLA microscope (Leica, Milan, Italy) equipped with an X-Y translator table, a digital camera (QICam, QImaging, Surrey, Canada), and an Intel Pentium 4, 2.60 GHz computer. We used *ad hoc* built-in image analysis software that automatically filtered, selected, and marked the outlines of the images of interest using color thresholds based on the levels of red, green and blue. All of the measurements were made at 10 × objective magnification. The Dioguardi Histological Metriser automatically selected and excluded Glisson's capsule from the computation of fibrosis by means of an appropriate algorithm (Figure 3).

The minimum and maximum scalars obtained empirically on the basis of the Dioguardi Histological Metriser measurements of 398 biopsies are shown in Table 1.

Table 1 Quantitative parameters automatically obtained with the Metriser

Parameter	min	max
Residual hepatocellular set		
Residual hepatocellular set (%)	67.97	99.59
Inflammation		
Inflammatory cell cluster space (%)	0	8.71
Pure inflammatory cell cluster space (%)	0	3.67
Extra-basin inflammatory space (%)	0	1.14
Fibrosis		
Area of Sample covered by fibrosis (%)	0	32
Islets magnitude		
$10\text{-}10^3$ (%)	8.9	100
$10^3\text{-}10^4$ (%)	0	58.15
$> 10^4$ (%)	0	87.4
Wrinkledness	0	1666
Tectonic Index		
Liver tissue	0	0.78
Low or no disorder	0	0.4
Middle disorder	0.4	0.6
High disorder	0.6	1

Data are expressed as percentage of true liver surface. The value 0 is obtained when no cell clusters were recognized. Quantitative evaluation of steatosis. At this phase of the research this quantitative parameter is still obtained with an ad hoc software.

Measuring the pathological structures

We took as a reference for the following measurements the physical transformation average of the areas of the studied histological section.

Residual parenchymal mass: We consider the surviving hepatocellular set that remains after necrotic viral destruction, together with the nodular regenerated hepatocytes.

This surviving part of the hepatocellular set (residual mass) is expressed as a percentage of the reference area using the formula:

$H_S = 100\% - A_I - A_F$

Where H_S is the area of the residual hepatocellular set, A_I the sum of the area of the inflammation basin, and A_F the area covered by fibrosis. Vacuolization is due to the accumulation of lipids within hepatocytes (Figure 4). In this phase of the research we included lipid vacuoles in the residual parenchyma. The extension of these cytoplasmic enclaves was measured separately, to define the steatosis grade.

Inflammation basin: We called the inflammation basin (Figure 5A) the classic liver tissue pattern characterized by various sets of spatial immune-cell aggregates[23]. We consider three components. (1) Inflammatory cell clusters

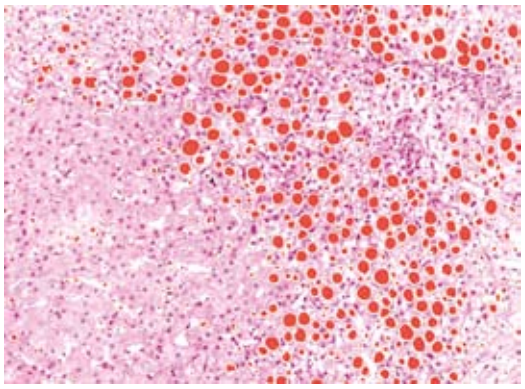

Figure 4 Computer-aided recognition of vacuolization, due to the accumulation of lipid vacuoles within hepatocytes. The image represents only a limited exemplificative area taken from the whole histological section (× 10).

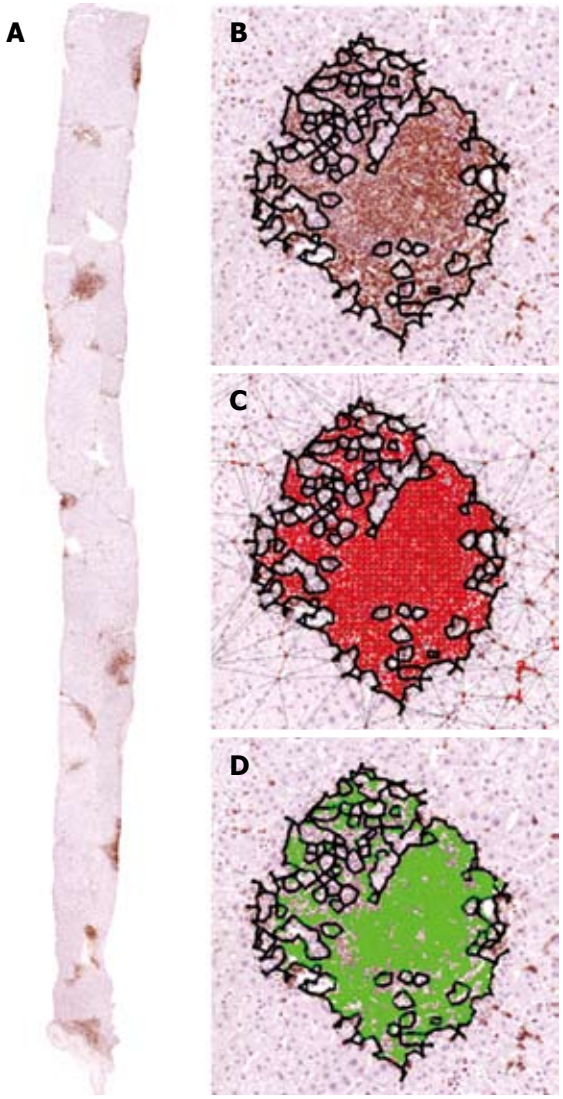

Figure 5 Liver inflammation (× 10). A: Morphological picture showing the inflammatory cell' clusters forming the inflammation basin; B: inflammatory cell cluster; C: discrimination of the cluster outline (black line) using the Delaunay' triangulation; D: pure intra-cluster inflammatory space covered by the inflammation cell bodies (green surface). The immunological staining was performed by treating the sections with monoclonal antibodies raised against LCA.

(Figure 5B). The boundaries of this dot-like pathological

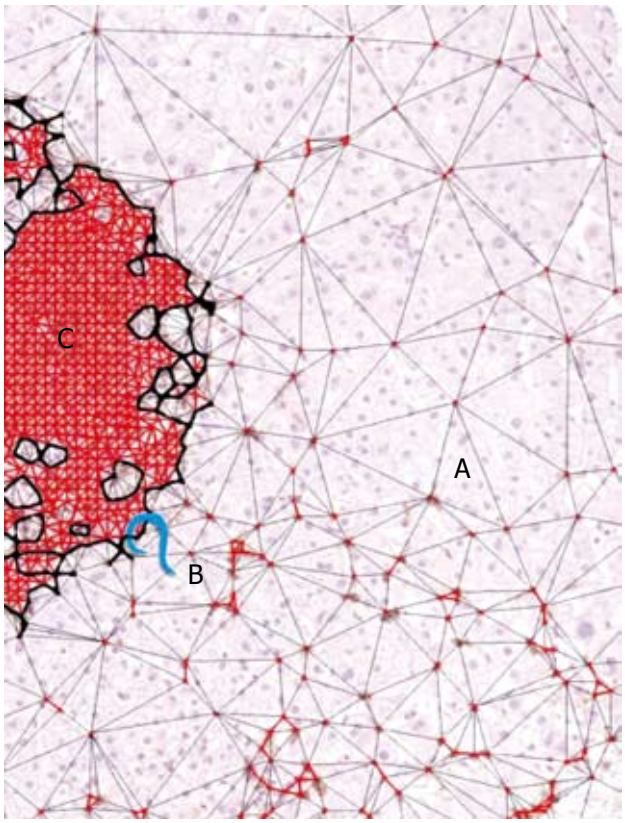

Figure 6 Liver inflammation (× 10). A: Extra-cluster inflammatory space, which is the sum of micro-areas covered by individual inflammatory cells represented by the nodes of the Delaunay' triangulation network; B: irregular outline (black line) in which lies the outermost cells distant each other no more than 20-μm; C: the irregular outline divided outermost cells from the inside resident cluster cells. The immunological staining was performed treating the sections with monoclonal antibodies raised against LCA.

structure are arbitrarily fixed using Delaunay's triangulation (Figure 5C), which defines their edges, as a continuous line, connecting the centers of the outermost cells (with a maximum distance of 20 μm)[23]. This line separates the intra-cluster inflammatory cells, from the immunologically evidenced parenchymal leukocytes throughout the tissue[23]. (2) Intra-cluster inflammatory space (Figure 5D), which is intra-cluster area covered by resident inflammatory cell bodies micro-areas[23]. (3) Extra-cluster inflammatory space, which is the sum of micro-areas covered by individual inflammatory cells that remain outside the clusters, within the liver tissue interstitium (Figure 6)[23].

Fibrosis: The fibrotic framework appears as a multifarious set of collagen islets (Figure 3). Three classes of collagen islets were arbitrarily identified on the basis of their area the first one included islets with an area of between 10 and 10^3 μm^2, the second are those with an area of between 10^3 and 10^4 μm^2, and the third are those with an area of > 10^4 μm^2[21] (Figure 7).

The wrinkledness of collagen islets is calculated using the formula:

$$W = \frac{P}{2\sqrt{\pi A}} - R$$

Where wrinkledness (W) is expressed as the ratio between the perimeter and area of an object[21], P is the frac-

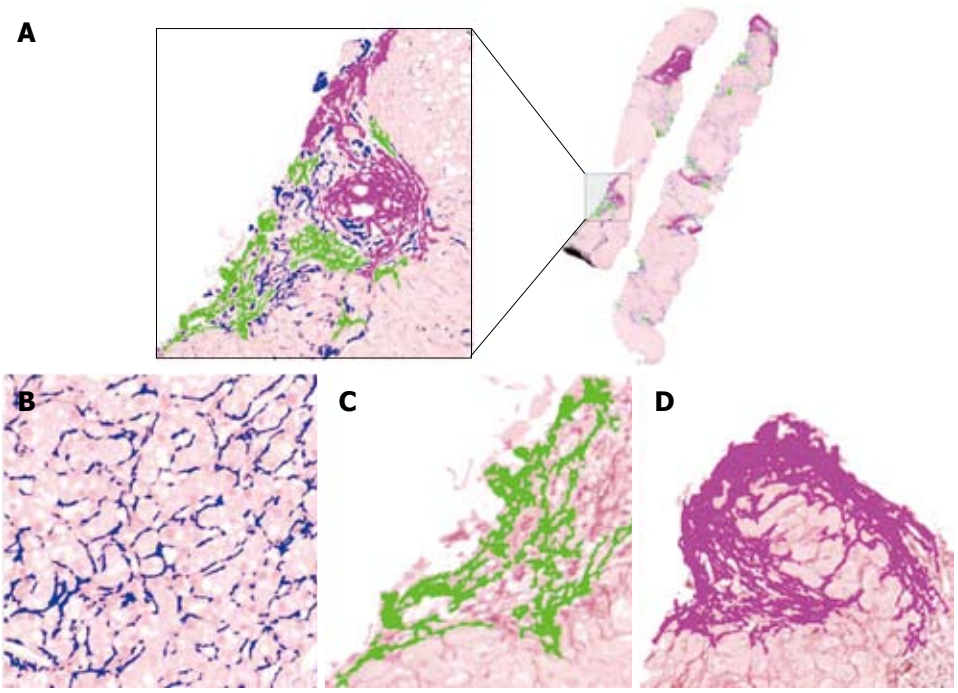

Figure 7 Liver collagen islets magnitude (× 10). A: The Histological Metriser Dioguardi distinguish and highlight with different colours three classes of Sirius-red stained collagen islets; B: islets with magnitudes arbitrarily fixed at 10-10^3 μm^2 (colored in blue); C: islets with magnitudes fixed at 10^3-10^4 μm^2 (colored in green); D: islets with magnitudes fixed at and > 10^4 μm^2 (coloured in pink).

tal-corrected perimeter of the collagen area, *A* the fractal-corrected collagen area, and *R* the roundness coefficient of the collagen islets[21].

Liver tectonics: Natural liver tectonics defines the organization of the intersecting elements of liver tissue. The tectonic order was quantitatively described by the Tectonic Index (*TCI*, which ranges from 0 to 1) obtained using the Euclidean and fractal dimensions and *TCI* was obtained from *H* using the conversion formula:

$TCI = 1 - H$

Where $H = D\gamma + 1 - D$, in which *D* is the fractal dimension and $D\gamma$ the Euclidean dimension.

TCI describes the loss of tissue organization or any deviation from natural order: a high *TCI* indicates a high degree of tissue disorder, and a low *TCI* indicates a low degree of tissue disorder. It can therefore be written:

$TCI = 1 - H = D - D\gamma$

Fractal dimension correction of the IS meter

The irregularity of collagen islets makes it impossible to measure them using IS linear units unless these units are corrected by means of fractal dimension[20,21]. This correction makes it possible to include details of shape that escape (or do not interact with) linear unit measurements at any given scale[20,21]. We derived the fractal dimension using the box-counting method[20,21,28,29]. Since the biological objects has been classified as "truncated fractals"[20,21,30] we used the fractal dimension to correct the reference units as a dilation factor[20,21].

Table 2 shows the differences between the uncorrected and fractal dimension-corrected IS measurements.

Table 2 Differences between the uncorrected and fractal-corrected IS measurements of fibrosis surface extension

n	Uncorrected fibrosis extension range (%)	Mean increase of fibrosis extension after IS meter correction (%)	min (%)	max (%)
193	0-3	25	0	65
156	3-10	10	5	18
49	10-40	4	2	6

n: Number of liver biopsy.

RESULTS

Dioguardi Histological Metriser resolution power

The Dioguardi Histological Metriser resolution was assessed by computing the surface area of liver fibrosis in 13 tissue sections, and repeating the measurements 10 times in order to define the instrument error. Two objects were considered distinct if, and only if, their values and 95% confidence intervals (twice the standard deviation of the experimental values) did not overlap. The Dioguardi Histological Metriser distinguished 68 different categories, as against the six of Ishak, the five of the METAVIR scoring system, and the four of Knodell's or Sheuer's methods. The mean distance between the data with no overlap was 0.786% (range: 0.056%-2.216%).

The selective power of the metrical quantifications defines the capacity of the method to distinguish small differences in magnitude.

Comparison of metrical and semi-quantitative data

A study a part, was performed to define differences of the metrical date concerning residual hepatocytic mass inflammation, fibrosis and liver tissue tectonics on the same patient. The digital images of 61 pairs of histological biopsies from patients with hepatitis C virus-dependent disease. The first measurement was made 4-15 years after of the interval (after the antiviral treatment). The aim was to study the date differences after a long and irregular time.

For each pair of biopsies, we studied: (1) the difference

Table 3 Residual parenchyma metrical and semi-quantitative case numbers

Δ metrical measure		Semi-quantitative evaluations				
		HAI	Scheuer	Ishak	METAVIR	Average
Increase	24	19	26	26	23	23.5
Stationarity	0	30	23	18	25	24.0
Decrease	37	12	12	17	13	13.5

Mean value of the four semi-quantitative scoring systems; Data refer to the difference between two biopsies taken at different times.

Table 4 Inflammatory basin metrical and semi-quantitative case numbers

Δ metrical measure		Semi-quantitative evaluations				
		HAI	Scheuer	Ishak	METAVIR	Average
Increase	35	16	16	23	7	15.5
Stationarity	1	25	22	19	36	25.5
Decrease	25	20	23	19	18	20.0

Mean value of the four semi-quantitative scoring systems; Data refer to the difference between two biopsies taken at different times.

Table 5 Fibrosis metrical and semi-quantitative case numbers

Δ metrical measure		Semi-quantitative evaluations				
		HAI	Scheuer	Ishak	METAVIR	Average
Increase	42	19	26	26	23	23.5
Stationarity	0	30	23	18	25	24.0
Decrease	19	12	12	17	13	13.5

Mean value of the four semi-quantitative scoring systems; Data refer to the difference between two biopsies taken at different times.

Table 6 Tectonic Index metrical and semi-quantitative case numbers

Δ of loss of the natural liver tissue order		Semi-quantitative evaluations				
		HAI	Scheuer	Ishak	METAVIR	Average
Increase	46	19	26	26	23	23.5
Stationarity	3	30	23	18	25	24.0
Decrease	12	12	12	17	13	13.5

Mean value of the four semi-quantitative scoring systems; Data refer to the difference between two biopsies taken at different times.

(Δ) between the scalar measurements performed with Dioguardi Histological Metriser; and (2) the changes of the semiquantitative evaluation with the four most widely used semi-quantitative methods (Knodell, Sheuer, Ishak, METAVIR).

Juxtaposing the quantitative variations in the metrical measurements with the time to the same parameters studied by means of the semi-quantitative methods in order to collate the results of the two scores, we found the following. (1) The metrical measurements of residual parenchyma gave, in comparison to semi-quantitative results, fewer indications of no change and more indications of decreases (Table 3). (2) The metrical measurements of inflammation gave more indications of increases, and fewer indications of no change than the semi-quantitative results (Table 4). (3) The metrical measurements of fibrosis gave more indications of increases and fewer indications of no change than the semi-quantitative results (Table 5). (4) The quantitative evaluations of the tectonic index of liver architecture gave more indications of increased disorder, and fewer indications of no change than the semiquantitative results (Table 6).

To compare the difference of the metrical scalars, with the ordinally numbered categories is not possible, because metrical measurements are *in continuum* (and thus additive with successive points separated by $\Delta = 0$), whereas semi-quantitative evaluations are discrete and not additive with intervals of $\Delta \neq 0$.

Differences between the metrical and semi-quantitative results

On the straight line of real numbers, we reported the value of the cases grouped by each category recognized with the current semi quantitative scoring systems.

The collate of the distribution of the data reported on the state shows the overlapping of the metrical measurements that correspond to patients classified in different semi quantitative categories (Knodell, Ishak, Sheuer, and METAVIR). This highlights the inadequacy of all four semi-quantitative methods in discriminating different states of liver fibrosis (Figure 8).

Classes of magnitude of collagen islets

Our method distinguished three classes of collagen islets with magnitudes arbitrarily fixed at 10-10^3 μm^2, 10^3-10^4 μm^2, and > 10^4 μm^2 (Figure 7). As the process of fibrosis is a progressive deposition of extracellular matrix that coalesces into islets that are subsequently thickened by matricial deposits, it can be speculated that thin islets indicate the initiation and persistence of inflammation.

Stad-ging and grading

Stad-ging indicates the part of the disease course already covered and the part that remains to be covered, before it reaches its end. It is established by placing the value of fibrosis (expressed as a scalar) on the ideal trajectory that indicates the phase of fibrosis, at the time of measurement (Figure 9)[21]. In brief, stad-ging indicates the tendency of the process to evolve in both senses from one state to another.

The staging and stad-ging of fibrosis are different insofar as the former indicates the current fibrotic state, and the latter indicates the phase of the process: i.e. the percentage of the course before collagen deposition reaches its maximum level of tolerance, which in our case, was empirically found to be 32% of fibrosis. The magnitude of inflammation defines the current status (grading) of the disease process.

DISCUSSION

The aim of this study was to test the means of rapidly

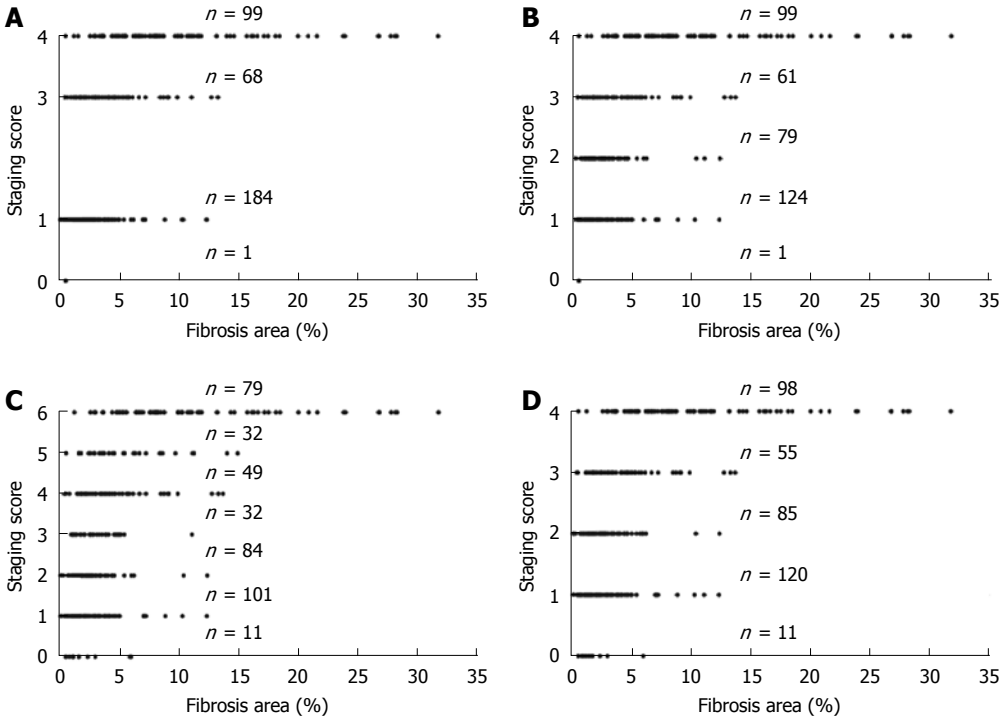

Figure 8 Comparison of the phase portraits obtained using the scalar values of fibrosis (%) calculated from each biopsy section, projected onto the state spaces. A: Knodell HAI; B: Sheuer; C: Ishak; and D: METAVIR categories (staging). All graphs highlight a considerable overlap of scalar data that corresponds to different categories. Forty-six cases for HAI, 34 for Sheuer, 10 for Ishak and 29 for METAVIR resulted uncertain to be classified in a unique category of severity.

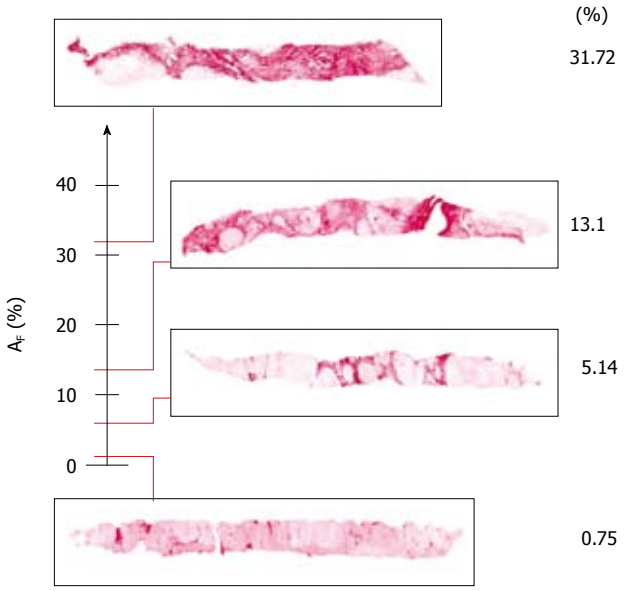

Figure 9 Stad-ging indicates the part of the disease course already covered and the part that remains to be covered before it reaches its end, and is established by placing the value of fibrosis (expressed as a scalar) on the ideal trajectory that indicates the phase of fibrosis at the time of measurement. A_F = fibrosis area (× 10).

making standardized and precise metrical measurements of pathological elements in a liver biopsy histological section using the rules of measurement theory.

The model used to make quantitative comparisons between the structural elements of the natural liver system and the same elements in liver tissue affected by chronic viral inflammation was constructed on the basis of a set of four observables, which were considered the prime structural elements of the system. Three of these (the hepatocyte sub-set, topical immune system and collagen support network) were taken as axioms for measuring the purely structural organized matter; the fourth (tissue tectonics) was taken as an axiom because it defines the harmony that orders the natural conformation of liver tissue. Recognizing these observables in their most strictly structural form makes it possible to define necrosis, the topical immune system, the collagen network and tissue tectonics in geometrical terms: (1) necrosis of the hepatocyte mass was considered a reduction, and steatosis an enlargement due to lipid deposition in the form of intercellular vacuoles; (2) inflammation was considered an enlargement of the topical immune cell system; (3) fibrosis an enlargement of natural liver collagen formed by the deposition of intercellular matrix; and (4) the transformations in liver tectonics as a reduction in the natural harmonic state of liver tissue that caused disorder in the organ's lobular structure.

This model allows the Dioguardi Histological Metriser to provide the following quantitative data: (1) the metrical extension of the residual hepatocellular set including the area of vacuoles pertinent to abnormal lipid accumulation; (2) the geometric measure of the inflammation basin (i.e. that part of the liver surface covered by inflammatory cell clusters), which distinguishes intra-basin space and extra-basin dispersed parenchymal leukocytes; (3) the magnitude of collagen islets, which were considered truncated or asymptotic planar fractals and classified into three degrees of magnitude; and (4) the TCI that quantifies alterations (disorders) in the organization of liver tissue.

To quantify these elements, the Dioguardi Histological Metriser uses three units of measurement. The first are the traditional IS units, which are used to measure the outlines of clusters and lipid vacuolization; the second are the traditional IS units corrected by the fractal dimension, which are used to measure irregular fractal collagen islets and the area of the residual hepatocellular set; and the third is the TCI, which is based on the inter-relationships between the Euclidean and fractal dimensions of liver tissue, and provides

Table 7 Side effects of liver biopsy sampling and other invasive techniques

Examination	Side effects		Ref.
	Serious complications (%)	Mortality (%)	
Liver biopsy	0.5	> 0.02	31
Oesophagogastroduodenoscopy		0.01	31
Colonoscopy	0.3	0.02	31-33
Flexible sigmoidoscopy	0.0001		32, 34
Endoscopic retrograde cholangiopancreatography	5-10	0.1-1	32, 35, 36
Percutaneous transhepatic cholangiography	3		32
Contrast agents	2-10		37-39

a quantitative estimate of the loss of natural tectonic order.

On the basis of our results, it is difficult not to consider biopsies a rich source of information regarding the course of chronic liver inflammation, despite their undeniable limitations[31]. Our perseverance in studying biopsies should be seen in the light of the risks[32-36] currently accepted in many fields of medical practice[37-39] (Table 7). Quantifying liver lesions in a biopsy sample raises many questions concerning the status and organization of natural and pathological liver systems that do not seem to be merely subsidiary matters to be dealt with within the confines of a specific investigation[1].

Why adopt a new measuring method in hepatology?

One fundamental question is whether hepatology researchers or practitioners need to adopt a metrical method of tissue analysis in order to confront the everyday problems they already solve in a less precise but what they still consider to be a satisfactory manner. Viral inflammation is a dynamic process influenced by a variety of factors that generate changes in the shapes (wrinkledness) of liver tissue lesions. However, studying the evolution of liver tissue status can use no more than one biopsy, which must therefore be examined in as much detail as possible, and this makes any attempt to improve precision crucial. It is clear that the status of the organ cannot be defined quantitatively without an appropriate technology that is capable of: (1) discriminating the most representative observables; (2) eliminating imprecise identifications and measurements; (3) using a suitable metrical unit for measuring the irregularly shaped elements of the tissue; (4) standardizing the measurement of previously unavailable histological elements; and (5) considering the smallest foci of inflammation and fibrotic islets that cannot be observed through an optical microscope.

The problem of the most representative observables was solved by the model, and that of the linear IS unit was solved by correcting it by the fractal dimension of the measured object[20,21]. The problem of standardizing the measurements was solved by constructing an innovative and completely automatic instrument that excludes human error, provides objective and reproducible results, and eliminates the need for the tedious work of light microscopic analysis (the automated analysis of an entire histological section takes place at a speed of 0.1 mm^2/s).

The problem of the representativeness of a biopsy fragment (which accounts for only 1/40 000-1/60 000 of the whole liver)[12] cannot be directly solved by our method which, however, can ensure objective and mathematical precision in measuring elements that may or may not be visible through a microscope.

At this point it has to be stressed that, although our metrical measures are rigorous and reproducible, their scalars provide definitions of magnitude and not interpretations, which remain the responsibility of pathologists and clinicians.

However, our machine can already describe a histological picture in verbal and repeatable terms, and thus provide a strictly morphological diagnosis. We can also say that we have begun to consider it in terms that make it more comparable with an intelligent collaborator than a sophisticated desk computer. Finally, our data come from machine-made metrical measurements of the pathological observables in a histological pattern, and are not hypotheses based on semi-quantitative methods that can only continue to generate new hypotheses.

This paper is closed with a dedication to Robert Rosen.

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COMMENTS

Background

Liver biopsy can be considered the gold standard for grading, staging and stad-ging the chronic liver disease. In addition, it remains a primary source for acquiring new knowledge about liver pathology.

Innovations and breakthroughs

This study introduced a new kind of liver biopsy measurement that bases the tissue state description on scalars, not with subjective interpretations (i.e. hypothesis). Furthermore, the Dioguardi Histological Metriser can describe a histological picture in verbal and repeatable terms, and thus provide a strictly morphological diagnosis.

Applications

The method, with opportune software, based on the same principles can be used for investigating also non-viral or inflammatory liver disease of other organs.

Peer review

This paper reports an impressive method for automated biopsy scoring.

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CLINICAL RESEARCH

Disruption of colonic barrier function and induction of mediator release by strains of *Campylobacter jejuni* that invade epithelial cells

Johannes Beltinger, Jo del Buono, Maeve M Skelly, John Thornley, Robin C Spiller, William A Stack, Christopher J Hawkey

Johannes Beltinger, Jo del Buono, Maeve M Skelly, John Thornley, Robin C Spiller, William A Stack, Christopher J Hawkey, Wolfson Digestive Diseases Centre, Nottingham University Hospital, QMC Campus, Nottingham NG7 2UH, United Kingdom
Author contributions: Stack WA and Hawkey CJ conceived and co-ordinated the study and raised funding; Beltinger J did the majority of the work, ably assisted by del Buono J and Skelly MM; Thornley J and Spiller RC kindly provided samples; All authors contributed to the design conduct and authorship of the paper which was originally drafted by Beltinger J.
Supported by The Medical Research Council (UK), No. G9716348
Correspondence to: Christopher J Hawkey, Wolfson Digestive Diseases Centre, Nottingham University Hospital, QMC Campus, Nottingham NG7 2UH,
United Kingdom. cj.hawkey@nottingham.ac.uk
Telephone: +44-115-9709918 Fax: +44-115-9422232
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Abstract

AIM: To study the mechanisms by which *Campylobacter jejuni* (*C. jejuni*) causes inflammation and diarrhea. In particular, direct interactions with intestinal epithelial cells and effects on barrier function are poorly understood.

METHODS: To model the initial pathogenic effects of *C. jejuni* on intestinal epithelium, polarized human colonic HCA-7 monolayers were grown on permeabilized filters and infected apically with clinical isolates of *C. jejuni*. Integrity of the monolayer was monitored by changes in monolayer resistance, release of lactate dehydrogenase, mannitol fluxes and electron microscopy. Invasion of HCA-7 cells was assessed by a modified gentamicin protection assay, translocation by counting colony forming units in the basal chamber, stimulation of mediator release by immunoassays and secretory responses in monolayers stimulated by bradykinin in an Ussing chamber.

RESULTS: All strains translocated across monolayers but only a minority invaded HCA-7 cells. Strains that invaded HCA-7 cells destroyed monolayer resistance over 6 h, accompanied by increased release of lactate dehydrogenase, a four-fold increase in permeability to [^{3}H] mannitol, and ultrastructural disruption of tight junctions, with rounding and lifting of cells off the filter membrane. Synthesis of interleukin (IL)-8 and prostaglandin E_2 was increased with strains that invaded the monolayer but not with those that did not.

CONCLUSION: These data demonstrate two distinct effects of *C. jejuni* on colonic epithelial cells and provide an informative model for further investigation of initial host cell responses to *C. jejuni*.

Key words: *Campylobacter jejuni*; Cell invasion; Cell culture; Chloride secretion; Colonocyte; HCA-7 cells; Membrane permeability; Monolayer; Mucosal barrier; Ussing chamber

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INTRODUCTION

Campylobacters are small (1.5-6.0 μm long and 0.2-0.5 μm wide) Gram-negative spiral rods. *Campylobacter jejuni* (*C. jejuni*), a foodborne organism contracted from untreated water, milk and meat, especially chicken, is one of the most important causes of bacterial diarrhea worldwide[1-4]. The clinical spectrum ranges from non-inflammatory watery diarrhea to an acute entero-colitis with neutrophilic invasion of the mucosa and bloody diarrhea mimicking ulcerative colitis.

Much work has been conducted on laboratory strains such as NCT11168, which has been completely geno-

typed. This has allowed a number of virulence factors to be identified, including a number of flagellar proteins, which not only enable chemotaxis towards mucus and amino acids and epithelial cell invasion[5-7], but also facilitate secretion of non-flagellar virulence proteins[6], O-linked glycosylation, which is required for optimal flagella function[7], proteins secreted *via* flagella that result in epithelial cell invasion and apoptosis[8-10], a cytolethal distending toxin (CLDT)[11] with DNAse activity[12], associated with apoptosis[13] and secretion of interleukin (IL)-8 and other chemokines[14,15] and a lipo-oligosaccharide that resembles human neuronal gangliosides, which may pre-dispose to autoimmune phenomena such as Guillain-Barre syndrome[16].

Clinical isolates vary in the extent to which they express these virulence factors. *C. jejuni* display heterogeneity in its ability to invade cells of the intestinal epithelial layer[17-21]. Estimates of the proportion of clinical isolates that are characterized by toxin production range from 12% to 100%[22]. Cell death may occur by a variety of mechanisms, not all involving CLDT[23]. Release of chemokines such as IL-8 seems to occur by CLDT-dependent and independent mechanisms[14], and it is unclear how far inflammatory responses to *C. jejuni* infection, such as secretion of chemokines, correlate with reported differences in the ability of the bacteria to invade epithelial cells, or how much this is due to responses of cells in the lamina propria, such as macrophages. Similarly, the extent to which secretory responses by epithelial cells can maintain a secretory diarrhea, which is characteristic particularly of childhood infection, is also unclear. One possibility is that *C. jejuni* induces synthesis of prosecretory compounds such as prostaglandins directly in epithelial cells[24-27]. An earlier study that failed to show this might have been flawed because it used CaCo2 cells, which do not readily express cyclo-oxygenases (COXs) or synthesize prostaglandins[28].

In order to investigate the direct effects of *C. jejuni* on colonic epithelial function *in vitro*, we therefore exposed the well-differentiated colonocyte line HCA-7, clone 29 to a panel of primary clinical isolates. Our data suggest two distinct patterns of interaction between *C. jejuni* and colonic epithelium, with a minority of strains invading colonic epithelial cells, which causes barrier destruction and induces elaboration of potentially pro-inflammatory mediators.

MATERIALS AND METHODS

Bacterial strains

Nineteen consecutive *C. jejuni* clinical strains from community patients with acute bacterial enteritis were isolated and characterized by the Laboratory of Public Health, University Hospital, Nottingham, UK. Of these, three fresh clinical isolates (strains 2801055, 2102011 and 1702030*)* were used for detailed studies along with the laboratory strain 12189 (a kind gift from Dr. J Ketley), originally isolated from a patient with diarrhea and passaged several times in the laboratory[28]. Strains were grown microaerobically for 48 h on chocolate agar plates prior to inoculation into tissue culture flasks containing liquid medium (RPMI 1640 supplemented with 4% Isosensitest broth and 5% FCS) (Sigma, UK). A stock of the original isolates was aliquoted and frozen at -80℃. For study of bacterial-epithelial cell interactions, bacteria were resuspended in 4% Isosensitest broth with 5% FCS. After static microaerobic incubation for 24 h, bacteria were pelleted, washed and resuspended in PBS.

The bacteria were diluted in Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK) to different concentrations (10^5-10^8 bacteria/mL). Determination of the number of bacteria was standardized by optical density at a wavelength of 570 nm. Growth curves for the different strains and titration and culture of serial dilutions was used to establish the number of bacteria in culture. This was done by incubating the different strains grown on a chocolate agar plates in Isosensitest broth (5% FCS) overnight. After centrifugation (7000 r/min, 5 min) and washing in PBS, bacteria were resuspended in 1 mL PBS, and serial diluted. Each dilution was measured in a spectrophotometer at a wavelength of 540 nm. Each sample was then serially diluted in a 96-well plate and dilutions incubated on chocolate agar plates. After overnight growth, bacterial colonies were counted and plotted against absorbance. The results were used to establish bacterial numbers before incubating epithelial cells.

Cell culture

Tumor-derived colonic epithelial cells, HCA-7, colony 29 (a kind gift from Susan Kirkland) were cultured as described previously[29]. Briefly, cells were grown in DMEM with 10% FCS, glutamine (0.29 mg/mL), ampicillin (8 μg/mL), penicillin (40 μg/mL), streptomycin (368 μg/mL) and non-essential amino acids in an atmosphere of 5% CO_2 at 37℃. For studies of bacterial cell interactions, normal medium was replaced with antibiotic-free medium for at least 24 h. Tests for mycoplasma contamination were not performed. For electrophysiological studies, cells (10^5 cells/membrane) were seeded on Snapwell or Transwell filters (polycarbonate membrane, pore size 0.45 μm, surface area 1 cm^2; Costar UK Ltd) and formed confluent monolayers within 8-10 d, as assessed by an epithelial volt-ohmmeter (EVOM; World Precision Instruments, Stevenage, UK)[29].

Effect of C. jejuni on barrier function

Bacteria were grown and added to the apical side of monolayers grown on Transwell or Snapwell filters at a concentration of 10^4-10^8 bacteria/monolayer. Transepithelial resistance was measured with an EVOM at various timepoints up to 24 h after inoculation with different concentrations of bacteria. These data were supported by detailed studies of filter-grown monolayers voltage-clamped in Ussing chambers (World Precision Instruments) and by assessment of [^{3}H] mannitol flux[26].

Ussing chamber studies

Confluent HCA-7 cell monolayers were inoculated on the apical side with varying amounts of strains of *C. jejuni*. After different time periods, filters were

placed in an Ussing chamber and voltage-clamped by continuous application of a short circuit current (SCC)[30]. Resistance (Ω cm^2) was measured under basal conditions and the change in short circuit current (ΔSCC μA/cm^2) after basolateral stimulation by bradykinin (10^{-6} mol/L), and finally after similar stimulation by carbachol (10^{-4} mol/L).

Invasion assay

Invasion of epithelial cells was investigated using a gentamicin invasion assay[18]. Bacteria were grown in 4% Isosensitest broth and 5% FCS. After static microaerobic incubation for 24 h, bacteria were pelleted, washed and resuspended in PBS. Bacterial number was assessed spectrophotometrically and 10^6 organisms added to the cell monolayer. Infected monolayers were incubated for up to 6 h at 37℃, washed and covered with tissue culture medium containing gentamicin (100 μg/mL). After 90 min, the integrity of the monolayer was checked microscopically, then washed in PBS and flooded with 1 mL 10 mL/L Triton X-100 for 5 min, to release intracellular bacteria. Dilutions and viable counts were made of the bacteria within the lysed monolayer. Positive control was a *Yersinia enterocolitica* invasive strain 8081c, and the negative control was *Escherichia coli* non-invasive strain HB101. When it became clear that monolayer destruction occurred with some bacteria, we modified the assay to correct for the number of remaining cells per monolayer. We calculated the number of cells remaining per monolayer by using a hemocytometer, before lysing to obtain counts of intracellular protected bacteria.

Lactate dehydrogenase (LDH) release

HCA-7 cells were incubated with *C. jejuni* for 4 h. Media from the apical reservoir of bacterium-exposed and control monolayers were then collected and analyzed for spontaneous LDH using a colometric assay (Sigma)[31]. In addition, total intracellular LDH concentration from HCA-7 cells was determined by addition of 1 mL 0.1% Triton X-100 to wells of bacterium-exposed and control monolayers. After vigorous pipetting to ensure lysis of all cells, the homogenate was then collected from each well and was also assayed for LDH.

[^{3}H] mannitol flux

Mannitol flux studies were performed in an Ussing chamber[32]. Mannitol (final concentration 5 mmol/L) was added to both sides of the monolayer in Krebs solution. After equilibration of the epithelial monolayer, [^{3}H] mannitol (1 μCi/mL) was added to the apical side. Radioactivity was counted on samples from the basolateral compartment at 15-min intervals for 60 min. Basolateral chamber volume was maintained by replacing the sample aliquot with an equal volume of fresh Krebs buffer. Apical to basolateral flux, expressed as mol·h/cm^2, was calculated by relating the accumulation of tritium in the basolateral chamber compared to the apical.

Electron microscopy (EM)

Filters with HCA-7 cells inoculated with 10^7 bacteria for 6 h where fixed by immersion in 2.5% glutaraldehyde (in 0.1 mol/L cacodylate buffer, pH 7.4). Subsequent processing was performed as described previously[25].

Bacterial translocation

Translocation was measured by inoculating the apical side of monolayers with 10^7 bacteria/0.5 mL. Medium from the basolateral compartment was collected after 2, 4, 6 and 8 h inoculation, diluted 10-fold and cultured on agar plates[33,34]. After each collection, membranes were transferred to fresh wells to establish the number of bacteria translocating over each 2-h period. The number of bacteria was established by counting colony-forming units at each time interval to establish the total number of bacteria translocated. Colony forming units where counted after microaerophilic incubation for 24 h to assess the time course of bacterial translocation.

IL-8 measurement

Level of IL-8 in the basolateral supernatant after 6 h incubation with different strains of *C. jejuni* and of control monolayers was measured using a quantitative ELISA[14] (Amersham, UK). Samples were pipetted into wells coated with specific antibody for IL-8, followed by incubation with a biotinilated antibody reagent. After extensive washing, a streptavidin-horseradish peroxidase conjugate was added and developed with 3,3',5,5'-tetramethylbenzidine substrate. After terminating the reaction, the optical density was read at 450 nm. Detection level was 25-1000 pg/mL.

PGE2 measurement

Aliquots of supernatant were prepared in the same way as for IL-8 release. PGE2 levels were measured by ELISA (Amersham), based on competition between unlabeled PGE2 and a fixed quantity of peroxidase-labeled PGE2 for a limited amount of PGE2-specific antibody[35].

Statistical analysis

Analysis of variance was used in experiments where time or dose was varied, to investigate the influence of cellular invasive and non-invasive bacteria and the interaction with time. For simple comparisons, unpaired *t* tests were used. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using PRISM (Graphpad, San Diego, CA, USA) or Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA).

RESULTS

Transepithelial resistance

Fourteen of the 19 clinical strains tested, as well as the laboratory isolate 12189 had no effect on transepithelial resistance up to 24 h. In contrast, the other five fresh clinical isolates abrogated monolayer resistance entirely by 6 h. These differences did not correlate with rates of translocation. Detailed studies, with strain 2801055 which abrogated transepithelial resistance, showed that decrease in transepithelial resistance varied with bacterial load and was

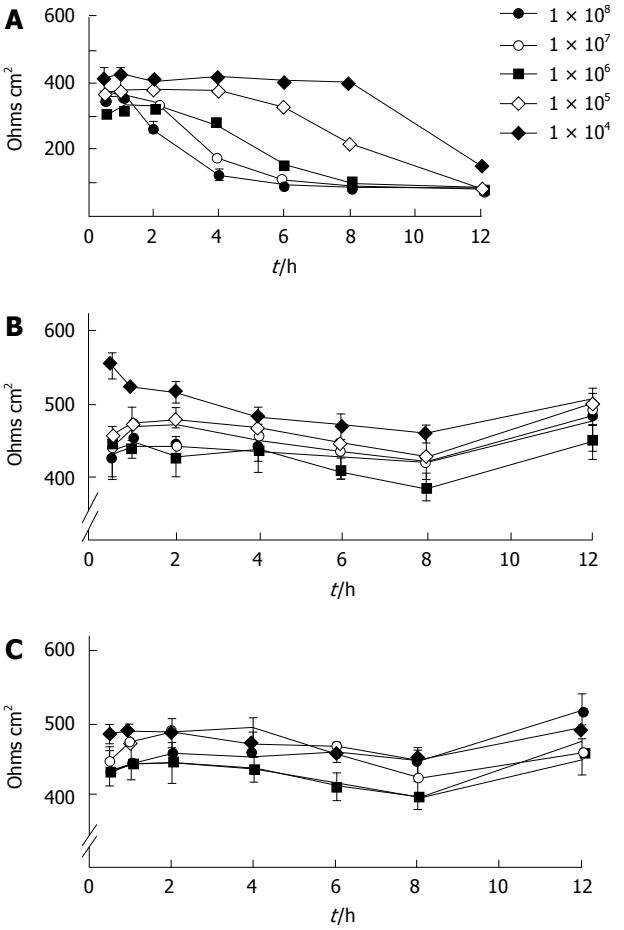

Figure 1 Time course and dose-response of changes in transepithelial resistance after inoculation of T84 cells with three different strains of C jejuni. A: Strain 2801055 reduced resistance to baseline over 6 h in a time- and dose-dependent manner [data are mean ± SE, n = 3 for each bacterial concentration; P < 0.05 compared with controls (uninfected monolayers)]. Baseline resistance across the filter membrane was 100 Ω.cm^2; B: Strain 12189 had no effect on resistance across the monolayer; C: Strain 2102011 had no effect on resistance across the monolayer.

time dependent, starting after 2 h of inoculation with 10^8 bacteria/0.5 mL (Figure 1). With strains 12189 or 2102011, no changes were seen at any time with any of the bacterial loads inoculated (Figure 1). Measurements in Ussing chambers mirrored those obtained with the EVOM with the different strain types. There was no significant difference in electrical resistance in monolayers infected with the strains 12189 and 2102011 at 4 and 8 h. However, strain 2801055 showed a time-dependent decrease in transepithelial resistance, with resistance falling to 71.3% and 17.7% of control at 4 and 8 h respectively (P < 0.05 *vs* control at 8 h).

Invasion of epithelial cells by C. jejuni

The standard gentamicin protection assay showed that 1.15% ± 0.05% of the positive control strain *Y. enterocolitica* invaded HCA-7 cells *vs* 0.0005% with the negative control strain *E. coli* HB101. Overall, *C. jejuni* showed levels of HCA-7 invasion that were intermediate between these values. Analysis of variance showed that the total number of *C. jejuni* per monolayer was higher for those that abrogated monolayer resistance compared to those that did not (P = 0.033), and that this increased with

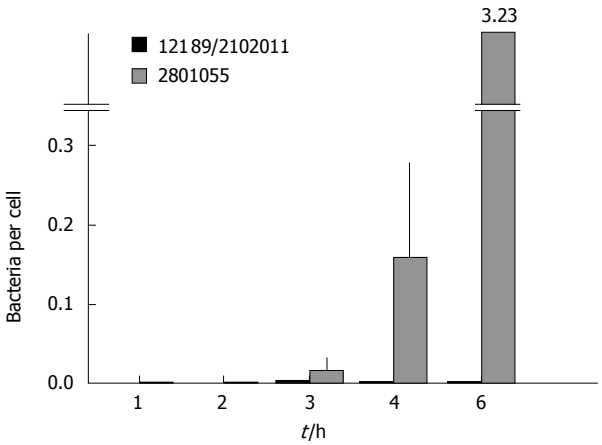

Figure 2 Invasion of monolayers of HCA-7 cells determined by a gentamicin protection assay, corrected for the number of cells remaining per monolayer. *C. jejuni* strain 2801055 invaded the cells of the monolayer in a time-dependent manner, while strains 12189 and 2102011 showed no cellular invasion. Data are mean ± SE, n = 4. Analysis of variance showed that the total number of *C. jejuni* per monolayer was higher for those strains that abrogated transepithelial resistance compared to those that did not (P = 0.033), and that this increased with time (P = 0.02).

time (P = 0.02). When the number of bacteria was related to the number of HCA7 cells remaining in the monolayer at the time of assessment, there was a substantial and highly significant difference between strains that destroyed and did not destroy the monolayer (Figure 2).

LDH release

LDH release from monolayers inoculated with the cell-invasive strain 2801055 was significantly increased after 24 h compared to inoculation with the non-invasive strains 12189 and 210211 (n = 4 per experiment, P < 0.05). LDH release with these strains did not differ from that seen with uninfected monolayers (Figure 3A).

Transepithelial mannitol flux

Cumulative flux data show that strains 12189 and 210211 did not change the flux of [^{3}H] mannitol, which indicated an intact paracellular resistance, whilst strain 2801055 increased the flux significantly (Figure 3B).

EM studies

In keeping with electrophysiological results, strains 12189 and 2102011 did not affect cellular morphology (Figure 4A and B). Monolayers infected with these strains showed close cell-to-cell contact, but bacteria were occasionally located in the pores of the filter membrane (Figure 4C). In contrast, strain 2801055 showed marked cellular and tight junction destruction at 6 h (Figure 4D). There was cell rounding and condensation of the plasma membrane (Figure 4E). In addition, cells were lifted off the filter membrane and multiple bacteria were seen between epithelial cells and the filter (Figure 4E and F).

Bacterial translocation

All strains tested translocated across the monolayer to become detectable after 2 h, with no obvious inter-strain differences.

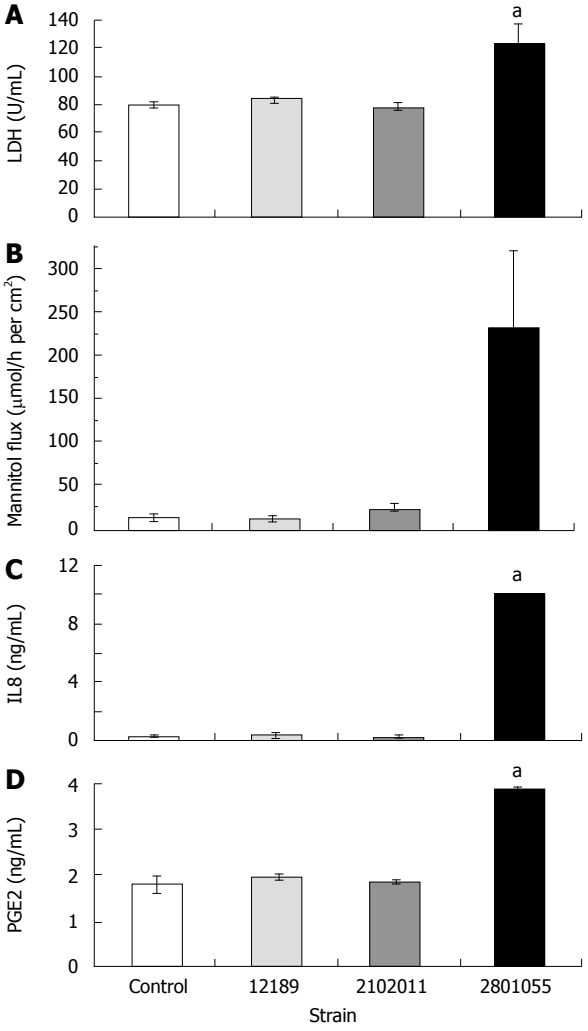

Figure 3 HCA-7 monolayer release of LDH, [^{3}H] mannitol flux and release of IL8 and PGE2 after inoculation with different strains of *C. jejuni*. All data are mean ± SE at 4 h, (n = 4). A: Strain 2801055 showed a significantly higher LDH release after 4 h ($^aP < 0.05$) incubation compared to the other two strains, which were similar to control values; B: Strain 2801055 induced a significantly higher flux rate across the monolayer after 4 h ($^aP < 0.05$) incubation compared to the other two strains, which were similar to control values; C: Strain 2801055 showed a significantly higher IL8 release after 4 h ($^aP < 0.05$) incubation compared to the other two strains, which were similar to control values; D: Strain 2801055 showed a significantly higher PGE2 release after 4 h ($^aP < 0.05$) incubation compared to the other two strains, which were similar to control values.

IL-8 production

IL-8 release from HCA-7 monolayers increased in response to inoculation with the invasive strain 2801055, whereas IL-8 release with the non-invasive strains 12189 and 210211 was not significantly different from that seen in uninfected monolayers (Figure 3C).

PGE2 release

There was a two-fold rise in PGE2 released after inoculation with the invasive strain 2801055 (n = 4, $P < 0.05$). By contrast, PGE2 levels for strains 12189 and 2102011 were similar to spontaneous PGE2 production in control monolayers (Figure 3D).

Bradykinin- and carbachol-induced secretion

Bradykinin 10^{-6} mol/L administered basolaterally induced a ΔSCC of 19.83 ± 3.25 μAmp/cm^2. ΔSCC responses to bradykinin were significantly enhanced by 30% compared with control (uninfected) monolayers at 4 h (n = 4, $P < 0.05$ for all 3 strains Figure 5A), but were lost at later time points, when there was a significant reduction with strain 2801055. The early increase was not seen in response to carbachol (Figure 5B), and at 8 h was significantly decreased with strains 2801055 and 2102011 (n = 4, $P < 0.05$).

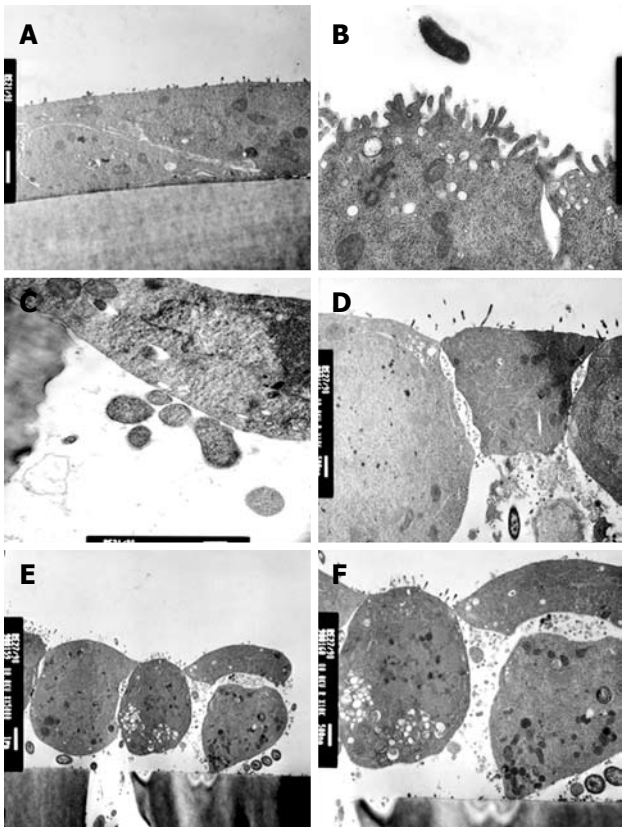

Figure 4 Comparison of the effects of strains 12189 and 2801055 on transmission electron micrograph appearance of monolayers of HCA-7 cells. A: Following inoculation of strain 12189, 10^7 bacteria/0.5 mL, on the apical side for 6 h, there was no disruption of monolayer integrity; B: There were normal tight junctions and normal apical microvilli; C: Close cell-to-cell contact is seen with occasional bacteria located in the pores of the filter membrane; D: Following inoculation of strain 12189, 10^7 bacteria/0.5 mL, on the apical side for 6 h, monolayer integrity was compromised with disruption of tight junctions; E: Monolayers showed condensation of the plasma membrane with lifting and rounding of cells off the supporting membrane. *C. jejuni* are also seen beneath a cell which is lifting off the membrane; F: Changes are seen at higher power.

DISCUSSION

In this study, we showed two distinct patterns of interaction between clinical isolates of *C. jejuni* and a colonic epithelial cell line. Strains that invaded epithelial cells were shown to destroy them, as demonstrated by a fall in transepithelial resistance and release of LDH. These processes were accompanied by release of IL-8 and PGE2. Strains that did not invade epithelial cells did not affect barrier properties or increase mediator production.

Translocation to the lamina propria[2,33] and a consequent interaction between bacterial antigens and antigen presenting cells, immunocytes and macrophages in the

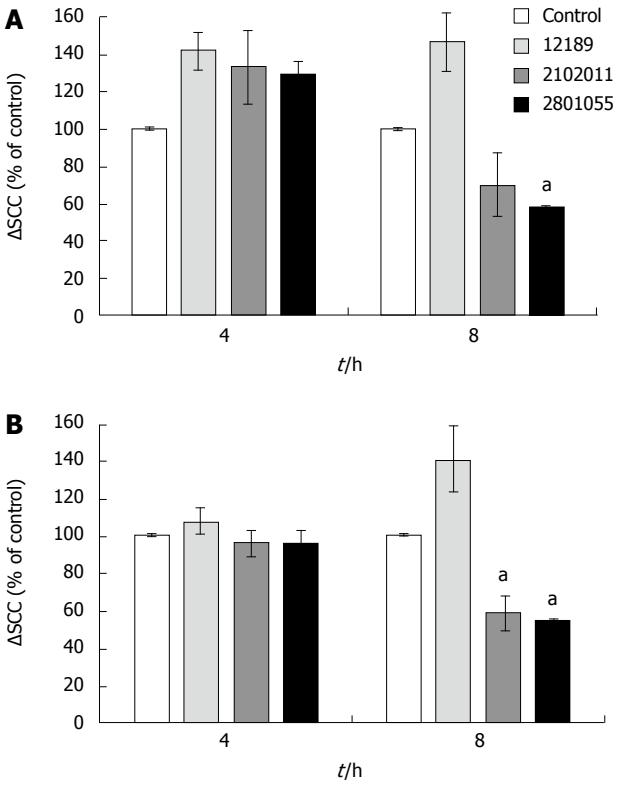

Figure 5 ΔSCC after stimulation with bradykinin (10^{-6} mol/L) and carbachol (10^{-4} mol/L) in monolayers inoculated with different strains of *C. jejuni*. Data are mean ± SE (n = 4) and are shown as percentage of control. A: Reduced chloride secretory response (represented by SCC) to bradykinin after 8 h inoculation with strain 2801055. $^aP < 0.05$; B: Reduced chloride secretory response to carbachol after 8 h inoculation with strains 2801055 and 2102011. $^aP < 0.05$.

lamina propria[36], is likely also to result in production of cytokines and chemokines. However our data show that epithelial cells can themselves be a source of mediators that could influence inflammatory and secretory processes in the case of strains that invade epithelial cells.

Although a paracellular route has been invoked to explain the ability of *C. jejuni* to invade the mucosa and achieve systemic infection, intracellular bacteria have been reported and a paracellular route of invasion inferred[37]. Using a modified gentamicin protection assay that allows for cell death, we were able to confirm cellular invasion by *C. jejuni*, and showed that this was associated with cytotoxicity and elaboration of pro-inflammatory and pro-secretory molecules. As yet it is uncertain whether intracellular invasion directly causes the associated release of IL-8 and PGE2, or whether this is a secondary consequence of cell death. A direct specific effect is possible since we have reported that IL-8 synthesis and PGE2 release from epithelial monolayers also occur in response to treatment with a boiled cell-free extract of *C. jejuni*[38], with induction of COX-2[39] and activation of nuclear factor-κB (NF-κB) and other relevant signaling pathways[40].

Enhanced release of IL-8 and other chemokines would play an important role in chemoattraction of neutrophils that characterizes some clinical infections with *C. jejuni*. Whether the increased prostaglandin synthesis that we observed with cellular invasion is sufficient under some circumstances to induce secretory diarrhea is more difficult to evaluate. We showed an early increase in bradykinin-induced secretion, as indicated by changes in SCC, with all strains tested, including non-invasive strains that did not stimulate prostaglandin synthesis. This increase in bradykinin-induced chloride secretion may therefore occur by prostaglandin-independent mechanisms. Direct epithelial action, *via* cAMP, cGMP, calcium mobilization, or induction of galanin or inducible nitric oxide are alternative mechanisms that are activated directly by bacterial enterotoxins or *via* signaling mechanisms that include NF-κB, which we and others have shown are upregulated by components of *C. jejuni*[24-27,40-43]. Destruction of the monolayer by strains that did invade epithelial cells and stimulate prostaglandin synthesis makes it difficult to evaluate whether enhanced prostaglandin synthesis by epithelial cells contributed to secretory diarrhea in these cases.

Campylobacter, like *Salmonella*, *Yersinia*, *Shigella* and *Listeria*, is an organism capable of translocation, as demonstrated by the current and previous monolayer studies and clinical features that include septicemia, Guillain-Barre syndrome and meningitis[16,33,34,41]. Previous studies have left unclear whether the main route is transcellular or paracellular. Both have been described. Our data suggest the translocation across the monolayer is common since all of the 19 clinical strains isolated from patients and one laboratory strain showed this property, regardless of whether they invaded epithelial cells and/or destroyed the monolayer. This suggests a dominant paracellular route of translocation, which is supported by our EM observations, which showed bacteria in the paracellular space. This appeared to occur efficiently, as judged by the rate of accumulation of bacteria on the basolateral side, and without gross disruption of tight or adherence junctions, as judged by the unchanged transepithelial resistance and mannitol permeability, seen with strains that translocated without epithelial cell invasion.

In the case of bacteria that invaded and destroyed the epithelial monolayer, translocation could be a crude consequence of its destruction, although bacteria that destroyed the monolayer did not appear to translocate faster than those that did not. Translocation results may differ according to the cell type used for epithelial monolayers. In previous studies that used differentiated CaCo2 cells, cellular invasion was not accompanied by the complete abrogation of monolayer resistance seen in our study and not all strains translocated[34]. We chose to use HCA7 cells in preference to CaCo2 cells because they have a differentiated large rather than small bowel phenotype and because they are capable of expressing COX-2 under induction conditions. Our data suggest that this is an informative model and cell line to study disease pathogenesis and signaling mechanisms[40].

COMMENTS

Background

Campylobacter jejuni (*C. jejuni*) is the commonest cause of bacterial diarrhea worldwide but its mode of pathogenesis is not clear.

Research frontiers

Since this work was done, the *Campylobacter* genome has been sequenced. Work following from the current study has investigated gene expression and has shown that chemokines play a central role.

Innovations and breakthroughs

The paper underlines the importance of allowing for cell destruction when doing gentamicin assays. Unlike many previous studies, this one used clinical isolates, which showed that there were two distinct patterns for the effect of *C. jejuni* on colonic epithelial cells. The cells themselves have the capacity to generate chemoattractant molecules, without necessary involvement of immune and other cells in the lamina propria.

Applications

Showing that *Campylobacter* spp. are cell invasive and stimulate production of chemoattractant mediators points to possible targets for treatment.

Terminology

Ussing chamber: Monolayers are grown on a permeable filter. When cells form tight junctions they cause resistance to an electrical current passed through the monolayer.

Peer review

The majority of previous studies regarding *C. jejuni* have been performed using a laboratory strain NCT11168. This manuscript is considered to contain attractive information for enhancing the understanding the mechanism of *C. jejuni* infection. It's an interesting paper.

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BASIC RESEARCH

Magnolol attenuates sepsis-induced gastrointestinal dysmotility in rats by modulating inflammatory mediators

Tie-Cheng Yang, Shu-Wen Zhang, Li-Na Sun, Hong Wang, Ai-Min Ren

Tie-Cheng Yang, Shu-Wen Zhang, Hong Wang, Ai-Min Ren, Intensive Care Unit and Medical Department of Infection, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China
Li-Na Sun, Department of Pharmacology, School of Chemical Biology and Pharmaceutical Sciences, Capital Medical University, Beijing 100069, China
Author contributions: Zhang SW and Yang TC designed the research; Yang TC, Sun LN, Wang H and Ren AM performed the research; Yang TC and Sun LN analyzed data; Yang TC and Zhang SW wrote the paper.
Supported by Beijing Municipal Science & Technology Commission Major Sci-tech Program, No. H020920050130
Correspondence to: Shu-Wen Zhang, Professor, Intensive Care Unit and Medical Department of Infection, Beijing Friendship Hospital, Capital Medical University, No. 95, Yong An Road, Beijing 100050, China. zsw401106@sina.com
Telephone: +86-10-63138749 Fax: +86-10-62384167
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Abstract

AIM: To investigate the protective effects of magnolol on sepsis-induced inflammation and intestinal dysmotility.

METHODS: Sepsis was induced by a single intraperitoneal injection of lipopolysaccharide (LPS). Male Wistar rats were randomly assigned to one of three treatment groups: magnolol prior to LPS injection (LPS/Mag group); vehicle prior to LPS injection (LPS/Veh group); vehicle prior to injection of saline (Control/Veh). Intestinal transit and circular muscle mechanical activity were assessed 12 h after LPS injection. Tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1) and inducible nitric oxide synthase (iNOS) mRNA in rat ileum were studied by RT-PCR 2 h after LPS injection. Nuclear factor-κB (NF-κB) activity in the intestine was also investigated at this time using electrophoretic mobility shift assay. In addition, antioxidant activity was determined by measuring malondialdehyde (MDA) concentration and superoxide dismutase (SOD) activity in the intestine 2 h after LPS injection.

RESULTS: Magnolol significantly increased intestinal transit and circular muscle mechanical activity in LPS-treated animals. TNF-α, MCP-1 and iNOS mRNA expression in the small intestine were significantly reduced after magnolol treatment in LPS-induced septic animals, compared with untreated septic animals. Additionally, magnolol significantly increased IL-10 mRNA expression in septic rat ileum. Magnolol also significantly suppressed NF-κB activity in septic rat intestine. In addition, magnolol significantly decreased MDA concentration and increased SOD activity in rat ileum.

CONCLUSION: Magnolol prevents sepsis-induced suppression of intestinal motility in rats. The potential mechanism of this benefit of magnolol appears to be modulation of self-amplified inflammatory events and block of oxidative stress in the intestine.

Key words: Sepsis; Motility; Cytokines; Magnolol; Lipopolysaccharide

Peer reviewer: Susumu Ohwada, Associate Professor, Department of Surgery, Gunma University Graduate School of Medicine, 3-39-15 Shoma-Machi, Maebashi 371-8511, Japan

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INTRODUCTION

Sepsis frequently occurs after trauma, burns, hemorrhage or abdominal surgery. It is a leading cause of morbidity and mortality in critically ill patients[1]. During sepsis, the most frequent complications within the gastrointestinal (GI) tract are ileus and mucosal barrier dysfunction[2]. Ileus plays an important role in the pathophysiology of sepsis by promoting bacterial stasis, bacterial overgrowth and bacterial translocation, which lead to the development of secondary infections and multiple organ failure[3].

Although common during sepsis, the etiology of ileus is still unclear. Current evidence supports the hypothesis that lipopolysaccharide (LPS) rapidly activates resident intestinal macrophages, which subsequently initiate a molecular and cellular inflammatory response that causes intestinal dysmotility[4-6]. Additionally, oxidative stress during sepsis may also be involved in this process[7]. Currently, there is no accepted pharmacological prevention or management of sepsis-induced intestinal

dysmotility. Blocking oxidative stress and modulating the inflammatory events might be helpful.

Magnolia officinalis, a traditional Chinese herb, is commonly used in the treatment of abdominal distention and vomiting associated with many clinical conditions. It has been reported to attenuate L-arginine-induced GI dysmotility in rodents[8], and to improve the electrical activity of GI smooth muscle during endotoxemia[9]. Recently, magnolol (5,5'-di-2-propenyl-1,1'-biphenyl-2,2'-diol), a principal constituent isolated from the bark of *Magnolia officinalis*, has been showed to attenuate peroxidative damage and to improve survival of rats with sepsis[10]. Treatment with magnolol after hemorrhagic shock can suppress the tumor necrosis factor-α (TNF-α) level and preserve interleukin-10 (IL-10) production in rats[11].

Thus, we have developed the hypothesis that through modulation of inflammatory cytokines during sepsis, magnolol may be helpful for treatment of sepsis-induced ileus. Therefore, the objective of the present study was to examine the capacity of magnolol pretreatment to prevent sepsis-induced intestinal dysmotility and to determine its effects on pro- and anti-inflammatory molecular responses in the local intestine.

MATERIALS AND METHODS

Animal preparation and experimental design

Male Wistar rats (250-300 g body weight) were obtained from the Academy of Military Medicine Sciences (Beijing, China). The rats were exposed to 12 h light and 12 h darkness each day, with free access to food and water. All experiments were performed in accordance with the institutional criteria for the care and use of laboratory animals in research. Sepsis was induced by a single intraperitoneal injection of LPS (*Escherichia coli*, O55: B5; Sigma, St Louis, MO, USA) at 20 mg/kg. Controls received intraperitoneal injections of saline.

Magnolol (National Institute for the Control of Pharmaceutical and Biological Products, China) was dissolved in 40% (v/v) propylene glycol and diluted to the desired concentration in normal saline. Final concentration of propylene glycol in the injected solution was < 4.0×10^{-3}% (v/v). The single dose used for the magnolol instillation was 10^{-5} g/kg, which was previously shown to be helpful for increasing survival of surgically induced sepsis[10]. Normal saline with 4.0×10^{-3}% (v/v) propylene glycol served as a vehicle.

Animals were randomly assigned to one of three treatment groups. LPS/Mag group: rats received magnolol (10^{-5} g/kg, intravenous bolus *via* the tail vein) 30 min before LPS injection; LPS/Veh group: rats received vehicle 30 min before LPS injection; Control/Veh: rats received vehicle 30 min before injection of saline. Preliminary results showed that intraperitoneal injection of LPS caused a profound suppression of intestine muscle contractile activity, which was both dose- and time-dependent. Furthermore, the effects of LPS are always rat strain specific and relate to the serotype of LPS[12]. In this study, we chose the 12-h time point for measurement of intestinal smooth muscle function. To elucidate the potential mechanism for magnolol preventing sepsis-induced ileus, we also evaluated changes in the chemokines and cytokines in the intestine 2 h after LPS injection, because the inflammatory response in the local intestine rapidly initiated by LPS is always responsible for GI dysmotility[4-6].

Intestinal transit

Twelve hours after LPS (or saline) was administered, the animals received an intragastric injection of 0.1 mL Evans blue (50 mg in 1 mL 0.9% NaCl). Then, the rats were killed by exsanguination 1 h later. Intestinal transit was determined by measuring the distance between the gastric pylorus and distal small intestine that was stained blue[13].

Measurement of muscle contractility

Circular muscle mechanical activity was assessed using full-thickness strips obtained from the ileum of each animal 12 h after LPS (or saline) injection. Muscle strips (2 × 10 mm) were placed in a mechanical organ chamber that was continuously perfused with pre-oxygenated Krebs-bicarbonate solution maintained at 37℃. One end of each strip was tied to a fixed post, and the other was attached to an isometric force transducer. After an equilibration period of 30 min, spontaneous mechanical contractions were recorded. The contractile responsiveness of muscle strips to the muscarinic receptor agonist bethanechol was also evaluated. Dose-response curves were generated by exposing the muscles to increasing concentrations of bethanechol (0.1-100 μmol/L) for 10 min; with intervening 20-min wash periods. Contractions were recorded, measured, and stored in a computer using a commercially available hardware and software package (TaiMeng Technology, Chengdu, China).

RT-PCR

To elucidate the potential mechanism of magnolol treatment blunting sepsis-induced intestinal dysmotility, mRNA for TNF-α, IL-10, monocyte chemoattractant protein 1 (MCP-1) and inducible nitric oxide synthase (iNOS) in rat ileum was assessed by RT-PCR.

Total mRNA was extracted with TRIZOL Reagent (GIBCO BRL, USA). Reverse transcription was performed using a Reverse Transcription System Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Primers were designed and purchased from AuGCT Biotechnology (Beijing, China). β-actin was used as an endogenous control. The sequences of the RT-PCR primers are listed in Table 1. PCR was performed with 25 μL reaction mixture of 1 μL RT product, 2 mmol/L $MgCl_2$, 0.03 U/L Taq DNA polymerase, 0.4 mmol/L dNTP, 0.1 μmol/L primer (endogenous control, target genes), and 1 × Taq DNA polymerase magnesium-free buffer. Then, the reaction

Table 1 Primer sequences

Gene	Primer sequences	Product size (bp)
β-actin	F 5' GAAATCGTGCGTGACATTA 3' R 5' TAGGAGCCAGGGCAGTAA 3'	349
TNF-α	F 5' GTAGCAAACCACCAAGCAG 3' R 5' GGTATGAAATGGCAAATCG 3'	211
IL-10	F 5' GCTATGTTGCCTGCTCTT 3' R 5' ATGCTCCTTGATTTCTGG 3'	307
MCP-1	F 5' ACTTGACCCATAAATCTGA 3' R 5' TGGAAGGGAATAGTGTAAT 3'	168
iNOS	F 5' TTGGGTCTTGTTAGCCTAGTC 3' R 5' TGTGCAGTCCCAGTGAGGAAC 3'	264

mixture was overlaid with two drops of mineral oil and incubated in a thermocycler (Eppendorf, Germany) programmed to pre-denature at 94℃ for 2 min, denatured at 94℃ for 30 s, annealed at 55℃ for 30 s, and extended at 72℃ for 30 s for a total of 30 cycles. The last cycle was followed by a final incubation at 72℃ for 6 min and cooling to 4℃. PCR products were electrophoresed on a 1.2% agarose gel and saved as digital images. Relative quantities of target gene mRNA were analyzed by Quantity One software (Bio-Rad Laboratories, USA), normalized with β-actin expression.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein of rat ileum was prepared by hypotonic lysis followed by high salt extraction[14,15]. Nuclear factor-κB (NF-κB) activity in the nuclear extract was analyzed using the EMSA kit according to the manufacturer's protocol (Gel Shift Assay System; Promega). In brief, an NF-κB oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') was end-labeled with [γ-32P] ATP and T4-polynucleotide kinase. Binding assays were performed in 10 μL binding reaction mixture that contained 10 μg nuclear proteins and [γ-32P]-labeled NF-κB oligonucleotides. The binding reaction mixture was incubated at room temperature for 20 min and then electrophoresed on 4% non-denaturing PAGE. After PAGE, the gels were dried and exposed to X-ray film. The autoradiograms were quantified by scanning densitometry, using Quantity One software (Bio-Rad US).

Detection of superoxide dismutase (SOD) and malondialdehyde (MDA) in ileum

To evaluate the antioxidative capacity of magnolol, MDA concentration and SOD activity in rat ileum were measured 2 h after LPS injection. Intestinal tissue samples were thawed, weighed and homogenized 1:9 (w/v) in 0.9% saline. The homogenates were centrifuged at 3000 r/min for 10 min at 4℃, and the supernatant was removed for the assay of MDA content, SOD activity and total protein.

Total intestinal protein concentration was determined using the Coomassie blue method, with bovine serum albumin as a standard. SOD activity and MDA level were detected with kits, according to the manufacturer's instructions (Jiancheng Bioengineering Ltd, Nanjing, China). Results were expressed as N/mg protein and nmol/mg protein, respectively.

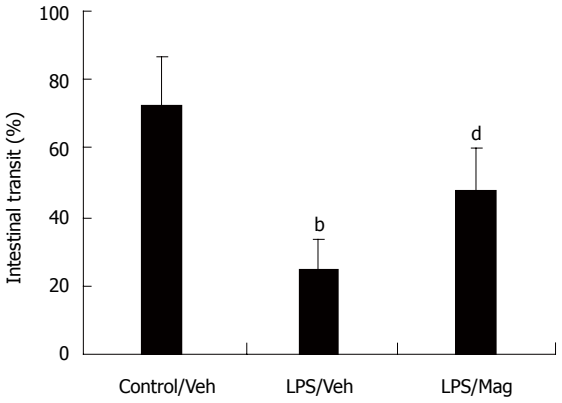

Figure 1 Magnolol prevents delayed small intestinal transit caused by LPS-induced sepsis. Data are shown as mean ± SE; $n = 6$. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{d}P < 0.01$ LPS/Mag *vs* LPS/Veh, LPS/Mag *vs* Control/Veh.

Statistical analysis

Data were expressed as mean ± SE. Statistical significance was determined by one-way ANOVA using SPSS 11.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Intestinal transit

As shown in Figure 1, LPS significantly delayed small intestinal transit from 74% ± 14% in control rats to 25% ± 9% in LPS rats. Pretreatment with magnolol significantly increased the transit in LPS animals, although this increase did not return to the control distribution pattern.

Changes in muscle contractility

The second series of experiments was designed to determine the effect of intravenous magnolol pretreatment on the intestinal musculature by measuring *in vitro* ileal circular muscle contractility from septic animals after LPS injection. Figure 2A shows the typical spontaneous contractility of circular muscle strips from three different animals. Analysis of the frequency of spontaneous contraction showed that muscle contractility in LPS-treated intestines was significantly lower than that in control tissues. Pretreatment with magnolol partly restored the spontaneous contractile pattern (Figure 2B).

Next, we evaluated the contractile response of muscle strips to the muscarinic receptor agonist bethanechol (0.1-100 μmol/L) using isometric force measurements.

As shown in Figure 3A, ileal circular muscle strips from LPS-treated animals showed significant impairment in the dose-response curve of bethanechol-stimulated muscle contraction. Magnolol treatment partly prevented LPS-induced impairment of ileal circular smooth muscle contractility. Figure 3B shows that, compared with

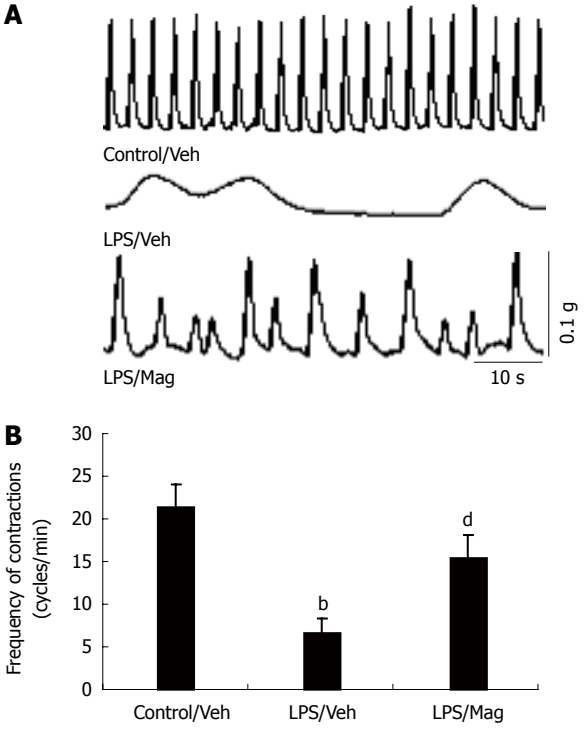

Figure 2 Change in spontaneous rhythmic contractions. A: Original traces of ileal circular muscle strip rhythmic contractions; B: Frequency of spontaneous rhythmic contractions. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{d}P < 0.01$ (LPS/Mag *vs* LPS/Veh) ($n = 6$).

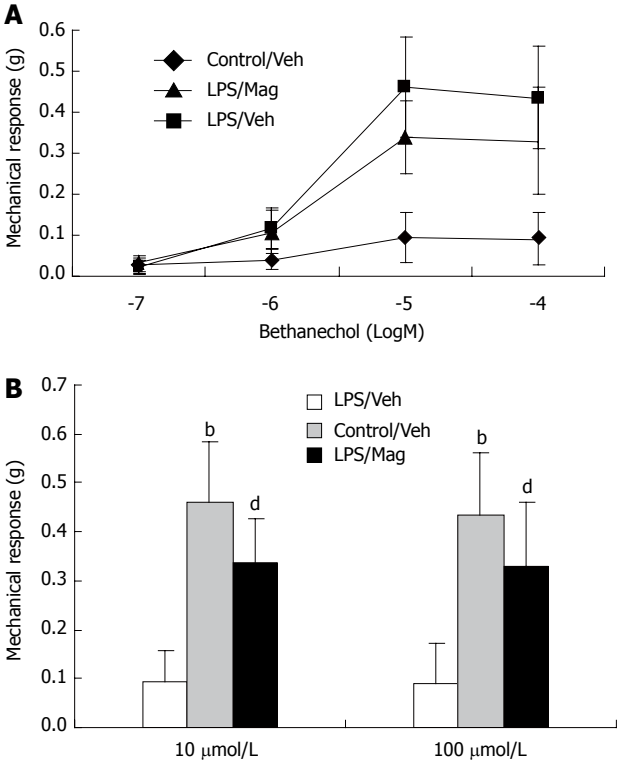

Figure 3 The contractile responsiveness of circular muscle strips to bethanechol. A: Bethanechol-stimulated dose-response curve; B: Circular muscle contractions at bethanechol concentration of 10 and 100 μmol/L. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{d}P < 0.01$ (LPS/Mag *vs* LPS/Veh) ($n = 6$).

controls, LPS significantly suppressed bethanechol-induced circular muscle contractions at bethanechol concentrations of 10 and 100 μmol/L. Magnolol treatment significantly increased the mechanical response of ileal circular muscles in LPS-treated animals.

The effect of magnolol treatment on GI motility of control rats was also evaluated. Neither intestinal transit nor circular muscle strip contractility was altered by magnolol (data not shown).

Molecular inflammatory responses

As shown in Figure 4, LPS induced a significant increase in TNF-α, IL-10 and MCP-1 mRNA levels in the ileum. Magnolol treatment significantly decreased LPS-induced TNF-α and MCP-1 mRNA expression. As for the anti-inflammatory mediator IL-10, magnolol significantly increased IL-10 mRNA expression in the ileum of LPS-treated animals.

iNOS has been shown to be the most important mediator of smooth muscle contraction during sepsis. Therefore, we also explored the effect of magnolol on iNOS mRNA expression in the ileum. Magnolol significantly suppressed LPS-induced iNOS mRNA expression.

NF-κB activity in rat intestine

NF-κB comprises a family of transcription factors that act as regulators of pro-inflammatory mediators[16]. We hypothesized that magnolol could potentially produce the above beneficial effects through decreased expression of NF-κB. As shown in Figure 5, LPS significantly induced activated NF-κB above control levels, and as hypothesized, magnolol significantly suppressed this response.

SOD and MDA in the small intestine

As shown in Figure 6, the MDA concentration in rat ileum, an index of lipid peroxidation, was significantly increased after LPS challenge compared with controls. Pretreatment with magnolol significantly decreased the MDA concentration. SOD activity in intestinal tissue decreased markedly in LPS-treated animals. Magnolol pretreatment caused a significant increase in SOD activity in rat ileum.

DISCUSSION

This study demonstrated the ability of magnolol, an antioxidant isolated from a Chinese herb, to prevent intestinal dysmotility in LPS-induced septic rats. It also provided evidence that the potential mechanism of action of magnolol results from both attenuation of peroxidative damage and modulation of the inflammatory response during sepsis.

Sepsis-induced ileus after complicated abdominal surgery, hemorrhagic shock, trauma and burns still causes morbidity and mortality in critically ill patients. Accumulating evidence has indicated that overwhelming pro-inflammatory and oxidative stress responses combined with diminished anti-inflammatory pathways are responsible for GI dysmotility during sepsis[4-7]. Unfortunately, there is no accepted pharmacological

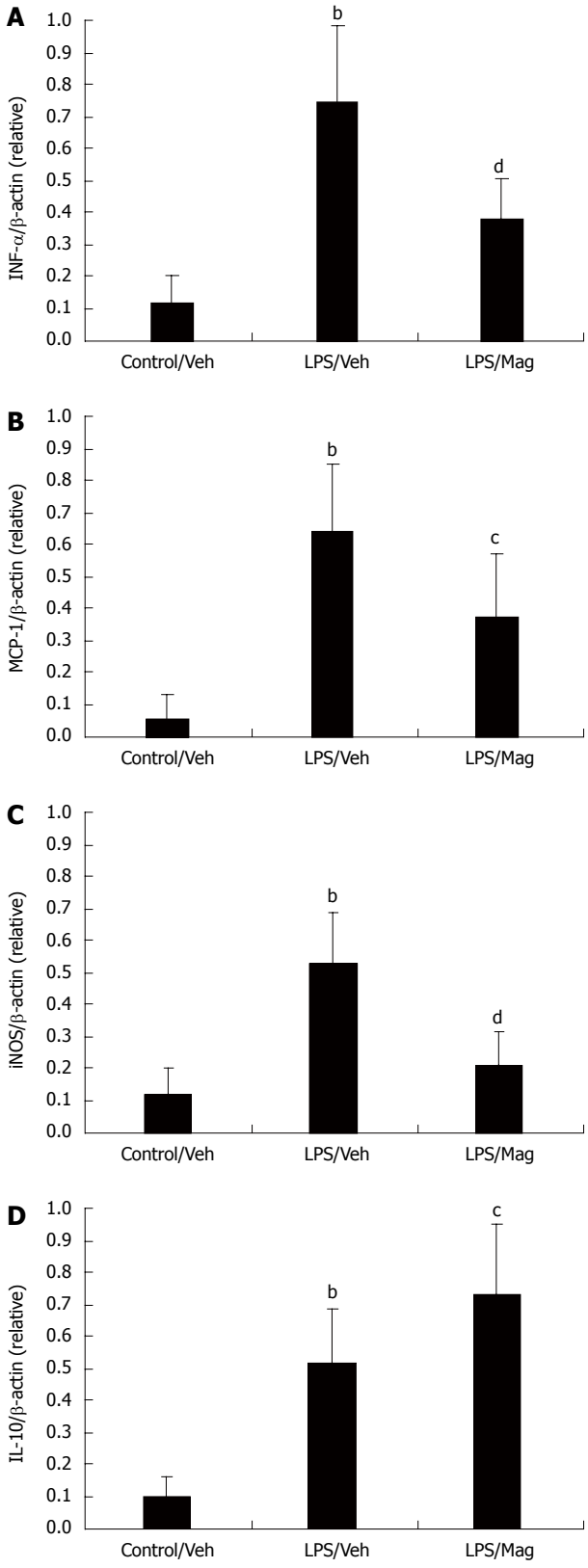

Figure 4 RT-PCR analysis of (A) TNF-α, (B) IL-10, (C) MCP-1, and (D) iNOS mRNA expression in rat ileum. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{c}P < 0.05$ (LPS/Mag *vs* LPS/Veh) ($n = 6$); $^{d}P < 0.01$ (LPS/Mag *vs* LPS/Veh).

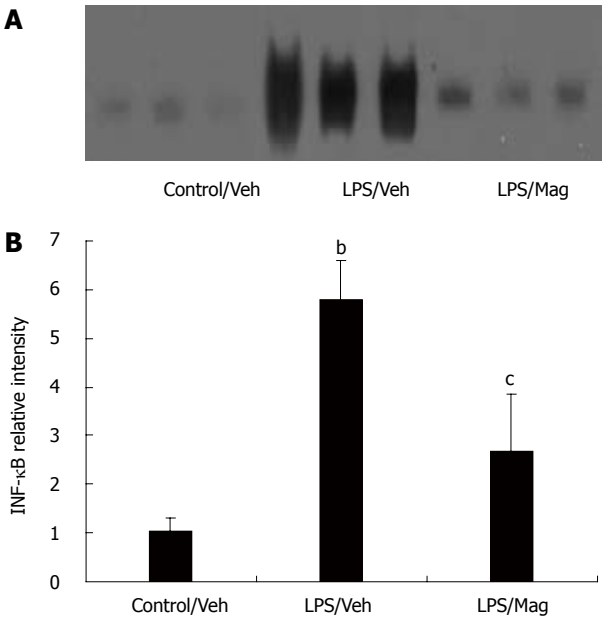

Figure 5 NF-κB activity in rat intestine. A: Representative EMSA gel; B: Scanning densitometry analysis of NF-κB activity. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{c}P < 0.05$ (LPS/Mag *vs* LPS/Veh) ($n = 3$).

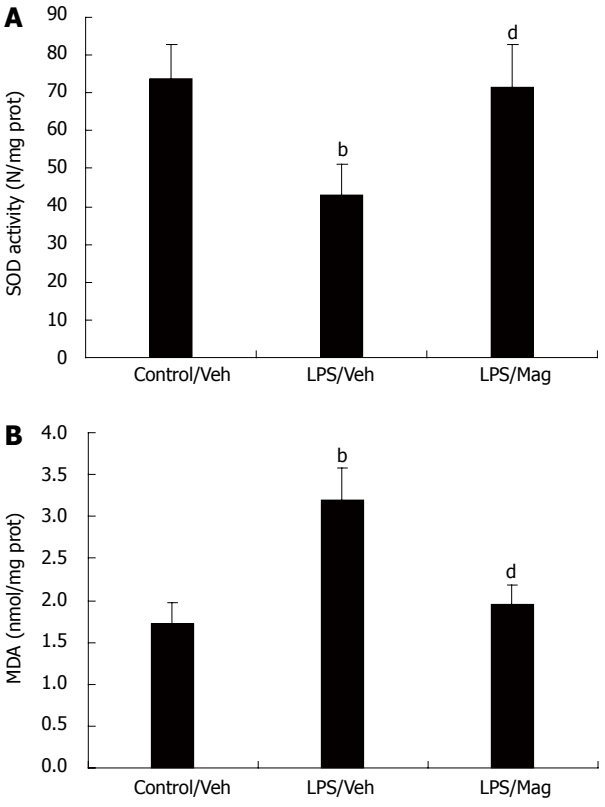

Figure 6 SOD activity (A) and MDA concentration (B) in rat ileum. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{d}P < 0.01$ (LPS/Mag *vs* LPS/Veh) ($n = 6$).

prevention or management of sepsis-induced ileus at present. The present study demonstrated that magnolol can partly restore the delayed intestinal transit caused by LPS. Additionally, magnolol treatment can prevent LPS-induced impairment of ileal circular smooth muscle contractility.

Numerous studies have demonstrated that exaggerated production of oxygen-derived free radicals in the face of defective antioxidative protection occurs in animals and humans with sepsis[17-19]. This

imbalance between pro- and anti-oxidants may produce oxidative stress, which ultimately leads to cellular injury and necrosis, *via* several mechanisms including lipid peroxidation, protein denaturation, and DNA damage. *Magnolia officinalis* has been used as a blood-quickening and stasis-dispelling agent in traditional Chinese medicine. Magnolol, a compound purified from the bark of *Magnolia officinalis*, has been shown to be 1000 times more potent than α-tocopherol in inhibiting lipid peroxidation in rat heart mitochondria[20], and 50 000 times more potent than glutathione, a well-known antioxidant. It can also exhibit free radical scavenging activity. Moreover, it has been reported to suppress superoxide anion production in myocardium exposed to ischemia and reperfusion[21]. In accordance with these studies, we found that magnolol significantly attenuated the intensity of lipid peroxidation and increased SOD activity in rat ileum during sepsis. The antioxidant properties of magnolol are proposed to underlie its beneficial effects during sepsis.

We considered that prevention of sepsis-induced intestinal dysmotility by magnolol was partly through interruption of the cycle of inflammatory events in the local intestine. To confirm this hypothesis, we performed semi-quantitative RT-PCR on ileal tissue for inflammatory cytokines TNF-α, MCP-1 and iNOS, which have been shown to participate in leukocyte recruitment and functional muscle impairment[4,5,22]. The anti-inflammatory mediator IL-10 was also evaluated in our experiment. We found that magnolol significantly suppressed the initial surge of TNF-α at the gene level and increased IL-10 expression in septic rat intestine. Pro-inflammatory cytokines, such as TNF-α, have been shown to be released early after an inflammatory stimulus[23]. The increase in pro-inflammatory cytokines is followed by an increase in anti-inflammatory cytokines, such as IL-10, which reflect the compensatory anti-inflammatory response syndrome[24]. It has been reported that IL-10 can inhibit cytokine production in monocytes by blocking LPS-induced NF-κB activation[25]. Additionally, IL-10 modulates the production of various chemokines (such as MCP-1) and prevents generation of NO by LPS-activated monocytes/macrophages[26-28].

MCP-1 is a potent chemoattractant that is capable of promoting monocyte recruitment into an inflammatory site, as well as activating monocytes and macrophages[29,30]. It has previously been shown that regulation of leukocyte recruitment and subsequent intestinal smooth muscle dysmotility during endotoxemia is mediated through MCP-1, and that a major source of MCP-1 is the dense network of resident muscularis macrophages[13]. In this study, we used MCP-1 mRNA as our marker of chemokine activity. As mentioned above, magnolol significantly reduced intestinal MCP-1 mRNA expression during LPS-induced sepsis.

NO is known to be the main inhibitory neurotransmitter of the GI tract, caused by the activity of the constitutive isoform of neural NO synthase (cNOS) within the enteric nervous system[31]. Besides, NO is produced at almost all sites of inflammation by leukocytes through the activity of iNOS[32]. Evidence indicates that NO from iNOS plays a pivotal role in mediating LPS-induced suppression of intestinal smooth muscle activity[4], and that up-regulation of iNOS activity is mediated by TNF-α and IL-1[5]. Additionally, NO and superoxide anions can join to form the toxic metabolite $ONOO^-$[33,34], which is also involved in the pathogenesis of sepsis-induced motility disturbances[7]. Magnolol has been reported to suppress the overproduction of NO and TNF-α in LPS-activated macrophages[35]. The results obtained in the present study provide support for this view. Pretreatment with magnolol significantly decreased iNOS mRNA expression in the intestine of the LPS-treated animals.

NF-κB is an inducible nuclear transcription factor that plays a central role in regulating the transcription of many pro-inflammatory cytokines[16], including TNF-α and IL-1β. Furthermore, intricate negative and positive feedback loops exist within NF-κB activation and cytokine expression. Pro-inflammatory cytokines activate NF-κB, but IL-10 deactivates NF-κB[36]. In the present study, we found that magnolol significantly suppressed NF-κB activation in the intestine of septic rats, which suggests that magnolol modulates inflammatory cytokines may be through intervention in the NF-κB signal transduction system. In addition, magnolol might also inhibit NF-κB activation through increasing IL-10 gene expression.

During sepsis, oxidative stress causes direct damage to cells and tissues and is involved with inflammatory cytokine production[17]. Suppression of cytokines by antioxidants has been demonstrated in previous studies. *N*-acetyl-cysteine has been shown to prevent the priming of increased expression of TNF-α mRNA after LPS[37]. Also, it has been reported that the free-radical-trapping compound phenyl *N-tert*-butylnitrone administered in LPS-induced sepsis promotes enhanced production of endogenous IL-10[38]. Additionally, the involvement of oxidative stress or oxygen free radicals in NF-κB activation has been suggested[39]. Therefore, we assume that magnolol modulation of cytokine synthesis may be related to its antioxidant properties. This is in agreement with previous studies that have shown that gut injury is partly prevented by antioxidants[40]. However, this has not been proven experimentally.

Although the findings of the present study predict a role for magnolol in a clinical setting, several problems should be mentioned. We did not use the cecal ligation and puncture (CLP) sepsis model in our study, which appears to be a reliable and clinically relevant animal model of the human septic condition, because abdominal surgery can also initiate an inflammatory cascade and ultimately lead to impairment of intestinal smooth muscle activity. More intricate pathophysiological mechanisms may be involved in the development of gut dysmotility in the CLP sepsis model[41]. Additionally, Zhang *et al*[42] previously reported that, *in vitro*, magnolol exerted an inhibitory effect on isolated ileum of guinea pigs. However, we found in our study that *in vivo,* magnolol treatment could prevent LPS-induced suppression of intestinal motility but had no

effect on control animals. These discrepancies suggest that the pharmacological properties of magnolol on GI motility might change when it is administered at different doses or *via* different routes. At the dose and route that we used in our study, the antioxidant effect of magnolol could be the important mechanism through which it ameliorates the severity of sepsis. Under other pathophysilogical conditions, whether magnolol could exert a similar effect is still not known. Other well-designed experiments are needed to further determine the clinical usefulness and safety of magnolol.

In conclusion, the data presented in this study suggest a protective role of magnolol in preventing sepsis-induced suppression of intestinal motility. The potential mechanism of this beneficial effect of magnolol appears to be modulation of the self-amplified inflammatory events and block of oxidative stress in the intestine.

COMMENTS

Background

During sepsis, gastrointestinal (GI) dysmotility occurs frequently. Accumulating evidence has indicated that overwhelming pro-inflammatory and oxidative stress responses combined with diminished anti-inflammatory pathways are responsible. Recently, magnolol, an antioxidant isolated from a traditional Chinese herb, has been showed to attenuate peroxidative damage and to improve survival of rats with sepsis. It can also suppress the TNF-α level and preserve IL-10 production in hemorrhagic shock in rats. Thus, the authors presumed that through modulation of inflammatory cytokines during sepsis, magnolol might be helpful for treatment of sepsis-induced ileus.

Research frontiers

Sepsis-induced GI dysmotility is a major problem in critically ill patients. The pharmacological intervention is difficult for the clinician to handle. In addition, there is a lack of controlled studies on which to build an evidence-based treatment concept for critically ill patients.

Innovations and breakthroughs

Currently, there is no accepted pharmacologic prevention or management of sepsis-induced GI dysmotility. Therefore, management remains largely supportive. Insights gained in this preliminary study might be helpful in producing an effective pharmacological intervention strategy.

Applications

This study provides the evidence that pretreatment with magnolol could attenuate sepsis-induced GI dysmotility. The potential mechanism of this benefit of magnolol appears to be modulation of the self-amplified inflammatory events and block of oxidative stress in the intestine.

Terminology

Sepsis is defined as infection plus systemic manifestations of infection. Cytokines: non-antibody proteins secreted by inflammatory leukocytes and some non-leukocytic cells, which act as intercellular mediators. Magnolol (5,5' -di-2-propenyl-1,1'-biphenyl-2,2'-diol), a principal constituent isolated from a traditional Chinese herb. Lipopolysaccharides (LPS) are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, act as endotoxins and elicit strong immune responses in animals.

Peer review

This preliminary study provides us with a new insight into management of sepsis-induced GI dysmotility. However, the pharmacological properties of magnolol may change when it is administered at different doses or *via* different routes. Other well-designed experiments are needed to further determine its clinical utility and safety.

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RAPID COMMUNICATION

Adverse events with bismuth salts for *Helicobacter pylori* eradication: Systematic review and meta-analysis

Alexander C Ford, Peter Malfertheiner, Monique Giguère, José Santana, Mostafizur Khan, Paul Moayyedi

Alexander C Ford, José Santana, Mostafizur Khan, Paul Moayyedi, Gastroenterology Division, McMaster University, Health Sciences Centre, Hamilton, Ontario L8N 3Z5, Canada
Peter Malfertheiner, Klinic fur Gastroenterologie, Hepatologie, and Infektiologie, Otto-v.-Guerike Universitat, Magdeburg, Germany
Monique Giguère, AxCan Pharma Inc., Mont St-Hilaire, Quebec, Canada
Author contributions: Ford AC, Santana J, Khan M, and Moayyedi P extracted and analysed the data; Ford AC drafted the manuscript; All authors revised the manuscript for important intellectual content.
Supported by A Grant from AxCan Pharma Inc
Correspondence to: Dr. Alexander C Ford, Gastroenterology Division, McMaster University Medical Centre, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada. alexf12399@yahoo.com
Telephone: +1-90-55212100-76409 Fax: +1-90-55214958
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Abstract

AIM: To assess the safety of bismuth used in *Helicobacter pylori* (*H pylori*) eradication therapy regimens.
METHODS: We conducted a systematic review and meta-analysis. MEDLINE and EMBASE were searched (up to October 2007) to identify randomised controlled trials comparing bismuth with placebo or no treatment, or bismuth salts in combination with antibiotics as part of eradication therapy with the same dose and duration of antibiotics alone or, in combination, with acid suppression. Total numbers of adverse events were recorded. Data were pooled and expressed as relative risks with 95% confidence intervals (CI).
RESULTS: We identified 35 randomised controlled trials containing 4763 patients. There were no serious adverse events occurring with bismuth therapy. There was no statistically significant difference detected in total adverse events with bismuth [relative risk (RR) = 1.01; 95% CI: 0.87-1.16], specific individual adverse events, with the exception of dark stools (RR = 5.06; 95% CI: 1.59-16.12), or adverse events leading to withdrawal of therapy (RR = 0.86; 95% CI: 0.54-1.37).
CONCLUSION: Bismuth for the treatment of *H pylori* is safe and well-tolerated. The only adverse event occurring significantly more commonly was dark stools.

Key words: Bismuth; Eradication therapy; *Helicobacter pylori*; Adverse events; Systematic review; Meta-analysis

Peer reviewer: Francis Mégraud, Professor, Department of Bacteriology, Universite Victor Segalen Bordeaux 2, 146 rue Leo Saignat, Bordeaux 33076, France

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INTRODUCTION

Bismuth salts have been used for centuries in medicine. From a gastroenterology perspective these drugs have been used to treat peptic ulcer disease, dyspepsia, parasitic infections, microscopic colitis, and infectious diarrhoea[1]. The discovery of *Helicobacter pylori* (*H pylori*) in 1983 by Warren and Marshall revolutionised the management of peptic ulcer disease[2], and led to a renewed interest in bismuth compounds, largely because bismuth was found to inhibit the growth of *H pylori* and was effective in eradicating the organism (when combined with antibiotics or in combination with antibiotics and acid suppression therapy[3,4]).

The first randomised controlled trial (RCT) of bismuth in *H pylori*-positive individuals suggested that bismuth was superior to erythromycin monotherapy in eradicating the infection[5]. A further RCT of 6 wk of colloidal bismuth subcitrate versus cimetidine, in *H pylori*-positive duodenal ulcer patients, demonstrated that bismuth successfully eradicated the bacterium in up to 50% of patients[6]. Subsequently, an RCT of both colloidal bismuth subcitrate and cimetidine, alone or in combination with tinidazole, confirmed that colloidal bismuth subcitrate and tinidazole cleared the infection in almost 75% of patients[7]. With the addition of a second antibiotic, tetracycline or amoxicillin, eradication rates in later RCTs exceeded 80%[8-10]. However, there were some problems associated with bismuth-based triple therapy, which included the number of tablets patients were required to take, the duration of therapy, and side effects such as altered taste, nausea, and diarrhoea.

There are a variety of bismuth salts currently available on the market. All are inorganic, poorly soluble and therefore less than 1% is typically absorbed systemically[11]. Blood concentrations of bismuth do rise when these compounds are ingested however, and there is therefore the potential for toxicity, though levels less than 50 μg/mL are unlikely to be associated with any meaningful toxicity in man[11]. In the 1970s, high doses of bismuth salts were used for long periods and were associated with neurotoxicity. In France, there were almost 1000 cases of bismuth-associated encephalopathy of which 72 were fatal[1]. The doses of bismuth used in *H pylori* eradication are administered for a much shorter duration, typically 1 to 2 wk. In a recent bioavailability study, where bismuth salts were given in combination with omeprazole for 6 d[12], plasma levels of bismuth remained well below 50 μg/mL, but a review of their safety profile would provide additional evidence that such low doses of bismuth, given for a short period of time, do not expose patients to undue risks. We have therefore conducted a systematic review and meta-analysis of available published literature to assess the magnitude of the risk of adverse events experienced when bismuth salts are used, either alone or in combination with one or more antibiotics, to eradicate *H pylori*.

MATERIALS AND METHODS

Outcomes assessed

Primary outcomes: The primary aim of this systematic review and meta-analysis was to assess the total number of adverse events occurring following treatment for *H pylori* with bismuth compounds, either alone, or in combination with antibiotics and/or acid suppression therapy, compared to treatment with antibiotics alone, acid suppression therapy alone, a combination of the two, or no treatment/placebo.

Secondary outcomes: The secondary aims were to evaluate the number of specific individual adverse events occurring and the number of withdrawals of therapy due to adverse events, and to assess the effect of long-term (defined as 1 mo or more) therapy on number of adverse events (both total number and by specific category) and withdrawals due to adverse events.

Eligibility criteria

Types of studies: In order to best estimate adverse events that were directly attributable to the use of bismuth, studies were only eligible for inclusion in this systematic review if they were RCTs that compared bismuth monotherapy with either acid suppression therapy alone, placebo, or no treatment, or compared bismuth compounds in combination with either antibiotics, or antibiotics and acid suppression therapy as part of a recognised efficacious eradication regimen with an identical dose and duration of antibiotics either alone or in combination with acid suppression therapy. We defined an efficacious bismuth-containing eradication regimen as any one of: bismuth triple therapy (bismuth in combination with two antibiotics); bismuth quadruple therapy (as for triple therapy, but with the addition of acid suppression therapy); or ranitidine bismuth citrate dual (with one antibiotic) or triple (with two antibiotics) therapy.

Types of participants: Patients were required to be *H pylori*-positive adults (over the age of 16 years) taking any bismuth compound for more than 1 d with a comparison group of *H pylori*-positive patients who were not taking bismuth.

Types of assessment: Bismuth toxicity had to be assessed and recorded using one or more of the following methods: medical databases; face-to-face interviews; telephone interviews; symptom diaries; or questionnaire in order for studies to be eligible for inclusion. The questionnaire used was not required to be previously validated but, if there were sufficient studies using questionnaires, we aimed to assess the impact of this in a sensitivity analysis.

Types of outcome measures: The proportion of patients that reported any adverse event and the proportion experiencing specific individual adverse events were assessed wherever trial reporting allowed this.

Search strategy and identification of eligible studies

Search strategy: Two authors performed searches of the medical literature to identify articles from MEDLINE (from 1966 up to October 2007), EMBASE (from 1988 up to October 2007), and the Cochrane Library and Current Contents electronic databases. RCTs using bismuth salts were identified using the medical subject heading term "bismuth". These studies were combined using the set operator and with papers that used a variety of free text terms including "Denol", "Pepto-Bismol", "bismuth", "subsalicylate", "tripotassium dicitrato bismuthate", "subnitrate", "subgallate", "ranitidine bismuth citrate", "pylorid", "quadruple therapy", "pylera", and "bismuth subcitrate potassium". There were no language restrictions, and papers published in abstract form only were also eligible for inclusion in the review. The abstracts of all papers identified by the initial search were evaluated for appropriateness to the study question, and all potentially relevant studies were retrieved and examined in greater detail to determine whether or not they met all eligibility criteria. The bibliographies of identified studies were then used to perform a recursive search of the literature to identify other potentially eligible studies. In addition, Digestive Disease Week, United European Gastroenterology Week, and European *H pylori* Study Group conference abstract books between 2000 and 2007 were hand-searched.

Selection of studies: Two reviewers screened all titles and abstracts of trials that were identified by the search strategy as being potentially eligible for inclusion in the systematic review to confirm or refute eligibility. This was performed using pre-designed eligibility forms. A third reviewer adjudicated where any disagreements arose, and a consensus view was taken.

Assessment of study quality: The quality of studies was assessed according to the following pre-defined criteria: method of assessment of occurrence of adverse events (interview, diary, and questionnaire), generation of randomisation schedule, method of allocation of concealment, and blinding of assessor as to patient allocation to therapy.

Data extraction

Data concerning total number of adverse events and number of specific individual adverse events were extracted on to specially developed forms by two reviewers and all data extraction was checked by a third reviewer. These verified data were then entered onto a Microsoft Excel spreadsheet (XP professional edition; Microsoft Corp, Redmond, WA, USA), and again this was double-checked by a third reviewer. Trial characteristics including setting (population-based, primary care, secondary care), country of origin, number of centres involved, duration of bismuth therapy and dosage schedule, type of bismuth compound, mean age of included patients, and proportion of male patients were recorded to allow exploration of potential reasons for any heterogeneity detected between trial results.

Data synthesis and statistical analysis

Data were extracted as dichotomous outcomes and pooled using a random effects model[13], where sufficient data were available. The impact of bismuth therapy on the incidence of total and specific individual adverse effects *versus* comparison regimen was expressed as a combined relative risk (RR) with a 95% confidence interval (CI). The number needed to harm with bismuth therapy to cause one adverse event, and a 95% CI, were calculated as the reciprocal of the risk difference from the meta-analysis, and where this was statistically significant the results were reported.

Due to differences in methodology, patient populations, and outcome measures between eligible trials, the results of individual studies can be very diverse and therefore when they are included in the same meta-analysis this may affect the accuracy of the overall result. This inconsistency within a single meta-analysis can be quantified with a statistical test of heterogeneity, to assess whether the variation across trials is due to true heterogeneity, or chance. This quantity is termed I^2, and its value ranges from 0 to 100 percent, with 0 percent representing no observed heterogeneity, and larger values indicating increasing heterogeneity. A value below 25 percent is arbitrarily chosen to represent low levels of heterogeneity[14]. Where the degree of statistical heterogeneity is greater than this, clinical reasons within individual trials that may account for some of this inconsistency can be explored. Wherever statistically significant heterogeneity existed between trial results in this systematic review, possible explanations were investigated informally using sensitivity analyses. These are exploratory only, and may explain some of the observed variability, but the results should be interpreted with caution.

All statistical analyses were performed using Stats Direct version 2.2.4 (Stats Direct Ltd, Sale, Cheshire, UK), which was used to generate Forest plots of pooled relative risks for total adverse event rates and specific individual adverse event rates by category, as well as funnel plots to assess for evidence of publication bias.

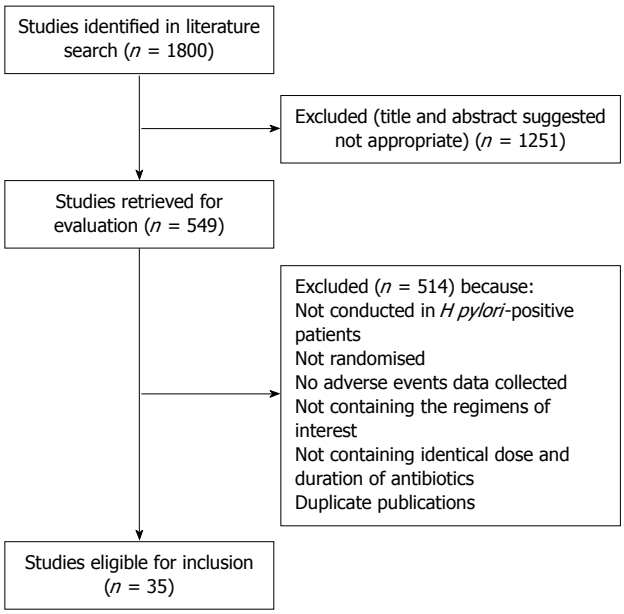

Figure 1 Flow diagram of assessment of studies identified in the systematic review.

RESULTS

Selection of eligible studies

The search strategy identified 1800 studies, of which 549 were possibly eligible. After reviewing the abstracts of these it became clear that 209 were RCTs of bismuth, and these were retrieved for further assessment. Of these, 35 were eligible for inclusion in the meta-analysis[7,15-48], reporting on 4763 *H pylori*-positive patients, 2435 of whom received bismuth or bismuth-based regimen, and 2328 received a comparison regimen (Figure 1). Thirty-three of the trials were found in fully published form, and two were only published as abstracts[29,44]. Seven of the RCTs used more than one bismuth-containing regimen[7,20,31,34,35,41,47].

Trial characteristics

Detailed trial characteristics are provided in Table 1. Nineteen of the trials were conducted in Europe[15-18,20,23-25,27-30,37,40,42-45,47], eight in the Far East[21,22,32,35,36,38,46,48], four in the USA[31,33,34,41], one in the Middle East[26], one in South America[19], one in Australia[7], and one was a multi-national study[39]. Eleven of the studies were multicentre RCTs[15,21,25,29,31,32,34,39,41,44,45]. Duration of bismuth therapy ranged from 7 to 56 d, with a total daily dose of between 400 mg and 2100 mg. Nineteen studies used ranitidine bismuth citrate[15,17,20,22,24,28-32,34,36,39-41,43,45,46,48], ten studies colloidal bismuth subcitrate[7,16,18,21,26,27,37,38,42,44], two studies tripotassium dictrato bismuthate[23,35], two studies bismuth subsalicylate[25,33], one study bismuth subnitrate[19], and one study both bismuth subnitrate and

Table 1 Characteristics of included studies

Study	Country	No. of centres	Bismuth compound used[1]	Duration of bismuth therapy (days)	Total dose (mg/d) used	Method of collection of adverse event data	Generation of randomization schedule provided	Method of concealment of allocation provided	Double-blind
Bujanda 2001[15]	Spain and Portugal	Multi-centre	RBC	7	800	Unclear	Yes	No	Yes
Burette 1992[16]	Belgium	1	CBS	10	480	Unclear	No	No	No
Buzas 2001[17]	Hungary	1	RBC	7	800	Unclear	No	No	No
Carpintero 1997[18]	Spain	1	CBS	42	480	Unclear	Yes	No	No
Carvalho 1998[19]	Brazil	1	BSN	14	1200	Unclear	No	No	No
Catalano 2000[20]	Italy	1	RBC	10	800	Questionnaire	Yes	No	Yes
Chuang 2001[22]	Taiwan	1	RBC	7	800	Unclear	No	No	Yes
Dal Bo 1998[23]	Italy	1	TDB	14	480	Unclear	No	No	No
Danese 2001[24]	Italy	1	RBC	7	800	Validated questionnaire	No	No	No
Eberhardt 1990[25]	Germany	4	BSS	28	1800	Unclear	No	No	No
Fakheri 2004[26]	Iran	1	CBS	14	480	Unclear	Yes	No	No
Forne 1995[27]	Spain	1	CBS	7	480	Diary cards	No	Yes	No
Gasbarrini 2000[28]	Italy	1	RBC	7	800	Validated questionnaire	No	No	No
Georgopoulos 1999[29]	Greece	3	RBC	7	800	Unclear	No	No	No
Gisbert 2000[30]	Spain	1	RBC	7	800	Unclear	Yes	No	No
Graham 1998[31]	USA	111	RBC	28	800	Unclear	No	No	Yes
Hung 2002[32]	Hong Kong	3	RBC	7	800	Diary	Yes	Yes	No
Lanza 1989[33]	USA	1	BSS	21	2100	Unclear	No	No	Yes
Lanza 1998[34]	USA	47	RBC	28	800	Diary cards	No	No	Yes
Liu 1999[35]	China	1	TDB	7	480	Diary	No	No	No
Mao 2000[36]	Vietnam	1	RBC	10	400	Diary	No	No	No
Marshall 1988[7]	Australia	1	CBS	56	480	Unclear	No	No	Yes
Masci 1995[37]	Italy	1	CBS	28 to 56	480	Unclear	Yes	No	Yes
Nafeeza 1992[38]	Malaysia	1	CBS	28	480	Unclear	No	No	Yes
Pare 1999[39]	Multi-national	Multi-centre	RBC	28	800	Unclear	Yes	No	Yes
Perri 2002[40]	Italy	1	RBC	7	800	Questionnaire	No	No	No
Peterson 1996[41]	USA	38	RBC	28	800	Unclear	No	No	Yes
Rokkas 1988[42]	UK	1	CBS	56	480	Unclear	No	No	Yes
Spadaccini 1998[43]	Italy	1	RBC	7	800	Face-to-face interview	No	No	No
Spiliadis 1998[44]	Greece	3	CBS	14	1200	Unclear	No	No	No
Spinzi 2000[45]	Italy	6	RBC	7	800	Face-to-face interview	No	No	No
Sung 1998[46]	Hong Kong	1	RBC	7	800	Telephone interview	No	No	No
Whitehead 2000[47]	UK	1	CBS and BSN	28	Unclear	Unclear	Yes	Yes	Yes
Wong 2001[48]	Hong Kong	1	RBC	7	800	Diary	Yes	Yes	No
Xiao 2001[21]	China	Multi-centre	CBS	7	480	Diary	No	No	No

[1]BSN: Bismuth subnitrate; BSS: Bismuth subsalicylate; CBS: Colloidal bismuth subcitrate; RBC: Ranitidine bismuth citrate; TDB: Tripotassium dictrato bismuthate.

colloidal bismuth subcitrate[47]. Comparison regimens were proton pump inhibitor or H_2-receptor antagonist (H_2-RA)-based eradication therapy in 23 studies[15,17-24,26-30,32,35,36,39,40,43,45,46,48], antibiotics alone in four studies[16,38,44,47], antibiotics or placebo in three studies[31,34,41], H_2-RA alone in two studies[25,37], placebo alone in two studies[33,42], and H_2-RA in combination with either one antibiotic or placebo in one study[7]. The mean age of individuals in included studies ranged from 36.7 years to 50.5 years, and the proportion of male patients varied between 32 percent and 78 percent. The number of participants in each RCT ranged from 20 to 530 individuals.

Trial quality

Thirteen of the trials were double-blind randomised studies[7,15,20,22,31,33,34,37-39,41,42,47], the remainder being either single-blind or open. Five of the single-blind trials specifically stated that assessors were blinded to treatment allocation[21,24,45,46,48]. Ten of the studies reported the method of generation of the randomization schedule[15,18,20,26,30,32,37,39,47,48], but only four the method of concealment of allocation[27,32,47,48]. Four of the studies recorded adverse events using a questionnaire[20,24,28,40], but only two of these stated that the questionnaire was validated[24,28]. Seven studies collected information concerning adverse events using a diary or diary cards[21,27,32,34-36,48], two *via* face-to-face interview[43,45], and one *via* telephone interview[46]. The remainder of trials did not state how they collected adverse events data.

Total number of adverse events with bismuth or bismuth-containing regimen versus comparison regimen

There were no serious adverse events such as death or neurotoxicity in either arm of any of the included

Table 2 Crude adverse event rates, and relative risk of adverse events

Adverse event	Number of trials	Total number of patients	Number of patients in bismuth arms	Number of patients in comparison arms	Number of adverse events in bismuth arms (%)	Number of adverse events in comparison arms (%)	Relative risk of adverse events with bismuth versus comparison regimen (95% CI)
Any	25	3180	1585	1595	431 (27.2)	419 (26.3)	1.01 (0.87-1.16)
Abdominal pain	13	2439	1221	1218	63 (5.2)	61 (5.0)	1.06 (0.64-1.74)
Dark stools	4	467	233	234	39 (16.7)	5 (2.1)	5.06 (1.59-16.12)
Diarrhoea	22	3406	1761	1645	124 (7.0)	113 (6.9)	1.01 (0.72-1.42)
Dizziness	8	1630	867	763	54 (6.2)	49 (6.4)	1.18 (0.81-1.72)
Headache	14	2433	1276	1157	41 (3.2)	28 (2.4)	1.31 (0.81-2.11)
Metallic taste	14	2475	1260	1215	124 (9.8)	116 (9.6)	1.02 (0.81-1.28)
Nausea and/or vomiting	20	3417	1767	1650	111 (6.3)	86 (5.2)	1.16 (0.89-1.52)
Leading to withdrawal of therapy	28	3951	2033	1918	33 (1.6)	38 (2.0)	0.86 (0.54-1.37)

RCTs. Twenty-five trials reported the total number of individuals experiencing any adverse event with bismuth or bismuth-containing regimens *versus* comparison regimen[15-20,23-27,30,32,33,35-40,42,44,45,47,48]. Three of these studies utilised more than one regimen[20,35,47], allowing 28 comparisons to be made. The relative risk of an adverse event with bismuth or bismuth-containing regimens versus comparison regimen was 1.01 (95% CI: 0.87 to 1.16) (Figure 2 and Table 2). There was statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 30.3\%$). The Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.16$). Sensitivity analysis according to trial setting, country of origin, dose of bismuth salt used, type of bismuth salt used, mean age of patients included in the study, and proportion of males included in the study failed to reveal any obvious explanation for the observed heterogeneity.

Number of specific individual adverse events with bismuth or bismuth-containing regimen versus comparison regimen

Abdominal pain: Thirteen trials reported the total number of individuals experiencing abdominal pain with bismuth or bismuth-containing regimens versus comparison regimen[17,18,20,21,24,26,28,30,34,39,40,46,47]. Three of these studies utilised more than one regimen[20,34,47], allowing 16 comparisons to be made. The relative risk of abdominal pain with bismuth or bismuth-containing regimens versus comparison regimen was 1.06 (95% CI: 0.64 to 1.74) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 22.0\%$), and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.15$).

Dark stools: Four trials reported the total number of individuals experiencing dark stools with bismuth or bismuth-containing regimens versus comparison regimen[17,42,46,48]. The relative risk of dark stools with bismuth or bismuth-containing regimens versus comparison regimen was 5.06 (95% CI: 1.59 to 16.12) (Figure 3 and Table 2). There was marginal statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 25.2\%$), but no obvious causes were found, and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.28$). The number of patients needed to harm with bismuth or bismuth-containing regimen versus comparison regimen to cause one case of dark stools was 7.5 (95% CI: 4 to 71).

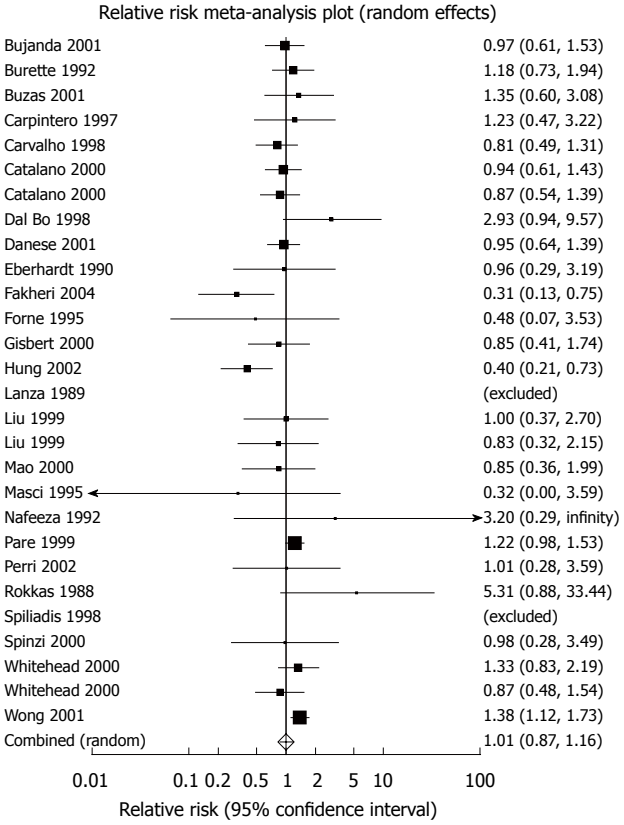

Figure 2 Forest plot of trials of bismuth or bismuth-containing regimens versus comparison regimen examining the effect on relative risk of any adverse event.

Diarrhoea: Twenty-two trials reported the total number of individuals experiencing diarrhoea with bismuth or bismuth-containing regimens versus comparison regimen[7,17,18,20,24-28,30-32,34,36,39-42,45-48]. Six of these studies utilised more than one regimen[7,20,31,34,41,47], allowing 28 comparisons to be made. The relative risk of diarrhoea with bismuth or bismuth-containing regimens versus comparison regimen was 1.01 (95% CI: 0.72 to 1.42) (Table 2). There was marginal statistically significant heterogeneity detected between trial results (heterogeneity

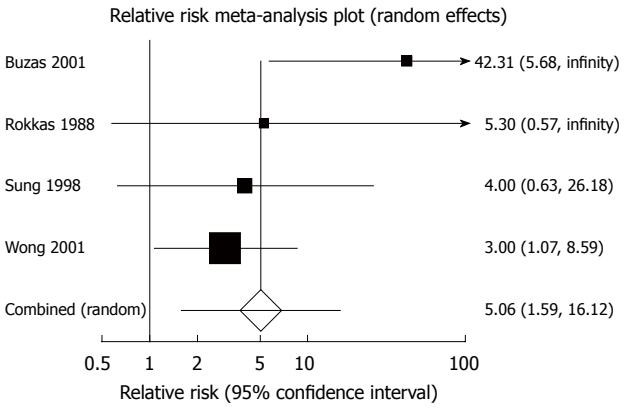

Figure 3 Forest plot of trials of bismuth or bismuth-containing regimens *versus* comparison regimen examining the effect on relative risk of dark stools.

test: $I^2 = 26.2\%$), but no obvious causes were found, and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.75$).

Dizziness: Eight trials reported the total number of individuals experiencing dizziness with bismuth or bismuth-containing regimens versus comparison regimen[21,26,31,32,35,41,46,48]. Three of these studies utilised more than one regimen[31,35,41], allowing 11 comparisons to be made. The relative risk of dizziness with bismuth or bismuth-containing regimens versus comparison regimen was 1.18 (95% CI: 0.81 to 1.72) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.20$).

Headache: Fourteen trials reported the total number of individuals experiencing headache with bismuth or bismuth-containing regimens versus comparison regimen[17,18,20,24-26,30,31,34,39-41,46,47]. Five of these studies utilised more than one regimen[20,31,34,41,47], allowing 19 comparisons to be made. The relative risk of headache with bismuth or bismuth-containing regimens *versus* comparison regimen was 1.31 (95% CI: 0.81 to 2.11) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.83$).

Metallic taste: Fourteen trials reported the total number of individuals experiencing metallic taste with bismuth or bismuth-containing regimens versus comparison regimen[17,20,24,27,30,34,35,39-42,45,46,48]. Four of these studies utilised more than one regimen[20,34,35,41], allowing 18 comparisons to be made. The relative risk of metallic taste with bismuth or bismuth-containing regimens versus comparison regimen was 1.02 (95% CI: 0.81 to 1.28) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), though the Egger test suggested there was funnel plot asymmetry ($P = 0.01$).

Nausea and/or vomiting: Twenty trials reported the total number of individuals experiencing nausea and/or vomiting with bismuth or bismuth-containing regimens versus comparison regimen[17,18,20,21,24-28,30-32,34,35,39-42,46,47]. Six of these studies utilised more than one regimen[20,31,34,35,41,47], allowing 26 comparisons to be made. The relative risk of nausea and/or vomiting with bismuth or bismuth-containing regimens versus comparison regimen was 1.16 (95% CI: 0.89 to 1.52) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), and the Egger test did not suggest that there was any evidence of funnel plot asymmetry ($P = 0.85$).

Withdrawal of therapy due to adverse events with bismuth or bismuth-containing regimen versus comparison regimen

Twenty-eight trials reported the total number of individuals who terminated therapy due to experiencing adverse events with bismuth or bismuth-containing regimens versus comparison regimen[16-18,20,22-32,34-37,39-43,45-48]. Six of these studies utilised more than one regimen[20,31,34, 35,41,47], allowing 34 comparisons to be made. The relative risk of withdrawal of therapy due to adverse events with bismuth or bismuth-containing regimens versus comparison regimen was 0.86 (95% CI: 0.54 to 1.37) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), though the Egger test suggested that there was evidence of funnel plot asymmetry ($P = 0.05$).

Effect of duration of bismuth therapy on incidence of adverse events

The duration of bismuth therapy was one month or more in eleven of the included studies[7,18,25,31,34,37-39,41,42,47]. There were sufficient trials to pool data to examine the effect of duration of therapy on total number of adverse events, some of the specific individual adverse events (including diarrhoea, headache, and nausea and/or vomiting), and withdrawal of therapy due to adverse events.

Total number of adverse events: Seven trials provided data on total number of adverse events in 945 individuals (467 of whom were assigned to bismuth)[18,25,37-39,42,47], and one study utilised more than one regimen allowing eight comparisons to be made[47]. The relative risk of an adverse event with bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 1.20 (95% CI:1.00 to 1.44), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

Diarrhoea: Nine studies provided data on the incidence of diarrhoea with one month or more of bismuth in 1601 patients (859 of whom were assigned to bismuth)[7,18,25,31,34,39,41,42,47], with five of the studies utilising more than one regimen allowing fourteen comparisons to be made[7,31,34,41,47]. The relative risk of diarrhoea with

bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 1.72 (95% CI: 1.14 to 2.60), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

Headache: Seven studies provided data on the incidence of headache with one month or more of bismuth in 1435 patients (778 of whom were allocated to bismuth)[18,25,31,34,39,41,47], with four of the studies utilising more than one regimen allowing eleven comparisons to be made[31,34,41,47]. The relative risk of headache with bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 1.39 (95% CI: 0.76 to 2.53), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

Nausea and/or vomiting: Eight studies provided data on the incidence of nausea and/or vomiting with one month or more of bismuth in 1501 patients (810 of whom were allocated to bismuth)[18,25,31,34,39,41,42,47], with four of the studies utilising more than one regimen allowing twelve comparisons to be made[31,34,41,47]. The relative risk of nausea and/or vomiting with bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 1.47 (95% CI: 0.87 to 2.48), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

Withdrawal of therapy due to adverse events: Nine studies provided data on the incidence of withdrawal of therapy due to adverse events with one month or more of bismuth in 1554 patients (837 of whom were allocated to bismuth)[18,25,31,34,37,39,41,42,47], with four of the studies utilising more than one regimen allowing thirteen comparisons to be made[31,34,41,47]. The relative risk of withdrawal of therapy due to adverse events with bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 0.86 (95% CI: 0.47 to 1.57), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

DISCUSSION

This is, to our knowledge, the first systematic review and meta-analysis to examine the safety profile of bismuth compounds used either alone or in combination with antibiotics for the treatment of *H pylori* infection, or *H pylori*-related diseases. This information is very important because there have been previous concerns surrounding the issue of potential for toxicity with use of the drug in some countries, particularly in France, where severe neurological adverse events related to the prolonged use of bismuth, given in large quantities, led to the complete withdrawal of all bismuth compounds. This is in contrast to much of the rest of the world, particularly North America, where these drugs are still available without prescription over the counter.

Serfontein *et al*[11], when reviewing blood bismuth levels in patients following administration of therapeutic bismuth formulations, concluded that levels less than 50 μg/mL were highly unlikely to be associated with any meaningful toxicity in man. The authors also reported site-specific toxicity issues depending on whether the complexes of bismuth were water or lipid soluble, the former being associated with renal toxicity, the latter with neurotoxicity. In both cases the doses used and the duration of treatment leading to such adverse effects were much greater than the ones used in the context of *H pylori* eradication therapy. When bismuth-based compounds are used in the treatment of *H pylori* they are usually only given for 1 to 2 wk at low doses, so it would be expected that in this situation the incidence of severe adverse events such as death or neurotoxicity would be lower. These data, with no reported deaths or neurotoxicity in any of the included RCTs, would support this hypothesis. Less serious adverse events are still important though, particularly from the patient's perspective. These may affect compliance with therapy, which is important as successful eradication of *H pylori* is likely to lead to successful ulcer healing and prevention of ulcer relapse[49], and may also improve symptoms in a small but significant proportion of those with functional dyspepsia[50,51]. In a previous analysis of factors that determine the likely success of *H pylori* eradication with bismuth-based triple therapies, patient compliance was shown to be the most important predictor of response[52].

No statistically significant difference was detected in the total number of side-effects between those receiving bismuth-based therapy and comparison regimen in this meta-analysis. In addition, there was no statistically significant difference detected in individual adverse events such as abdominal pain, diarrhoea, dizziness, headache, metallic taste, and nausea and/or vomiting with bismuth compounds versus comparison regimen. Finally, there was no statistically significant increase detected in the number of individuals requiring cessation of therapy as a direct result of adverse events with bismuth-based therapy versus comparison regimen. The number of individuals reporting dark stools with bismuth was significantly higher, though there were fewer studies reporting this adverse event, which probably explains the wider confidence interval. This is unlikely to have any serious consequences related to patient safety, but it is still important to warn patients that this is an expected side-effect of therapy. This observation also has implications for the successful blinding of patients allocated to bismuth therapy in double-blind RCTs.

Total number of side-effects did appear to increase slightly when only those trials that used one month or more of bismuth therapy were included in the meta-analysis, though this did not achieve statistical significance. Diarrhoea was significantly more common with bismuth compounds when only studies using more than one month of therapy were included, but no statistically significant difference was detected in the incidence of other adverse events reported, where there were sufficient trials to examine this issue. Again, there was no increase in cessation of therapy in individuals

assigned to bismuth-based therapy, even if treatment was for one month or more. As mentioned earlier, most current bismuth-based *H pylori* eradication regimens are given for 1 or 2 wk only, so these observations related to longer duration of bismuth therapy are unlikely to have any significant implications in the majority of patients.

The strengths of this systematic review and meta-analysis are that it has been conducted using rigorous methodology and contains a large number of RCTs, which have provided data from in excess of 2000 patients for most of the analyses. In addition, the fact that the data of interest to this meta-analysis were not the primary endpoint of any of the included trials means that the results of the current study are likely to be free from publication bias, as evidenced by the funnel plots for many of the outcomes we assessed. Disadvantages, as with any systematic review, arise from the methodology of the trials included. Many studies were not double-blind and few reported that assessors were blinded to the treatment allocation of the patients either, and this may have led to under-reporting of adverse events in those assigned to "active treatment" with bismuth therapy. Most studies also failed to report either the method of generation of the randomization schedule or the method of concealment of allocation. Finally, only four of the studies used a questionnaire to collect adverse event data, and only two stated that this questionnaire was validated. This may mean that adverse event data were inaccurate in many of the trials and we cannot exclude the possibility that this may have biased the results of the current meta-analysis towards the null hypothesis.

In summary, this systematic review and meta-analysis provides strong evidence that bismuth compounds used either alone, or in combination with antibiotics and acid-suppression therapy, for the treatment of *H pylori* are safe and well-tolerated. The only observation of note was that dark stools were significantly more common in those assigned to bismuth-based therapies.

COMMENTS

Background

Bismuth compounds are often used as part of eradication therapy for *Helicobacter pylori* (*H pylori*). There are concerns about toxicity of these compounds in some countries, particularly as a result of their potential neurological sequelae.

Research frontiers

Data concerning toxic effects of bismuth compounds are mainly derived from studies that have used these compounds at a high dose for a prolonged period of time. We conducted a meta-analysis of adverse events resulting from a 1 to 2-wk course of bismuth based *H pylori* eradication therapy.

Innovation and breakthroughs

The current study demonstrated that bismuth compounds, when used short-term for 1 to 2 wk in *H pylori* eradication therapy, are safe. The only adverse event that occurred more frequently in patients receiving bismuth was dark stools.

Applications

Potential adverse events from bismuth compounds in a 1 to 2-wk course of *H pylori* eradication therapy are now quantified. Gastroenterologists can be assured that these compounds are safe to use.

Terminology

The number needed to harm is the number of patients that would need to be treated with bismuth compounds for one patient to experience an adverse event.

Peer review

There are now problems in obtaining satisfactory eradication rates of *H pylori* with PPI-based triple therapies, so the use of bismuth containing regimens has been recommended as a potential first line therapy in the Maastricht guidelines. Furthermore, there are now new bismuth combinations commercially available. For these reasons it is important to be sure of the safety of bismuth compounds.

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RAPID COMMUNICATION

Changing patterns of hepatitis A prevalence within the Saudi population over the last 18 years

Faleh Al Faleh, Suliman Al Shehri, Saleh Al Ansari, Mohammed Al Jeffri, Yaqoub Al Mazrou, Ahmad Shaffi, Ayman A Abdo

Faleh Al Faleh, Ayman A Abdo, Department of Medicine, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia
Suliman Al Shehri, Saleh Al Ansari, Ministry of Education, Riyadh, Saudi Arabia
Mohammed Al Jeffri, Yaqoub Al Mazrou, Ministry of Health, Riyadh, Saudi Arabia
Ahmad Shaffi, Department of Community Medicine, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia
Author contributions: All authors contributed to the design of the protocol, conduct of the study and writing of the manuscript. Al Faleh F, Al Shehri S, and Abdo AA also did the fieldwork; Shaffi A performed all the statistical analyses.
Supported by Grant Number 113-27-AT ON6/6/2007 from King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia
Correspondence to: Ayman A Abdo, Department of Medicine, College of Medicine, King Saud University, PO Bob 2925(59), Riyadh 11461, Saudi Arabia. aabdo@ksu.edu.sa
Telephone: +966-1-4671215 Fax: +966-1-4671217
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Abstract

AIM: To determine the seroprevalence of Hepatitis A (HAV) amongst Saudi children and compare it with previously reported prevalence data from the same population.

METHODS: A total of 1357 students were randomly selected between the ages of 16 and 18 years (689 males and 668 females) from three different regions of Saudi Arabia (Madinah, Al-Qaseem, and Aseer) and tested for anti-HAV-IgG.

RESULTS: The overall prevalence of anti-HAV-IgG among the study population was 18.6%. There was no difference between males and females but there was a significant difference in the seroprevalence (P = 0.0001) between the three different regions, with Madinah region showing the highest prevalence (27.4%). When classified according to socioeconomic status, lower class students had a prevalence of 36.6%, lower middle class 16.6%, upper middle class 9.6%, and upper class 5.9% (P = 0.0001). Comparing the current study results with those of previous studies in 1989 and 1997 involving the same population, there was a marked reduction in the overall prevalence of HAV from 52% in 1989, to 25% in 1997, to 18.6% in 2008 (P < 0.0001).

CONCLUSION: Over the last 18 years, there has been a marked decline in the prevalence of HAV in Saudi children and adolescents. The current low prevalence rates call for strict adherence to vaccination policies in high-risk patients and raises the question of a universal HAV vaccination program.

Key words: Hepatitis A; Saudi Arabia; Epidemiology; Prevalence; Serology

Peer reviewer: Sammy Saab, MD, MPH, AGAF, Departments of Medicine and Surgery, Pfleger Liver Institute, 200 UCLA Medical Plaza, Suite 214, Box 957302, Los Angeles, CA 90095-7302, United States

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INTRODUCTION

Hepatitis A (HAV) is a major health problem worldwide and, like other enteric infectious diseases, is classically an infection of childhood. Although acute infection commonly passes unnoticed, a significant proportion of patients may have fulminant liver failure, especially patients with liver cirrhosis or immune deficiencies. Generally, its prevalence pattern varies from one population to the other and is closely related to the socioeconomic conditions of sanitation and hygiene. An improvement in sanitation and living standards in many areas of the world has caused the epidemiology of HAV to rapidly evolve. As such, with an improvement in living conditions, more clinical cases are being diagnosed owing to the increased age of those susceptible, which is paradoxical to childhood infection where the majority of infections are subclinical[1-5]. The availability of safe and efficacious vaccines against HAV has made it

increasingly important to understand the epidemiology of HAV in a given area before a strategy for the use of the vaccine is advised or implemented[6-8]. In particular, the epidemiological data from the previous decade may no longer be valid now.

Understanding the epidemiological shift in HAV seropositivity is of strategic importance to a nation's healthcare system. In countries that dramatically improve their socioeconomic status and standards of living, the susceptible pool may increase rapidly to such an extent that HAV becomes a major public health concern. The epidemiology of HAV in developed countries is characterized by low prevalence rates among children with a large group of susceptible adults being negative for anti-HAV.

Two decades ago, studies performed in Saudi Arabia indicated the HAV prevalence rate to be in the range of 90%-100% amongst the adult population[9-13]. However, later studies showed a consistent decline in anti-HAV prevalence rate[14,15]. In the years 1989 and 1997, community-based studies revealed that the overall prevalence rate of HAV infection among children of age 1-12 years had reduced dramatically from 52% (1989) to 25% (1997)[16,17].

We aimed to evaluate the epidemiological shift in HAV serostatus within the adolescent population of three predefined regions of Saudi Arabia and compare it to previously published data.

MATERIALS AND METHODS

Study population

We selected our sample from a population of 10-12th grade school students (corresponding to the age of 16-18 years) in three regions. These regions were as follows: (1) The Aseer region: school population of 25 512, 13 996 males and 11 516 females; (2) Madinah: school population of 23 852, 12 133 males and 11 719 females; and (3) The Al-Qaseem region: school population of 16 067, 7974 males and 8093 females. These regions were selected because they represented low, medium, and high prevalence rates in our previous studies. The sample was selected using a stratified random sampling technique where the Kingdom was stratified into three strata according to the previous endemicity of infection. The proportional allocation method was used to determine the recruited number of students in each stratum. Within the stratum, the sample was proportionally allocated according to gender. In every region, schools served as the sampling units. From the list of schools in the region, one or more male schools and one or more female schools that satisfied the required sample size were randomly selected.

A total of 1357 students (689 males, 668 females) from these three regions of the Kingdom of Saudi Arabia (KSU), were randomly selected. The socioeconomic status of this population was also stratified (low, middle and high class). King Abdulaziz City for Science and Technology approved the protocol of this study, and informed consent was obtained from the parents as well as from the students participating in the study.

Data collection, blood sampling and testing

Fieldwork for this study was undertaken in December 2007 and January 2008. Demographic data were recorded, and a venous blood sample (5-10 mL) was taken from each student. Serum was separated by centrifugation, coded, and stored at -70℃ until tested. Blood samples were tested for anti-HAV-IgG using EIA kits ADVIA Centaur system.

The socioeconomic status of each student was taken to be representative of that of the father and/or the mother and classified using a socioeconomic status 3-point scoring system derived according to the type of house (mud-built = 1, apartment or ordinary house = 2, villa = 3), number of rooms in the house (1-2 rooms = 1, 3-4 rooms = 2, 5 and more = 3), number of family members (4 or less = 3, 5 = 3, 6 = 2, 7 or more = 1), father and mother education (primary grade or less = 1, secondary/high school = 2, university or equivalent = 3), parent occupation (laborer = 1, farmer or office clerk = 2, trader = 3). An overall score of less than 10 from a maximum of 21 was classified as representative of a low socioeconomic status, 10-15 as low middle, 15-17 as high middle and above 17 as high class.

Statistical analyses

Data was entered in MS Excel and analyzed using SPSS Pc+ version 16.0 statistical software. Descriptive statistics (proportional) were used to summarize the categorical variables. χ^2 test followed by analysis of residuals was used to calculate the statistical association between two categorical variables. χ^2 test for trend was used to calculate the significance of proportions of categorical variables at different time points. P value of < 0.05 was considered statistically significant.

RESULTS

The blood samples of 1357 students (aged 16 to 18 years; 689 males and 668 females) were collected and analyzed. The overall prevalence rate of anti-HAV-IgG among the population study was 18.6%.

Association between anti-HAV-IgG values and gender

A significant association between gender and anti-HAV serostatus was seen. The proportion of males who were anti-HAV positive (21%) was significantly higher when compared with females (16.2%) ($P = 0.021$). The adjusted residuals of the frequencies were also statistically significant when compared with the 5% standard normal deviate value (1.96) (Table 1).

Association between anti-HAV-IgG values and regions

A significant association was observed between the area (Aseer, Madinah, and Al-Qaseem) and anti-HAV serostatus. The proportion of subjects from Madinah who were anti-HAV positive (27.4%) was significantly higher compared with samples from other areas (Aseer: 13.5% and Al-Qaseem: 13.9%; $P < 0.0001$). The adjusted residuals of the frequencies of Madinah area (6.2)

Table 1 Prevalence of anti-HAV within the study population and its association with gender

Gender	Anti-HAV (%)		*P* value
	Positive	Negative	
Male (*n* = 689)	145 (21)	544 (79)	0.021
Female (*n* = 668)	108 (16.2)	560 (83.8)	

Table 2 Prevalence of anti-HAV and its association with the socioeconomic status of study population

Socioeconomic status	Anti-HAV(%)		*P* value
	Positive	Negative	
Low class (*n* = 239)	88 (36.8)	151 (63.2)	< 0.0001
Lower middle class (*n* = 880)	146 (16.6)	734 (83.4)	
Upper middle class (*n* = 136)	13 (9.6)	123 (90.4)	
High class (*n* = 102)	6 (5.9)	96 (94.1)	

were also statistically significantly higher when compared with the 5% standard normal deviate value (1.96) (Table 2).

Association between anti-HAV-IgG values and socioeconomic status

A significant association between social status of the sampled population and anti-HAV status was found. The proportion of low class subjects who were anti-HAV positive (36.8%) was significantly higher compared with subjects of other classes (lower middle class: 16.6%; upper middle class: 9.6% and high class: 5.9%; $P < 0.0001$). The adjusted residuals of the frequencies of low class (7.9) were also statistically significantly higher when compared with the 5% standard normal deviate value (1.96) (Table 3).

Comparison of anti-HAV-IgG of previous studies performed in 1989[16] and 1997[17], with the present study

There was a high statistically significant trend for decreased prevalence of HAV infection in all three areas over the three time points. Among the three areas (Al-Qaseem, Aseer and Madinah), the Al-Qaseem area had a significantly decreased prevalence of HAV infection (61.1% in 1989, 31.5% in 1997 and 13.8% in 2007-2008). The decrease in prevalence of HAV infection in Aseer area from 1997 to 2007-2008 was only 5.4% and in Madinah for the same period was only 1.2%, whereas in Al-Qaseem the decrease was 17.7% during the same period (Table 4).

DISCUSSION

The results of this study show a marked decline in the endemicity of HAV within the Saudi population in the age range of 16-18 years. This trend is highlighted by the dramatic linear decline from 53% in 1987 to 25% in 1997 and finally to 18.6% in the present study. Other studies from the region have shown a similar trend.

Table 3 Prevalence of anti-HAV and its association with the region of study population

Region	Anti-HAV(%)		*P* value
	Positive	Negative	
Aseer (*n* = 532)	72 (13.5)	460 (86.5)	< 0.0001
Al-Qaseem (*n* = 332)	46 (13.9)	286 (86.1)	
Madinah (*n* = 493)	135 (27.4)	358 (72.6)	

Table 4 Prevalence of HAV infection in KSA over the last 18 years (1989[16], 1997[17], and 2008)

Region	1989 prevalence (%)	1997 prevalence (%)	2007-2008 prevalence (%)	*P* value
Al-Qaseem	126/201 (61.1)	71/225 (31.5)	46/332 (13.8)	< 0.00001
Aseer	212/476 (44.5)	78/411 (18.9)	72/532 (13.5)	< 0.00001
Madinah	208/350 (59.4)	83/317 (26.2)	135/493 (27.4)	< 0.0001
Total	546/1027 (53.1)	232/953 (24.3)	253/1357 (18.6)	

Al Muneef and colleagues found a hepatitis A seroprevalence of 28.9% in 2399 Saudi children two years ago[18]. However, the present study which was performed on the same population cohort three times over the past 18 years showed a graded but dramatic decline in prevalence. In addition, this study was performed in three areas of different endemicity within the country, representing different levels of socioeconomic development.

HAV endemicity is closely linked to improvements in sanitation and living conditions in the population. In the case of Saudi Arabia, the Saudi government's real-estate bank has helped to build 851 000 housing units through a government loan from 1974 to 2003[19].

According to the official human development report in 2003, the Saudi per Capita GDP increased from 1145 USD in 1970 to 10 853 USD in 2002 and the life expectancy at birth has changed from 53.9 years (1970-1975) to 70.9 in 2000[19].

We believe therefore, that the vast improvement in the socioeconomic status of the Saudi population is the factor most likely to be responsible for this decline. Furthermore, the overall reduction in illiteracy within the Saudi population from greater than 90% in the 1960s to 13.4% in 2007-2008 (7% in males and 19.8% in females)[20] is likely to have contributed to this decline.

A difference was also observed in the prevalence rates of anti-HAV between the three different regions of the KSA in our study, in effect reflecting the different stages of economic development of these regions. The role of socioeconomic status in the study population in determining the level of HAV prevalence was also demonstrated in this study (Table 3) similar to previous publications[16-17].

Previous community-based studies conducted in the Saudi population have shown differing gender-based results in HAV seropositivity. Al-Rashed showed no difference in the anti-HAV prevalence rates between male and female populations[17], while in another study, Khalil *et al*[21] showed a higher seropositivity for Saudi males. The dif-

ference between male and female prevalence rates among this age group is likely to be related to the greater exposure of the male population to HAV infection sources in the community. For instance, the eating habits of the Saudi male population are certainly more gregarious compared to the female population. Similarly, the local culture of less female co-habitation and social interaction may also play a role in reducing their exposure to infection sources.

Finally, this study indicated that more than 82% of the adolescent population of Saudi Arabia is susceptible to symptomatic HAV infection. This could occur either by exposure to infected persons, either where they live or upon travel to high endemic areas either within or outside the country. Outbreaks of symptomatic acute HAV infection have been recently reported within increasing pools of susceptible populations within the country[22-24]. This high susceptibility of the young population represents a continuous challenge to the healthcare system of the country.

Recently, an HAV vaccine has been introduced in many countries as part of an Extended Program of Immunization (EPI). Several studies have demonstrated the efficacy and safety of the vaccine[25-28] and some authorities have recommended its universal implementation in certain populations[29]. Therefore, the recent decision by the Saudi Ministry of Health to introduce the HAV vaccine as part of the EPI program starting from 2008, to children of 18-24 mo of age is certainly timely. The effect of this strategy needs to be studied in future community-based studies, where the results of the present study could well serve as a reference point for comparative analysis.

COMMENTS

Background

Hepatitis A (HAV) is a major health problem worldwide. Generally, its prevalence pattern varies from one population to the other and is closely related to the socioeconomic conditions of sanitation and hygiene. An improvement in sanitation and living standards in many areas of the world has caused the epidemiology of HAV to rapidly evolve.

Research frontiers

This research group took blood samples from school students aged 16-18 years in three different regions of the country after consent of the parents and students. It was found that the prevalence of hepatitis A in this population was 18.6% compared to 25% in 1997 and 52% in 1989. There was also a link between hepatitis A and socioecomonic status with children from a lower socioeconomic status having a higher prevalence.

Innovation and breakthroughs

This study confirmed the findings of other studies in Saudi Arabia and other developing countries showing a reduced rates of hepatitis A with improved socioeconomic status.

Applications

This study is important because it compares current prevalence rates with previous rates from the same community. The reported low rates in the current study calls for strict adherence to vaccination policies in high-risk patients and raises the question of a universal HAV vaccination program.

Peer review

HAV infection is an important topic, and continues to be a source of morbidity and mortality. Al Faleh elegantly describes how the HAV seroprevalance has decreased in Saudi Arabia, and appropriately raises concerns about an increasingly susceptible population.

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RAPID COMMUNICATION

Prevalence of celiac disease in Iranian children with idiopathic short stature

Jalal Hashemi, Eskandar Hajiani, HBB Shahbazin, Rahim Masjedizadeh, Navab Ghasemi

Jalal Hashemi, Eskandar Hajiani, Rahim Masjedizadeh, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran
HBB Shahbazin, Division of Endocrinology and Diabetes center, Department of Internal Medicine, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran
Navab Ghasemi, Department of Internal Medicine, Ahwaz Jundishapur University of Medical Sciences, Golestan Hospital, Ahwaz, Iran
Supported by A Grant from the Ahwaz Jondishapoor University of Medical Science, Ahwaz, Iran
Correspondence to: Eskandar Hajiani, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Ahwaz Jundishapur University of Medical Sciences, Golestan Hospital, PO Box 89, Ahwaz, Iran. ehajiani@ajums.ac.ir
Telephone: +98-611-5530222 Fax: +98-611-3340074
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Abstract

AIM: To determine the prevalence of celiac disease (CD) in children with idiopathic short stature (ISS) and the diagnostic value of immunoglobulin (Ig) A G antigliadin antibodies (AGA) and transglutaminase (TTG) antibodies for CD.

METHODS: A total of 104 children (49 male, 55 female) with ISS without a specific etiology were studied. Extensive endocrine investigations had shown no abnormalities in any subject. Anthropometric parameters and IgA AGA and IgA TTG antibodies were evaluated in this study group. These antibodies were measured by enzyme-linked immunosorbent assay. All patients were referred for an endoscopic intestinal biopsy. The biopsy samples were classified according to revised Marsh criteria (UEGW 2001).

RESULTS: We detected positive IgA TTG antibodies in 36 and IgA AGA in 35 of these patients. Thirty one IgA TTG antibody positive and 28 IgA AGA positive subjects showed histological abnormalities compatible with celiac disease (33.6%). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value for IgA AGA were found to be 80%, 88.4%, 77.8% and 89.7%, respectively. Sensitivity, specificity and PPV for IgA TTG antibodies were 88.6%, 94.2% and 88.6%, respectively.

CONCLUSION: We conclude that the prevalence of celiac disease is high in patients with ISS and it is important to test all children with ISS for celiac disease by measuring serologic markers and performing an intestinal biopsy.

Key words: Celiac disease; Growth disorders; Transglutaminases; Antibodies; Gliadin

Peer reviewer: Ian D Wallace, MD, Shakespeare Specialist Group, 181 Shakesperare Rd, Milford, Auckland 1309, New Zealand

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INTRODUCTION

Short stature is one of the most common causes for referrals to pediatric endocrinologists. Many of these patients have no identifiable medical abnormality and are classified as idiopathic short stature (ISS). For most of these patients, it is believed that genetic variations are the underlying cause. Short stature is a well-known feature of pediatric celiac disease (CD)[1]. In recent studies, CD was considered to be a more common cause of short stature in otherwise healthy children than growth hormone deficiency[2,3]. In other studies, CD has been found without typical gastrointestinal symptoms in some cases of short stature[4-26]. Moreover, some studies suggested an association between CD and growth hormone deficiency[27]. Diagnosis of CD depends on the demonstration of a flat or almost flat jejunal mucosa in biopsy specimens from the small intestine and regeneration of the mucosa after a gluten-free diet[5]. It has been suggested that patients with untreated CD have circulating antibodies against gliadin, and antiendomysium antibodies (anti-EMA) have proven to be a reliable screening test for CD, even in asymptomatic patients[6,7]. The immunofluorescence test is technically difficult to

interpret, with large interobserver variability. In addition, esophageal tissue from monkeys is a common substrate, and the testing is time consuming. Transglutaminase (TTG) antibodies are also highly sensitive and specific and since IgA antibodies to TTG can be examined by enzyme-linked immunosorbent assay (ELISA), they are easier to use as screening antibodies compared with EMA testing[9]. The purpose of the present study was to evaluate prospectively the clinical, laboratory and histologic features of CD and the sensitivity, specificity and positive and negative predictive values (PPV and NPV, respectively) of antibodies against gliadin and TTG in 104 children with a diagnosis of ISS but with no specific etiology.

MATERIALS AND METHODS

A total of 104 children (55 female, 49 male) with ISS and height less than the 2nd percentile adjusted for age and sex, but without specific etiology were enrolled in the study from November 1, 2003 to September 1, 2005 at Ahwaz Jundishapour University Hospitals. Ages ranged from 2 to 18 years. The height, weight and weight for height measurements had been recorded for all patients at presentation and the patients and their parents answered a CD-specific questionnaire used for data collection. All children were being followed at the Department of Endocrinology of Golestan Hospital and had undergone an extensive negative endocrine investigation which included: concentrations of serum electrolytes and glucose, sweat test, total proteins and albumin, determination of immunoglobulin A (IgA), assessment of liver and renal function (determined by standard methods), and hormonal evaluation through the measurement of thyroid-stimulating hormone, free-thyroxin, and growth hormone.

All etiologic factors known to produce growth retardation had also been excluded, e.g, diabetes mellitus, hematological and liver disease, renal failure, fetal growth failure, diseases of bone metabolism, and chromosomal abnormalities. When no cause of the short stature was found, additional investigations were performed by measuring the serum levels of IgA anti-TTG antibodies and IgA antigliadin antibodies (AGA). AGA was measured by a commercial ELISA assay (ELISA-Biosystem, Madrid, Spain). A serum dilution of 1:100 was used and the results were reported in terms of arbitrary units (AU/mL). An IgA AGA $\geqslant$ 20 AU/mL was considered positive. A commercial ELISA. (Orgentec) kit was used to measure anti-TTG antibodies and a titer of more than 1/10 was considered positive. Intestinal biopsies were obtained from all 104 patients with endoscopic grasp forceps (who had negative or positive results for anti-TTG antibody). Four to six biopsy specimens were taken from the second and third parts of the duodenum. Formalin-fixed biopsy specimens stained with hematoxylin and eosin were studied with the use of light microscopy. The slides were examined and confirmed by a pathologist experienced in CD. Mucosal lesions were classified according to the criteria of Marsh[8] as: (1) type 0, normal mucosa, pre-infiltrative lesions; (2) type 1, normal mucosal architecture with epithelial lymphocyte infiltration, infiltrative lesions; (3) type 2, hypertrophic crypts with epithelial lymphocyte infiltration, hyperplastic lesions; and (4) type 3, typical flat mucosa, destructive lesions. The research protocol was reviewed and approved by the Medical Ethics Committee of the Ahwaz Jundishapour University Hospitals. Written informed consent was obtained from the children's parents.

Table 1 The age, weight, height, short stature and BMI of patients (mean ± SD)

Group	Age	Weight	Height	Short stature (*n*)		BMI
				> 2 SD	> 3 SD	
Without CD	16.6 ± 6.5	38.7 ± 13.4	140 ± 17.1	54	15	19 ± 3.5
With CD	16.9 ± 7.1	37.9 ± 13.1	137.6 ± 13.1	30	5	19.1 ± 3.1
Total	16.8 ± 6.7	38.5 ± 13.2	139.2 ± 17.3	84	20	19.1 ± 3.5

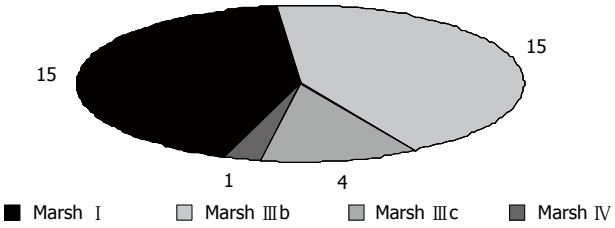

Figure 1 Histological findings of celiac disease.

Statistical analysis

The results are reported as mean ± SD. Statistical analysis was performed by the unpaired Student *t*-test (GraphPad Prism Software Incorporated), with the level of significance set at $P < 0.05$.

RESULTS

The most frequent symptom was diarrhea ($n = 13$) followed by abdominal pain and distention ($n = 3$) in patients with CD and the patients affected by CD did not differ from those without CD in any of the symptoms. A family history of CD was detected in two patients (5.7%). At diagnosis, in the CD patient group, mean weight was 37.9 ± 13.1 and mean height was 137.6 ± 13.1. In this group, short stature of > 2 SD and > 3 SD was found in 30 patients (85.7%) and 5 patients (14.3%), respectively ($P > 0.05$, Table 1).

Small intestine biopsies were performed in all 104 patients with ISS. Duodenal mucosal histopathology was normal in 69 patients. Histopathologic analysis showed evidence of abnormalities compatible with CD in 35 cases (33.6%).

The following histological findings were obtained: (a) 15 of 35 patients had normal mucosal architecture with epithelial lymphocyte infiltration and (b) 15 cases had hypertrophic crypts with epithelial lymphocyte infiltration and partial villous atrophy and (c) five cases showed subtotal or total villous atrophy (Figure 1).

Therefore, the prevalence of properly diagnosed CD

Table 2 Relationship between positive and negative IgA AGA, and IgA TTG antibodies and histological evidence of celiac disease (*n*)

Lab group	IgA AGA		IgA TTG antibodies	
	Positive	Negative	Positive	Negative
Without CD	8	61	4	65
With CD	28	7	31	4
Total	36	68	35	69

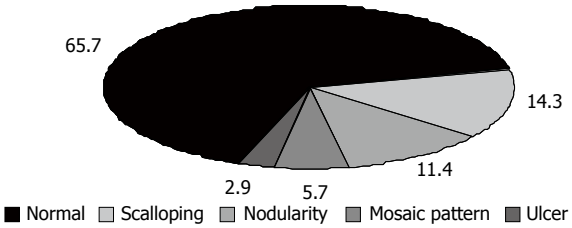

Figure 2 Endoscopic features.

among patients with ISS in this study was 33.6% (35 of 104 patients). IgA AGA, and I IgA TTG antibodies were found in 80% (n = 28), and 88.6% (n = 31) of patients with ISS, respectively. Specificity and the positive predictive value (PPV) for TTG antibodies were found to be 94.2% and 88.6% for CD in the group of patients with ISS in this study. Table 2 shows the relationship between positive and negative IgA AGA, and IgA TTG antibodies and histological evidence of CD. IgA AGA: sensitivity 80%, specificity 88.4%, PPV: 77.8%, negative predictive value (NPV) 89.7%; IgA TTG antibodies: sensitivity 88.6%, specificity 94.2%, PPV 88.6%, NPV 94.2%. The endoscopic features are summarized in Figure 2.

DISCUSSION

Screening for CD in the general population indicates a prevalence of 1:300 to 1:100. About 50% of these children are completely symptomless but because of these figures some experts suggest CD screening for all adults[28] and children[29]. In a British population-based study on short stature, where CD was not specifically investigated, the prevalence of CD was 2:180. In children with short stature and no gastrointestinal symptoms who were investigated for CD, the prevalence increased to 2%-8%. When other (endocrine) causes for short stature are excluded, the prevalence might rise as high as 59%[30,31]. Although CD was once thought to be rare in Iran, several recent reports have cleared this misconception[23,24]. Dr. Shahbazkhani reported that the minimum prevalence of gluten sensitivity among apparently healthy urban Iranian blood donors is 1/166[25]. However, all of these reports deal with typical presentation of CD.

We performed a prospective study on newly diagnosed cases of CD in a group of short stature patients in the south-West of Iran in 2003-2005. Despite the presence of clinical signs of CD during childhood in more than one-third of the patients, the disease remained undiagnosed for many years. This late diagnosis may lead to short stature and low female fertility.

In the last 20 years the clinical picture of CD has changed considerably. The classic form of CD now accounts for a small and systematically shrinking percentage of cases, while atypical forms that present with few or no symptoms are the majority[11]. Short stature is a well-recognized complication of CD[12] although Cacciari *et al*[13] found that adult height is normal in patients who experienced their first symptoms of CD during adulthood. Adult height was shorter only in patients who had symptoms during childhood. This study demonstrated the prevalence and clinical features of CD (n = 35) in a group of 104 patients with ISS. Ages ranged from 2 to 18 years and the mean age of diagnosis was 16.9 years, similar to the results of the study by Mäki *et al*[14]. The age at onset of symptoms appeared to modify the clinical picture. Patients with an earlier onset of CD have a typical clinical picture, whereas patients with delayed onset have atypical presentation, such as short stature.

According to our findings, the prevalence of biopsy-proven CD was 33.6% in the group of ISS children, thereby justifying screening for this disease in all children with short stature. The proportion of CD in cases with ISS ranged from 18.6% to 59.1% in other studies[15,16]. The mechanism of growth retardation is not clearly understood in patients with CD; nutritional deficiencies especially zinc deficiency, low serum somatomedin activity and defects in growth hormone secretion have been proposed as underlying mechanisms[17-19]. An association between CD and autoimmune disorders, such as type I diabetes, autoimmune thyroid disease, and Sjögren's syndrome, has been well documented in the literature[20]. These conditions were not detected in patients in the present study. Susceptibility to CD is determined by genetic factors, which is confirmed by the occurrence of multiple cases of CD in the same family. The prevalence of CD found among first degree relatives is approximately 10%[21]. Screening of siblings in the present study showed that only two siblings (5.7%) had CD. The tests used for CD in this study were IgAAGA and IgA TTG antibodies. The total IgA level was determined also, because CD is associated with IgA deficiency. Antiendomysial antibody and anti-TTG antibodies have been shown to have a high sensitivity and specificity for the diagnosis of CD and correlate well with villous atrophy in untreated patients[32], but false-negative results have been obtained for patients with IgA deficiency[22]. A jejunal biopsy remains the gold standard for the diagnosis of CD but both serologic and histopathologic parameters of CD were investigated in the patients in this study. The sensitivity and specificity of serologic tests were variable. The TTG antibody test has been shown to have a higher sensitivity and specificity for the diagnosis of CD in our patients. Our patients were also tested for IgA deficiency and all were found to have normal IgA values. However, negative results for these tests would not exclude CD. Shamir *et al*[10], using multiple serological strategies

to diagnose silent CD, demonstrated that using any serological marker alone, including EMA antibodies detected by immunofluorescence, would underestimate the prevalence of CD[10]. We found that seven cases with partial villous atrophy had normal AGA, and four of this subgroup had normal anti-TTG antibodies also. Anti-TTG antibodies seem to be more specific but both measurements had limitations in the diagnosis of CD. Our data support the view that there is no single test or measurement that can identify all subjects with CD and ISS. Histological findings of CD showed a spectrum ranging from type 1 mucosal lesions to total villous atrophy type 4 in our study. Fifteen of 35 CD patients (42.9% of CD cases) had mild mucosal abnormality without villous atrophy, 15 (42.9% of CD cases) had partial villous atrophy, four (10.3% of CD cases) had subtotal villous atrophy and one (2.9% of CD cases) had total villous atrophy. According to histological findings, if we limited the diagnosis of CD to cases with villous atrophy, only 20 of 106 ISS cases in our study would have CD (19.3% of all cases) which is obviously more common than the general population.

In conclusion, the possibility of CD should be kept in mind as the prevalence of CD is high in patients with ISS. The patients affected by CD did not differ from those without CD in any of the symptoms. Patients with ISS should be evaluated for CD even in the absence of typical clinical symptoms. It is important to test all children with ISS for CD by measuring anti-EMA IgA or anti-TTG antibodies and performing an intestinal biopsy.

Clinical bottom line: In 33.6% patients with idiopathic short stature, CD may be the underlying cause. Investigation of CD is recommended in the diagnostic assessment of a short child with no endocrinological abnormality.

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COMMENTS

Background

Short stature is one of the most common causes for referrals to pediatric endocrinologists. Many of these patients have no identifiable medical abnormality and are classified as idiopathic short stature (ISS). For most of these patients, it is believed that genetic variations are the underlying cause. Short stature is a well known feature of pediatric celiac disease (CD).

Research frontiers

The prevalence of CD is high in patients with ISS and it is important to test all children with ISS for CD by measuring serologic markers and performing an intestinal biopsy.

Peer review

This is a well written manuscript with a good abstract. In the text they mentioned jejunal biopsies as the gold standard for diagnosis.

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RAPID COMMUNICATION

Gluten sensitive enteropathy in patients with iron deficiency anemia of unknown origin

Farhad Zamani, Mehdi Mohamadnejad, Ramin Shakeri, Afsaneh Amiri, Safa Najafi, Seyed Meysam Alimohamadi, Seyed Mohamad Tavangar, Ardeshir Ghavamzadeh, Reza Malekzadeh

Farhad Zamani, Mehdi Mohamadnejad, Afsaneh Amiri, Gastrointestinal and Liver Disease Research Centre, Iran University of Medical Sciences, Tehran, Iran
Ramin Shakeri, Seyed Meysam Alimohamadi, Reza Malekzadeh, Digestive Disease Research Centre, Tehran University of Medical Sciences, Tehran 14117-13135, Iran
Safa Najafi, Ardeshir Ghavamzadeh, Hematology, Oncology, BMT research Center, Tehran University of Medical Sciences, Tehran 14117-13135, Iran
Seyed Mohamad Tavangar, Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran 14117-13135, Iran
Author contributions: Zamani F and Mohamadnejad M participated in conception, design, undertaking of the study and manuscript writing; Shakeri R and Amiri A participated in data collection, data analysis and manuscript writing; Malekzadeh R participated in conception and experimental design of study. Other authors participated in data collection.
Supported by Local funds from Digestive Disease Research Centre, University of Tehran and Gastrointestinal and Liver Disease Research Centre, Iran University of Medical Science
Correspondence to: Reza Malekzadeh, Professor, Digestive Disease Research Center, Shariati Hospital, North Kargar Ave, Tehran 14117-13135, Iran. malek@ams.ac.ir
Telephone: +98-21-88012992 Fax: +98-21-88012992
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Abstract

AIM: To determine the prevalence of gluten sensitive enteropathy (GSE) in a large group of patients with iron deficiency anemia (IDA) of obscure origin.

METHODS: In this cross-sectional study, patients with IDA of obscure origin were screened for GSE. Anti-endomysial antibody (EMA) and tissue transglutaminase antibody (tTG) levels were evaluated and duodenal biopsies were taken and scored according to the Marsh classification. The diagnosis of GSE was based on a positive serological test and abnormal duodenal histology. Gluten free diet (GFD) was advised for all the GSE patients.

RESULTS: Of the 4120 IDA patients referred to our Hematology departments, 206 (95 male) patients were found to have IDA of obscure origin. Thirty out of 206 patients (14.6%) had GSE. The mean age of GSE patients was 34.6 ± 17.03 (range 10-72 years). The female to male ratio was 1.3:1. Sixteen patients had Marsh 3, 12 had Marsh 2, and 2 had Marsh 1 lesions. The severity of anemia was in parallel with the severity of duodenal lesions. Twenty-two GSE patients (73.3%) had no gastrointestinal symptoms. Fourteen GSE patients who adhered to GFD without receiving iron supplementation agreed to undergo follow up visits. After 6 mo of GFD, their mean hemoglobin levels (Hb) increased from 9.9 ± 1.6 to 12.8 ± 1.0 g/dL ($P < 0.01$). Interestingly, in 6 out of 14 patients who had Marsh 1/2 lesions (e.g. no villous atrophy) on duodenal biopsy, mean Hb increased from 11.0 ± 1.1 to 13.1 ± 1.0 g/dL ($P < 0.01$) while they did not receive any iron supplementation.

CONCLUSION: There is a high prevalence (e.g. 14.6%) of GSE in patients with IDA of obscure origin. Gluten free diet can improve anemia in GSE patients who have mild duodenal lesions without villous atrophy.

Key words: Gluten sensitive enteropathy; Iron deficiency anemia; Anti-Tissue transglutaminase antibody; Anti-endomysial antibody; Gluten free diet

Peer reviewer: Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, University Hospital Heraklion, Department of Gastroenterology, PO Box 1352, 71110 Heraklion, Crete, Greece

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INTRODUCTION

Gluten sensitive enteropathy (GSE) is an autoimmune enteropathy due to food gluten intolerance in genetically predisposed people[1]. While GSE was thought to be a rare disease in the past and was believed to be essentially a disease of Europeans[2-5], recent screening studies showed that GSE is one of the most frequent genetically based diseases which occurs worldwide, with a prevalence ranging from 1:85 to 1:500 in different populations[6-9].

Several categories of GSE have recently emerged, including: monosymptomatic, oligosymptomatic, atypical (without gastrointestinal symptoms), silent, potential and latent form[10,11]. Iron deficiency anemia (IDA) is a commonly observed sign in GSE and is the only abnormality in 40% of patients[12]. In fact, only a minority of GSE patients present with classical malabsorption symptoms of diarrhea and weight loss, whereas most patients have subclinical or silent forms in which IDA can be the sole presentation[13].

In an extensive evaluation of the gastrointestinal tract in patients with IDA in order to identify a source of bleeding, the origin of bleeding cannot be detected in a significant minority of patients. In some of these patients IDA could be the result of diseases that impair iron absorption in the absence of bleeding[14,15]. Gluten sensitive enteropathy is one of these disorders which causes chronic inflammation in the bowel surface, leading to infiltration of T-lymphocytes, hyperplasia of crypts, villous atrophy and reduction of the bowel absorption surface for various nutrients such as iron[16].

Considering the broad spectrum of clinical manifestations of GSE, including anemia, osteoporosis, dermatitis herpetiformis, neurologic disorders and life-threatening complications such as non Hodgkin's lymphoma, small intestinal adenocarcinoma, esophageal cancer, and melanoma, early diagnosis of GSE is essential[17-20].

The present study was conducted to estimate the prevalence of GSE in a large group of patients with IDA of unknown origin by use of two highly sensitive and specific serological tests. We also present the follow-up data of those GSE patients who adhered strictly to a GFD and agreed to undergo follow up visits.

MATERIALS AND METHODS

Subjects

In this prospective study we evaluated all 4120 patients with IDA referred to the Hematology departments of Shariati Hospital, and Firoozgar Hospital from April 2003 to September 2007. Iron deficiency anemia was defined as: hemoglobin concentration less than 13.5 g/dL in men and less than 11.5 g/dL in women; mean corpuscular volume (MCV) less than 80 fl; and ferritin level less than 30 ng/mL.

Methods

Patients were evaluated in 6 steps. In step 1, patients with the following conditions were excluded from the study: known malignancies, hematological diseases (hemolytic anemia, aplastic anemia, thalassemia and myelodysplasia), known chronic diseases (e.g. chronic renal failure, chronic infectious disease, severe cardiac and respiratory disease, collagen vascular disease and chronic liver disease), pregnancy, heavy menstrual flow (cycles $\geqslant$ 7 d), menometrorrhagia, drug addiction, alcoholism, gastric surgery, and obvious blood loss (e.g. melena, hematochezia, hematuria, recurrent epistaxis). In this step 3559 patients were excluded and 561 were entered into the next step.

In step 2, patients were offered the chance to participate in the study, and a questionnaire was completed by each patient. Ninety-four patients declined to enter the study, and 467 patients entered into the next step. Informed consent was obtained from each patient and documented under institutional guidelines and oversight.

In step 3 all patients underwent colonoscopy. Patients with likely sources of blood loss, including any mass lesions, polyps greater than 1.5 cm, five or more vascular ectasias, histologically-proven inflammatory bowel disease, ischemic colitis, or solitary rectal ulcer were excluded. In this step 108 patients were excluded, and 359 patients were entered into the fourth step.

In step 4, all remaining patients underwent upper gastrointestinal endoscopy to exclude sources of blood loss, including varices, peptic ulcer, mass lesions, polyps greater than 1.5 cm in diameter, five or more vascular ectasias, or erosive gastritis. If none of the above lesions were detected, three biopsy specimens were taken from the second part of the duodenum. One hundred and forty seven patients were excluded in step 4.

In step 5, the remaining 212 patients underwent small bowel barium study. Six patients with abnormal small bowel series were excluded in this step.

Thus, from the 4120 patients with IDA who entered the Hematology department, 206 patients were found to have IDA of obscure origin.

In step 6, venous blood samples for tissue transglutaminase antibody (tTG) and endomysial antibody (EMA) were obtained from the 206 remaining patients with IDA of obscure origin. The duodenal biopsy specimens were fixed immediately in formalin solution for 4-8 h at room temperature and were routinely processed for conventional histological evaluation. The biopsy specimens were read by one expert histopathologist and the histological damage of duodenum was expressed based on Modified Marsh classification[3]: 0: Normal mucosal structure without significant lymphocytic infiltration; 1: Lymphocytic enteritis (more than 30 lymphocytes/100 epithelial cells); 2: Lymphocytic enteritis and crypt hyperplasia; 3A: Partial villous atrophy; 3B: Subtotal villous atrophy.

The levels of antibodies against IgA tTG were determined by ELISA using human recombinant tTG as the antigen (Orgentec Diagnostika GmbH, Mainz, Germany). Serum samples were diluted to 1:100 with distilled water, incubated with antigen for 30 min at room temperature, washed three times, and subsequently incubated for another 30 min with antihuman IgA. Optical density was read at 450 nm. Results were expressed in arbitrary units (AU) according to the reference calibrator. The cut-off value for a positive outcome was considered to be 10 AU, according to the instructions on the kit. IgA EMA assay was performed using an indirect immunofluorescence technique. The result was considered positive when bright green reticular fluorescence of smooth muscle was detected by fluorescence microscopy. Total serum IgA was measured in patients with negative tTG and EMA results to exclude IgA deficiency as a cause of false-negative tTG and EMA.

Table 1 Hemoglobin (Hb), Mean Corpuscular Volume (MCV) and Ferritin in GSE patients as compared with other anemic patients

	GSE	Other IDA patients[1]
Hb (g/dL)	9.8 ± 1.7	9.3 ± 2.0
MCV	74.0 ± 9.2	69.1 ± 12.6
Ferritin (ng/mL)	12.4 ± 9.8	11.2 ± 24.2

[1]*P* value was not significant compared to GSE patients (independent *t*-test).

Table 2 Mean hemoglobin level among patients with various degrees of duodenal lesions

MARSH classification	No. of GSE patients	Mean Hemoglobin level
1	2	11.2 ± 1.6
2	12	10.9 ± 1.2[b]
3	16	8.68 ± 1.5[b,d]

[b]*P* < 0.001 compared to Marsh 1 group (independent t-test), [d]*P* < 0.001 compared to Marsh 2 group (independent *t*-test).

The presence of positive tTG or EMA plus abnormal duodenal histology (e.g. Marsh 1, 2 or 3) was defined as gluten sensitivity enteropathy (GSE). All GSE patients were referred to our nutrition clinic and advised to follow a strict gluten free diet, but iron supplementation was withheld. Patients were followed up after 6 mo. Adherence to GFD was assessed in the follow up visit.

Statistical analysis

Data are presented as mean ± SD or percentage. Statistical analysis was performed using SPSS software version 15 and *t*-test for comparison of the means of quantitative variables. $P < 0.05$ was considered statistically significant.

RESULTS

From the 206 patients with IDA of obscure origin, 95 were men with a mean age of 37.6 ± 19.8 years, and 111 were women with a mean age of 39.1 ± 14.4 years.

Serological findings

Serological screening tests showed 31 patients had one or two positive tests. Twenty eight patients had positive tTG, and 23 had positive EMA. In 20 patients both tests were positive. None of the patients with negative serological tests was IgA-deficient.

Biopsy findings

Thirty-eight patients had abnormal duodenal histology. Sixteen patients had Marsh 3, 15 had Marsh 2 and 7 had Marsh 1. Among 38 patients with abnormal duodenal histology, 8 patients (3 with Marsh 2, and 5 with Marsh 1) had negative serologic tests. Eight patients who had abnormal duodenal histology but negative serological tests were not considered to have GSE.

GSE patients

Thirty out of 206 (14.6%) of the patients had GSE. The mean age of these patients was 34.6 ± 17.03 (range 10-72 years). The female/male ratio was 1.3:1. Thirty-one patients were positive for one or two serologic tests, but one of the tTG-positive patients had normal duodenal histology. Among 30 GSE patients, three had negative tTG, and seven had negative EMA. The mean duration of anemia before the diagnosis of GSE was 3.6 ± 1.4 years. These patients had been treated with oral iron for a mean duration of 1.9 years. Anemia improved in only 8 patients (26.8%) treated with oral iron supplementation before GSE diagnosis. Four patients (13.3%) had a family history of prolonged anemia of unknown cause in first degree relatives.

Six patients (20%) mentioned flatulence, two (6.7%) had intermittent diarrhea and one (3.3%) had dermatitis herpetiformis. There were no gastrointestinal symptoms in 22 GSE patients (73.3%).

The mean age of the GSE patients was not significantly different from other IDA of obscure origin patients (34.6 ± 17.0 *vs* 39.3 ± 17.1 years, respectively).

In Table 1, mean Hb, MCV and ferritin in GSE patients are compared with other patients with IDA of obscure origin. There were no statistically significant differences between the patient groups.

In GSE patients, the decrement in hemoglobin level was parallel to the severity of duodenal lesion. Patients with Marsh 3 lesions had more severe anemia (Table 2).

Sensitivity and specificity of the serologic tests

We calculated the sensitivity and specificity of the serological tests based on our definition of GSE (e.g. positive tTG or EMA, plus abnormal duodenal histology). The sensitivity and specificity of IgA tTG-Ab for diagnosing GSE were 90% and 98.5% respectively. Also, the sensitivity and specificity of EMA for diagnosing GSE were 76.6% and 100%, respectively.

Follow up

All the GSE patients were referred to our Nutrition Clinic, and GFD was advised for all of them. Iron supplementation was not started in the patients. The patients were invited for a follow up visit 6 mo after the diagnosis.

Four GSE patients were lost to follow up. Seven patients did not strictly adhere to GFD. For five other patients, iron supplementation was started at other clinics during the follow up period. Thus, we present the follow up data of 14 patients who strictly adhered to GFD and did not use iron supplementation during the 6 mo follow up period.

Mean hemoglobin increased from 9.9 ± 1.6 to 12.8 ± 1.0 g/dL ($P < 0.001$), and mean serum ferritin level increased from 12.0 ± 6.0 to 22.1 ± 7.9 ng/mL.

Interestingly, in 6 patients with Marsh 1/2 lesions (e.g. without villous atrophy) mean Hb increased from 11.0 ± 1.1 to 13.1 ± 1.0 g/dL ($P = 0.002$), and mean serum ferritin level increased from 16.5 ± 4.3 to 25.9 ± 6.2 ng/ml ($P = 0.014$). Demographic and clinical data are presented in Table 3.

Table 3 Demographic and clinical data of the 6 patients with Marsh 1/2 lesions that adhered to GFD

Patient No.	Gender	Age	Marsh classification	Hb level before GFD (g/dL)	Hb level 6 mo after GFD (g/dL)	Ferritin level before GFD (ng/mL)	Ferritin level 6 mo after GFD (ng/mL)
1	Male	29	1	12.8	14.0	19	24.6
2	Male	10	2	10.5	13.5	13.7	34.0
3	Female	17	2	9.5	12.2	20	23.5
4	Male	22	2	11.5	13.0	18	31.0
5	Male	30	2	11.1	14.2	9	16.2
6	Female	38	2	10.4	11.8	19	26.0

DISCUSSION

In this prospective study, we found GSE as the cause of IDA of obscure origin in a significant proportion (14.6%) of patients. Various rates of prevalence of GSE in IDA patients have been reported among different studies[21-24]. This discrepancy may be explained by patient selection criteria in the different studies.

In our study, the prevalence of GSE is amongst the highest rates reported. One reason is that we evaluated GSE among highly selected patients in whom the cause of IDA could not be identified after extensive evaluation. Also, we considered patients with positive serological tests and milder degrees of duodenal mucosal lesions (e.g. Marsh 1 or 2) as having GSE.

Physicians may fail to consider GSE as a cause of IDA when gastrointestinal symptoms are absent or nonspecific. In this study, most GSE patients (73.3%) did not report any gastrointestinal symptoms. In our study, there were no differences in demographic characteristics or hematological indices between GSE patients and other patients with anemia of obscure origin to help to distinguish them (Table 1). In GSE patents, the hemoglobin level was inversely correlated with the severity of the histological injury. Patients with Marsh 3 lesions had the most severe anemia, consistent with the role of impaired intestinal absorption in the pathogenesis of IDA. We found marked improvement of anemia in 14 patients who adhered to GFD but did not use iron supplementation. Many authors consider the presence of villous atrophy (e.g. Marsh 3) as one of the major criteria for diagnosing celiac disease (CD)[25,26]. In order to avoid this controversy in the definition of CD, we used the term "gluten sensitive enteropathy" rather than celiac disease to describe patients with any degree of intestinal damage together with positive serologic tests. In this study, we showed a significant objective improvement in hemoglobin level with GFD alone in patients with positive serology but no villous atrophy (e.g. Marsh 1 or 2). Our study suggests that restriction of the diagnosis of CD to patients with overt villous atrophy will exclude some patients who might benefit from GFD.

In this study, we used a human recombinant protein-based tTG test, which has higher sensitivity and accuracy than a guinea pig protein-based tTG test[27]. However, neither tTG nor EMA was 100% sensitive. We found 7 patients with positive tTG, negative EMA and intestinal damage. On the other hand, we found 3 patients with negative tTG, positive EMA, and duodenal lesions. While some guidelines suggest that either EMA or tTG is sufficient for identifying patients with CD[27,28], our study provides evidence that both tTG and EMA should be used for diagnosing CD.

In this study we did not evaluate GSE in all patients with IDA. One may speculate that some patients who were excluded before step 5, may have had GSE as well as another cause of IDA. The prevalence of GSE in IDA has been reported in previous studies[21-23]. In fact, the aim of our study was to evaluate GSE in a large population of patients with IDA of obscure origin. In a population based study done in Iran, the female to male ratio of GSE was 1 to 1.1[9]. Thus, the female to male ratio found in our study represents the ratio of the disease found in the general population of the country.

In conclusion, celiac disease should be considered in any patient with unexplained IDA, even if they do not have any gastrointestinal symptoms. Furthermore, GFD can improve anemia in IDA patients who have positive tTG/EMA and mild duodenal lesions without villous atrophy.

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COMMENTS

Background

Iron deficiency anemia (IDA) is the only abnormality in 40% of patients diagnosed with gluten sensitive enteropathy (GSE). The majority of patients with GSE don't present with classical malabsorption symptoms such as diarrhea and weight loss. In this study we determined the prevalence of GSE in patients with IDA in Iran.

Research frontiers

Recent screening studies shows that GSE is one of the most commongenetic based diseases which occurs worldwide, with a prevalence of ranging from 1:85 to 1:500 in different populations. Various rates of prevalence of GSE in IDA patients have been reported among different studies probably due to different patient selection criteria in these studies.

Innovation and breakthroughs

Iron deficiency anemia is a common presentation of GSE and can be the sole presentation of the disease. Gluten free diet (GFD) might improve mild duodenal damage (e.g. Marsh 1 or 2) without villous atrophy.

Applications

According to our results, identification of anemic patients with underlying GSE is of great importance. Since IDA can be the sole manifestation of GSE, by diagnosing GSE and giving GFD to patients, we can both prevent the complications of GSE and probably cure IDA without iron supplementation.

Terminology

GSE is an autoimmune enteropathy triggered by the ingestion of gluten-containing grains in susceptible individuals.

Peer review

This is a well-written, well analyzed and scientifically accurate manuscript. The paper is balanced in every part, the reference list is adequate and the conclusions are clear.

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RAPID COMMUNICATION

Inhibitory effect of modified citrus pectin on liver metastases in a mouse colon cancer model

Hai-Ying Liu, Zhi-Liang Huang, Guo-Hua Yang, Wei-Qun Lu, Nan-Rong Yu

Hai-Ying Liu, Zhi-Liang Huang, Guo-Hua Yang, Wei-Qun Lu, Nan-Rong Yu, Department of Abdominal Surgery, Affiliated Tumor Hospital of Guangzhou Medical College, Guangzhou 510095, Guangdong Province, China
Author contributions: Liu HY designed the research; Huang ZL, Yang GH, Lu WQ and Yu NR performed the research; Liu HY and Huang ZL analyzed the data and wrote the paper.
Correspondence to: Hai-Ying Liu, MD, PhD, Department of Abdominal Surgery, Affiliated Tumor Hospital of Guangzhou Medical College, #78 Hengzhigang Road, Guangzhou 510095, Guangdong Province, China. gzdlhy@yahoo.com.cn
Telephone: +86-20-83595032-3129 Fax: +86-20-83598205
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Abstract

AIM: To discuss the expression of glactin-3 in liver metastasis of colon cancer and its inhibition by modified citrus pectin (MCP) in mice.

METHODS: Seventy-five Balb/c mice were randomly divided into negative control group (n = 15), positive control group (n = 15), low MCP concentration group (n = 15), middle MCP concentration group (n = 15) and high MCP concentration group (n = 15). CT26 colon cancer cells were injected into the subcapsule of mouse spleen in positive control group, low, middle and high MCP concentrations groups, except in negative control, to set up a colon cancer liver metastasis model. The concentration of MCP in drinking water was 0.0%, 0.0%, 1.0%, 2.5% and 5.0% (wt/vol), respectively. Liver metastasis of colon cancer was observed after 3 wk. Enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of galectin-3 in serum. Expression of galectin-3 in liver metastasis was detected by immunohistochemistry.

RESULTS: Except for the negative group, the percentage of liver metastasis in the other 4 groups was 100%, 80%, 73.3% and 60%, respectively. The number of liver metastases in high MCP concentration group was significantly less than that in positive control group (P = 0.008). Except for the negative group, the median volume of implanted spleen tumor in the other 4 groups was 1.51 cm^3, 0.93 cm^3, 0.77 cm^3 and 0.70 cm^3, respectively. The volume of implanted tumor in middle and high MCP concentration groups was significantly smaller than that in positive control group (P = 0.019; P = 0.003). The concentration of serum galectin-3 in positive control and MCP treatment groups was significantly higher than that in the negative control group. However, there was no significant difference between them. Except for the negative control group, the expression of galectin-3 in liver metastases of the other 4 groups showed no significant difference.

CONCLUSION: Expression of galetin-3 increases significantly in liver metastasis of colon cancer, which can be effectively inhibited by MCP.

Key words: Pectin; Colonic neoplasms; Metastasis; Liver; Mice

Peer reviewer: Xin-Yuan Guan, Professor, Department of Clinical Oncology, The University of Hong Kong, Room L10-56, 10/F, Laboratory Block, 21 Sassoon Road, Pokfulam, Hong Kong, China

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INTRODUCTION

Liver metastasis is the main cause that impacts the therapeutic effect and postoperative prognosis of colorectal cancer. Inhibiting liver metastasis is beneficial to the therapeutic effect and postoperative prognosis of colorectal cancer[1]. Galectin-3, a carbohydrate-binding protein on tumor cell surface, is closely related to cell to cell adhesion, aggregation of cancer cells *in vitro*, tumor growth and metastasis *in vivo*[2,3]. Galectin-3 is highly expressed in a variety of metastatic cancer cells[4]. Galactosyl, a main component of modified citrus pectin (MCP), can specifically inhibit tumor growth and metastasis *in vivo* and galectin-3-mediated functions *in vitro*[5]. Few studies are available dealing with the inhibitory effects of MCP on cancer metastasis. The aim of this study was to discuss the inhibitory effect of MCP on liver metastasis in a rat colon cancer model.

MATERIALS AND METHODS

Cell lines

Mouse colon adenocarcinoma cell line (CT-26), preserved

and passaged in our biotechnology laboratory, was cultivated in RPMI-1640 culture medium containing 10% new born calf serum, penicillin G and streptomycin at 37℃ in an 5% CO_2 incubator containing 50 mL/L CO_2.

Animals

Seventy-five 6-8 wk old Balb/c female mice, offered by Guangdong Medical Laboratory Animal Center (certification No. 2006A019), weighing 20-25 g, were used in this study. The mice were free from specified-pathogens. Experiments were performed in the SPF Animal Laboratory.

Drugs and reagents

MCP was provided by Centraxinc International, Inc (Francisco, USA). Mouse galectin-3 ELISA kit was provided by R&D Company (Minneapolis, USA). Mouse galectin-3 affinity purified pol was purchased from Jingmei Biotech Co, Lid (Shanghai, China).

Main Equipments

U.S Beecher tissue microarray meter, ST360 auto ELIASA were purchased from Kehua (Shanghai, China).

Establishment of mouse model of liver metastasis of colon cancer

Seventy-five Balb/c mice were randomly divided into negative control group, positive control group, low MCP concentration group, middle MCP concentration group and high MCP concentration group. The concentration of MCP in drinking water was 0.0%, 0.0%, 1.0%, 2.5% and 5.0% (wt/voL), respectively. CT26 cells in exponential growth with sufficient NS were used to mix up into a suspension (1×10^6/mL). The mice were anesthetized with 4% chloral hydrate (10 mL/kg) by injecting into their abdominal cavity and an abdominal wall incision paralleling the left subcostal margin was then made. Laparotomy was performed and 0.05 mL of CT-26 suspension was injected into the spleen. A same volume of NS was injected into the abdominal cavity of mice in the negative control group. The incision was closed with #1 suture. All mice continuously received MCP dissolved in drinking water from the 2nd d after operation, to the necropsy day 21. A same volume of distilled water was given in negative control group. All mice had free access to food and water during the experiment.

Observation

After a 3-wk observation, the eyeball of mice was removed to collect 0.5-1.0 mL peripheral blood. All mice were killed by decapitation. The abdominal cavity was opened to observe primary neoplasms of the spleen and record the volume and number of neoplasms (volume = $ab^2/2$, a = max diameter, b = min diameter). The total volume was recorded if there were more than 2 neoplasms. The number of liver metastases was calculated. All neoplasms were identified with HE staining. Liver metastasis was divided into 4 grades as previously described[6]: grade 0: no liver metastasss; grade Ⅰ: 1-5 liver metastases; grade Ⅱ: 6-10 liver metastases; grade Ⅲ: more than 10 liver metastases.

ELISA analysis of galectin-3

Blood sample was centrifuged at 3000 r/min for 5 min to separate serum. The isolated serum was stored at ≤ -20℃. The serum sample was diluted in a diluent at 1: 20. In brief, 100 μL of a diluent was added into each well of a plate and incubated for 2 h at room temperature, and the plate was washed with a washing buffer. One hundred μL of detection antibody was added into each well of a plate, incubated for 2 h at room temperature, and the plate was washed with a washing buffer. One hundred μL of a working diluent of streptavidin-HRP was added into each well of a plate, incubated for 20 min at room temperature in the dark, the plate was washed. Finally, 100 μL of a substrate solution was added into each well of a plate, incubated for 20 min at room temperature in the dark, and 50 μL of a stop solution was then added into each well of the plate. A microplate reader was used to read the absorbance at 450 nm, then a standard curve was plotted and a formula was used to fit the OD of standard samples.

Liver metastasis tissue microarray

Liver tissue sections were stained with HE to select typical nidi, such as a region rich of neoplasms but lack of necrosed areas and bleeding. A tissue microarray meter was used to perforate into a paraffin block (25 mm × 25 mm × 20 mm). The diameter of each hole was 1.2 mm, and the distance between two holes was 1.0 mm. Fifty holes were arranged in 10 lines and 5 arrays. A 1.2-mm long puncture needle was used to draw out the marked typical tissue core and to transfer it to a certain location on the paraffin block. Forty-seven metastasis samples were arranged into 2 paraffin blocks. Each sample included 2 marked cores. The tissue array paraffin was kept on a 55℃ copper board for 30 min. The paraffin block was pressed gently to array the tissue cores and cooled at room temperature. The arrays were sliced quickly after pre-cooled at 4℃ for 4 h.

Immunohistochemistry analysis

The tissue sample sections were stained with galectin-3 immunohistochemistry following the instructions provided with galectin-3 affinity purified polyclonal antibody. The sections were deparaffined and hydrated. After washed with PBS, the sections were incubated with 3% hydrogen dioxide for 10 min at room temperature, with antibody for 10 min at room temperature, with EnVision for 30 min at room temperature, finally with DAB for color development. The results were judged double-blindly by 2 pathologists. The level of galectin-3 expression was classified into negative (-), weakly positive (+), positive (++) and strong positive (+++) as previously described[7].

Statistical analysis

All the data were analyzed by SPSS10.0 Software. Tumor

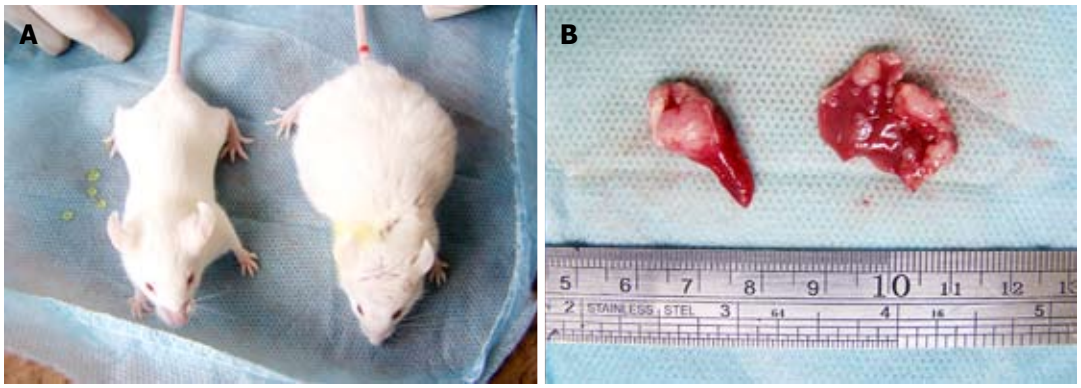

Figure 1 Mouse model of liver metastastic colon cancer. A: Tumor-bearing and healthy mice; B: Primary spleen tumor and liver metastasis.

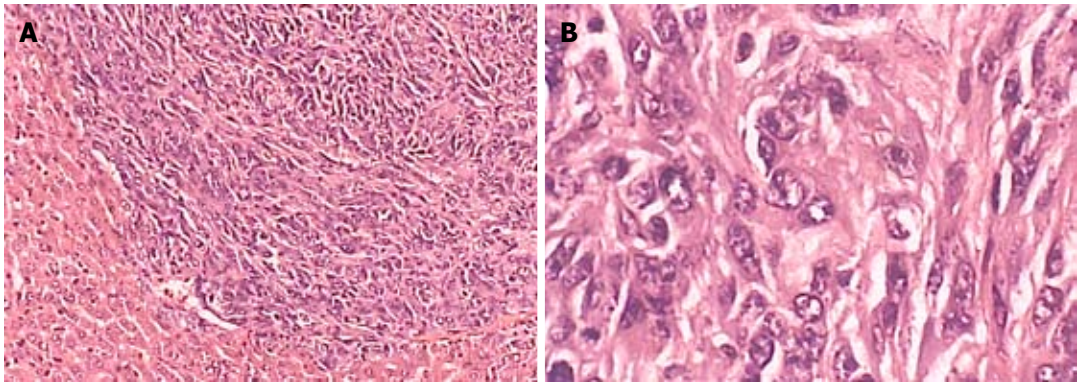

Figure 2 Liver metastastic colon cancer tissue sections stained with HE (A × 200, B × 400).

Table 1 Liver metastases

Groups	*n*	Numbers of liver metastases				χ^2	*P*
		0 (0)	I (1-5)	II (6-10)	III (> 10)		
Positive control group	15	0	2	7	6		
1.0% MCP group	15	3	6	2	4	3.996	> 0.05
2.5% MCP group	15	4	7	1	3	7.069	> 0.05
5.0% MCP group	15	6	4	2	3	8.052	< 0.05[1]

[1]The number of liver metastases in high MCP concentration (5.0%) group was significantly less than that in positive control group ($P = 0.008$).

Table 2 Volume of primary spleen tumor (cm^3)

Groups	*n*	*M*	mean ± SD	χ^2	*P*
Positive control group	15	1.51	1.71 ± 1.29		
1.0% MCP group	15	0.93	1.28 ± 0.68	2.955	> 0.05
2.5% MCP group	15	0.77	0.90 ± 0.55	8.083	< 0.05[1]
5.0% MCP group	15	0.70	0.76 ± 0.30	7.989	< 0.05[1]

[1]The volume of primary spleen tumor in middle and high MCP concentration groups was significantly smaller than that in positive control group ($P = 0.003$).

volume, number of liver metastases, concentration of galectin-3 in serum and tissue were analyzed by non-parametric test.

RESULTS

Mouse living status

No mouse died during the 3-wk experiment period. Some mice were found to have tumor mass bulging on the abdominal wall. Some of the cancer-carrying mice appeared signs of mental depression, such as reduced activity, slow response, gloomy hair color, loss of appetite (Figure 1).

Metastatic liver cancer

Except for the negative control group, the liver metastatic rate for the other 4 groups treated with high, middle and low MCP concentrations was 100%, 80%, 73.3% and 60%, respectively. The number of liver metastases in high MCP concentration group was significantly less than that in low and middle MCP concentration groups ($P < 0.05$) (Table 1).

Volume of primary spleen tumor

The median volume of implanted spleen tumor in high, middle and low MCP concentration groups was 1.51 cm^3, 0.93 cm^3, 0.77 cm^3 and 0.70 cm^3, respectively. No tumor was found in negative control group. The volume of tumor in high MCP concentration group was significantly lower than that in middle and low MCP concentration groups ($P < 0.05$) (Table 2, Figure 2).

Concentration of galectin-3 in serum

The concentration of galectin-3 in serum samples calculated according to the standard regression formula was (14.63 ± 10.08) ng/mL in negative control group, (91.01 ± 22.94) ng/mL in positive control group, (82.75 ± 20.33) ng/mL in low MCP concentration group, (79.01 ± 17.64) ng/mL in middle MCP concentration group and (85.94 ± 15.52) ng/mL in high MCP concentration

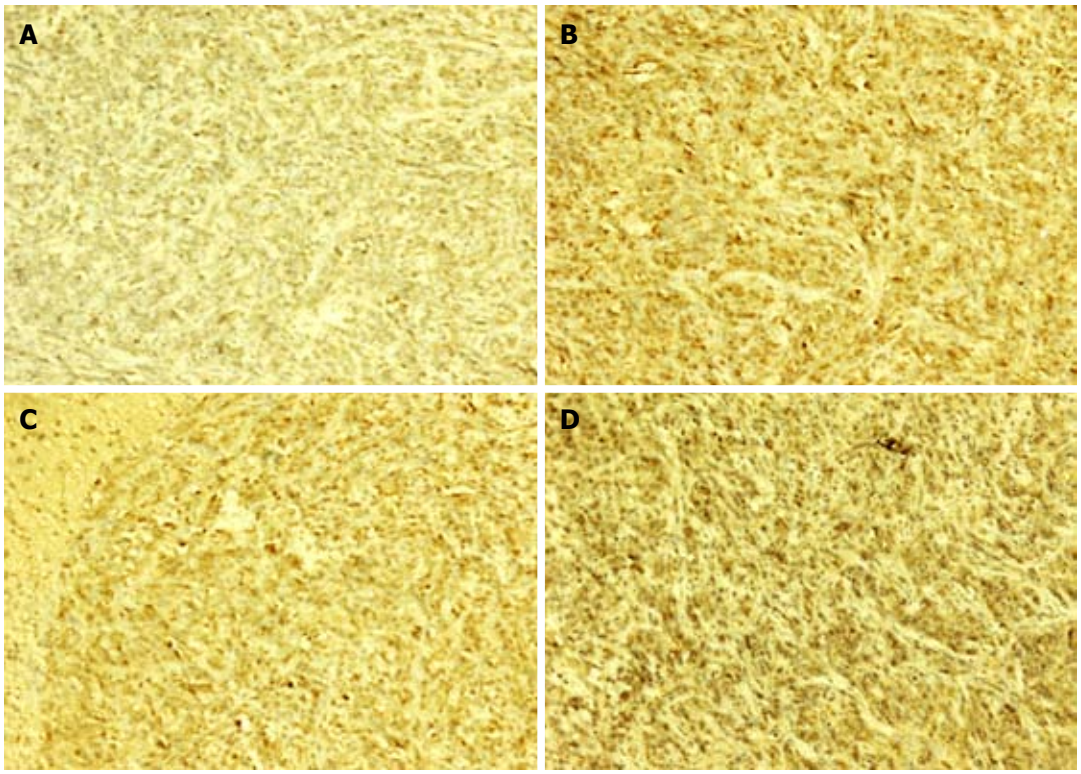

Figure 3 Expression of galectin-3 (× 200) in positive control group (A),1.0% MCP concentration (B), 2.5% MCP concentration group (C), and 5.0% MCP concentration group (D).

Table 3 Serum concentration of galectin-3 (μg/mL)

Groups	*n*	mean ± SD	*H*	*P*
Negative control group	15	14.63 ± 10.08	9.37	< 0.01[1]
Positive control group	15	91.01 ± 12.94		
1.0% MCP group	15	82.75 ± 20.33		
2.5% MCP group	15	79.01 ± 17.64	4.34	
5.0% MCP group	15	85.94 ± 15.52		

[1]The serum concentration of galectin-3 in negatives control group was significantly lower than that in positive control group ($P < 0.01$).

Table 4 Expression of galectin-3 in liver metastasis

Groups	*n*	Expression of galectin-3				*H*	*P*
		(-)	(+)	(++)	(+++)		
Positive control group	15	5	6	2	2	0.52	$P = 0.170$
1.0% MCP group	12	4	3	2	3		
2.5% MCP group	11	2	6	2	1		
5.0% MCP group	9	3	4	1	1		

group, respectively. The results indicate that the concentration of serum galectin-3 in positive control group and MCP treatment groups was significantly higher than that in negative control group ($P < 0.01$, Table 3).

Expression of galectin-3 in liver metastasis

Brown cells in cytolymph under microscope were considered positive cells. The percentage of positive cells in metastatic liver tissue showed that galectin-3 had no significant difference in liver metastases positive control and MCP treatment groups (Figure 3, Table 4).

DISCUSSION

Liver metastasis of colon cancer includes tumor cell infiltration, exfoliation, adhesion, aggregation and invasion, which invole carbohydrate-mediated recognition proteins, such as the galectins. Adhesion of tumor cells to tumor embolus and anchorage of tumor cells to blood vessel endothelium or basement membrane are the two crucial steps of liver metastasis of colon cancer. Different galectins expressed in different steps of metastasis cascade might play a crucial role in tumor progression[8]. Galectin-3, a member of the lectin family, is a multifunctional oncogenic protein which regulates cell growth, adhesion[9], proliferation and apoptosis, as well as cell-cell interaction and angiogenesis[10-13]. A large body of evidence has confirmed that metastatic cancer cells significantly express galectin-3, and high expression of galectin-3 can be detected in both primary and metastatic lesions[14], even in blood[15], showing a strong relation with cancer growth and metastasis[16-18]. Moreover, the expression of galectin-3 can be used as a diagnostic and prognostic marker of colorectal cancer[19-21]. Therefore, if the function of galectin-3 is blocked, the progression of adhesion and aggregation can be intercepted, which may stimulate the development of novel drugs for the targeted treatment of colorectal cancer and other cancers[22].

MCP is a non-digestible, water-soluble polysaccharide fiber derived from citrus fruits, and also a complex polysaccharide rich in galactosyl residues. MCP can specifically inhibit carbohydrate-binding protein as a high affinitive ligand[23]. When the concentration of MCP reaches an adequate level, galectin-3 protein on the surface of cancer cells would be almost completely blocked by MCP molecules. As a result, the procession of adhesion

and aggregation between cancer cells will be intercepted. In addition, MCP can inhibit morphogenesis of endothelial cells and angiogenesis by blocking galectin-3, thus intercepting cancer cells to absorb nutrition from vessels and cancer progression[24,25]. However, there is no evidence that MCP attacks cancer cells directly or indirectly with or without toxicity and side effects[26]. *In vitro* experiments have shown that MCP is able to inhibit adhesion of cancer cells to laminin and homotypic aggregation[27]. Animal experiments also showed that oral MCP can inhibit the growth and metastases of rat prostate cancer cells[28], human breast cancer[5] and melanoma cells[29-31].

The results of our study show that MCP could effetely inhibit the growth and metastasis of implanted colon cancer in mouse spleen. The number of liver metastases and tumor volume in high MCP concentration group were significantly less and smaller than those in control group, indicating that MCP can inhibit the growth and metastasis of colon cancer in a dose-dependent manner, which is consistent with the reported data[5,28-30]. In contrast, low MCP concentration group showed no significant difference in colon cancer growth and liver metastasis, which may be due to the lack of samples and the low sensitivity of non-parametric statistics. Further studies are needed to clarify the role of MCP concentration in this regard.

ELISA and immunohistochemistry analysis have shown that MCP does not impact glactin-3 concentration and expression in liver metastatic cancer cells, but inhibits liver metastasis *in vitro*[30]. The possible mechanism is that MCP only blocks out galectin-3 molecules on the surface of cancer cells, but does not intercept the expression or secretion of cancer cells. It was recently reported that galectin-3 can be used as a reliable diagnostic marker of colorectal cancer and is one of the target proteins in cancer treatment[22].

In conclusion, MCP can effectively inhibit the growth of colon cancer and liver metastasis by intercepting the adhesion and aggregation of cancer cells. MCP, as a natural polysaccharide derived from fruits and a nontoxic drug, may pave a new way in controlling the growth and metastasis of colon cancer and other cancers. The role of MCP and chemotherapy in controlling and curing liver metastatic colon cancer needs further study.

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COMMENTS

Background

Galectin-3 is a carbohydrate-binding protein closely related with cancer growth and metastasis. Studies have shown that galectin-3 is over-expressed in different types of cancer. Dietary components play an important role in cancer progression and metastasis, carbohydrate-mediated recognition processes participate in cancer progression. Modified citrus pectin (MCP), a non-digestible and water-soluble polysaccharide fiber derived from citrus fruits, can inhibit galectin-3-mediated function *in vivo* and *in vitro*.

Research frontiers

The role of galectin-3 in cancer growth and metastasis is an important field in tumor research. Several experimental studies have reported the specific inhibitory effect of MCP on different cancer cells in xenograft models. The present study investigated the effect of MCP on colon cancer growth and liver metastasis *in vivo*. In addition, the expression of galectin-3 was also studied to describe the possible anticancer mechanism of MCP.

Innovations and breakthroughs

Few experiments have been conducted to observe the effect of MCP on preventing cancer growth and metastasis *in vivo*. This study showed the inhibitory effect of MCP on liver metastasis of colon cancer in a mouse model. Besides, the expression of galectin-3 in tumor tissue and serum was tested to describe the galectin-3 status during MCP treatment.

Applications

This study demonstrated that galectin-3 was over-expressed in liver metastasis of colon cancer and oral MC could inhibit cancer growth and metastasis in a mouse model. The results show that galectin-3, as a potential marker and therapeutic target of colorectal cancer, played an important role in prevention and treatment of cancer.

Terminology

Citrus pectin is a complex polysaccharide fiber derived from the pulp and peel of citrus fruits. Citrus pectin is rich in galactosyl, a ligand for galectin-3, when it is modified by high-pH and temperature. MCP inhibits galectin-3 function when citrus fruits are combined with galectin-3.

Peer review

In this manuscript, the authors studied the inhibitory effect of MCP on liver metastasis in a mouse colon cancer model. The study demonstrated that MCP inhibited liver metastasis by suppressing the function of galectin-3. The study is well designed and the results are reliable.

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RAPID COMMUNICATION

Melatonin protects liver from intestine ischemia reperfusion injury in rats

Jian-Yi Li, Hong-Zhuan Yin, Xi Gu, Yong Zhou, Wen-Hai Zhang, Yi-Min Qin

Jian-Yi Li, Xi Gu, Wen-Hai Zhang, The Fifth Department of General Surgery, Shengjing Affiliated Hospital of China Medical University, Shenyang 110004, Liaoning Province, China
Hong-Zhuan Yin, The Second Department of General Surgery, Shengjing Affiliated Hospital of China Medical University, Shenyang 110004, Liaoning Province, China
Yong Zhou, The Sixth Department of General Surgery, Shengjing Affiliated Hospital of China Medical University, Shenyang 110004, Liaoning Province, China
Yi-Min Qin, Department of General Surgery, The First People's Hospital of Shenyang, Shenyang 110004, Liaoning Province, China
Author contributions: Li JY and Zhang WH contributed equally to this work; Li JY, Zhang WH, Yin HZ, Gu X, and Zhou Y designed the research; Li JY, Yin HZ, Gu X, Zhou Y, and Qin YM performed the research; Yin HZ provided the analytic tools; Li JY, Yin HZ, Gu X, and Zhou Y analyzed the data; Li JY, Yin HZ and Zhang WH wrote the paper.
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Correspondence to: Dr. Wen-Hai Zhang, Department Five of General Surgery, The Shengjing Affiliated Hospital of China Medical University, 36 Sanhao Street, Heping District, Shenyang 110004, Liaoning Province, China. zhangwh@sj-hospital.org
Telephone: +86-24-83955232
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Abstract

AIM: To investigate the protective effect of melatonin on liver after intestinal ischemia-reperfusion injury in rats.

METHODS: One hundred and fifty male Wistar rats, weighing 190-210 g, aged 7 wk, were randomly divided into melatonin exposure group, alcohol solvent control group and normal saline control group. Rats in the melatonin exposure group received intraperitoneal (IP) melatonin (20 mg/kg) 30 min before intestinal ischemia-reperfusion (IR), rats in the alcohol solvent control group received the same concentration and volume of alcohol, and rats in the normal saline control group received the same volume of normal saline. Serum samples were collected from each group 0.5, 1, 6, 12, and 24 h after intestinal IR. Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with an auto-biochemical analyzer. Serum TNF-α was tested by enzyme-linked immunosorbent assay (ELISA). Malondialdehyde (MDA) in liver was detected by colorimetric assay. Pathological changes in liver and immunohistochemical straining of ICAM-1 were observed under an optical microscope.

RESULTS: The levels of ALT measured at various time points after intestinal IR in the melatonin exposure group were significantly lower than those in the other two control groups ($P < 0.05$). The serum AST levels 12 and 24 h after intestinal IR and the ICAM-1 levels (%) 6, 12 and 24 h after intestinal IR in the melatonin exposure group were also significantly lower than those in the other two control groups ($P < 0.05$).

CONCLUSION: Exotic melatonin can inhibit the activity of ALT, AST and TNF-α, decrease the accumulation of MDA, and depress the expression of ICAM-1 in liver after intestinal IR injury, thus improving the liver function.

Key words: Melatonin; Intestinal ischemia-reperfusion injury; Liver; TNF-α

Peer reviewer: Adrian G Cummins, MD, Department of Gastroenterology and Hepatology, 28 Woodville Road, Woodville South, 5011, South Australia, Australia

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INTRODUCTION

Bowel transplantation is still the only definite therapy for bowel dysfunction although it is more difficult than other organ transplantations at the final stage of bowel dysfunction[1]. Small intestine with high immunogenicity has the highest reject reaction rate in all organ transplantations[2]. Although more and more powerful nonspecific immunosuppressive agents have been used in recent years, the rate of graft rejecting reaction is still higher than 50% in intestine[3]. Current clinical data show that combined liver and small intestine transplantation decreases both acute and chronic rejection compared with simple small intestine transplantation[4]. Small intestine transplantation, the best therapy for bowel dysfunction at present, cannot replace total parenteral nutrition (TPN) due to its severe rejecting reaction. Long term

TPN therapy may induce liver injury and some patients with intestinal dysfunction can accompany hepatic diseases. Since combined liver and intestine transplantation can prevent further liver injuries[5], systematic research should be done to identify the mechanism of hepatic injury caused by intestinal ischemia-reperfusion (IR) and detect the possible intervention which can lessen the injury. Melatonin can protect liver from intestinal IR injury due to its powerful ability to clear free radicals[6]. The objective of this study was to determine the possible protective effect and mechanisms of melatonin in intestinal IR models and provide evidence for its clinical application in spare-part surgery.

MATERIALS AND METHODS

Materials

One hundred and fifty healthy male Wistar rats, weighing 190 to 210 g, aged 6-7 wk, were randomly allocated into melatonin disposure group, alcohol solvent control group and normal saline (NS) control group. All rats were raised for at least 1 wk before operation in a 12 h dark and 12 h light cycle, with free access to food and water. One gram of melatonin (Sigma Company, USA) was dissolved in alcohol solvent (40%) and kept at a subambient temperature. Rats in the melatonin exposure group received 20 mg/kg intra-peritoneal melatonin diluted by NS to 1/10 of the incipient concentration 30 min before intestinal IR. Rats in the alcohol solvent control group received the same concentration of alcohol and NS. Thiopental sodium (40 mg/kg) was injected into biceps femoris of rats 45 min before the intestinal IR model was established. A model of pan-small intestinal IR injury was established by occlusion of the superior mesenteric artery (SMA) for 30 min. Serum samples were collected from rats in each group after reperfusion for 0.5, 1, 6, 12 and 24 h. A paraformaldehyde (PFA, 40 g/L) phosphate buffer (0.1 mol/L, pH = 7.3) was provided by Laboratory of Shengjing Hospital, China Medical University (Shenyang, China). Correlated biochemical agents were bought from Boehringer Mannheim Company in Germany. MDA kits were purchased from Nanjing Jiancheng Biological Engineering Research Center. TNF-α kit was bought from Shenzhen Jingmei Biological Engineering Limited Company (China). ICAM-1 kit was purchased from Serotech Company in UK. OLYMPUS-BX60 optical microscope and photograph system, HITACHI-7600A automatic biochemistry analyzer and SAKURA paraffin section cutter, were provided by Laboratory of China Medical University (Shenyang, China).

Methods

Blood was obtained by trans-diaphragmatic cardiac puncture, each sample was centrifuged for 5 min at 3000 r/min with the clear supernatant remained. ALT and AST were detected with an automatic biochemistry analyzer. Serum TNF-α was determined by ELISA. MDA in liver tissue homogenate obtained from the central part of medial lobes was assayed by colorimetry. ICAM-1 in liver cells was tested by immunohistochemistry and observed under an optical microscope. Cells (including endotheliocytes in liver sinusoid and hepatocytes) with fine yellow particles in cytoplasm were defined as positive. The number of ICAM-1 positive cells per one high power field was calculated, the mean value was expressed as mean ± SD. All parameters were analyzed by variance analysis and SNK test using SPSS 13.0.

RESULTS

Serum ALT

After reperfusion for 12 and 24 h, the levels of ALT in the exposure group at each time point were significantly lower than those in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between alcohol solvent and NS control groups (Table 1).

Serum AST

After reperfusion for 12 and 24 h, the levels of AST in the melatonin exposure group were significantly lower than those in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between the alcohol solvent and NS control groups (Table 1).

Serum TNF-α

After reperfusion for 12 and 24 h, the levels of TNF-α in the melatonin exposure group were significantly lower than those in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between the alcohol solvent and NS control groups (Table 1).

MDA in liver tissue homogenate

After reperfusion for 6, 12 and 24 h, MDA in the melatonin exposure group was significantly lower than that in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between the alcohol solvent and NS control groups (Table 1).

ICAM-1 stained cells in liver tissue

After reperfusion for 6, 12 and 24 h, the positive rate of ICAM-1-stained cells in the melatonin exposure group was significantly lower than that in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between the alcohol solvent and NS control groups. The positive cells were mainly liver parenchymal cells located near the sinus hepaticus and sinusoid endothelial cells of liver (Figure 1A-F). The number of positive cells in the alcohol solvent and NS control groups increased gradually and reached its peak at 12 h, and then decreased. The similar trend occurred in the melatonin exposure group, but the extent was much lower than that in the other two control groups (Table 1).

DISCUSSION

Serum ALT and AST levels are generally accepted as the most sensitive indexes of acute hepatic injury and AST appears at the later phase[7]. After reperfusion, the ALT levels in the three groups gradually decreased with the time and were lower in the melatonin exposure group than in the other two control groups ($P < 0.05$),

Table 1 ALT, AST, TNF-α, MDA and ICAM-1 in rats with ischemia-reperfusion injury (mean ± SD)

Group	Time (h)	Serum ALT (μkat/L)	Serum AST (μkat/L)	Serum TNF-α (pg/mL)	MDA in liver (nmol/g)	ICAM-1 in liver (%)
Melatonin	0.5	1.8 ± 0.6[a]	1.8 ± 0.6[a]	8.2 ± 2.9	23.7 ± 4.3	2.9 ± 1.2
	1	1.6 ± 0.5[a]	1.6 ± 0.5[a]	10.9 ± 3.0	28.3 ± 5.1	3.0 ± 0.9
	6	1.5 ± 0.5[a]	1.5 ± 0.5[a]	14.8 ± 3.8[a]	42.0 ± 5.2[a]	4.0 ± 1.4[a]
	12	1.4 ± 0.4[a]	1.4 ± 0.4[a]	18.0 ± 3.3[a]	55.2 ± 5.4[a]	11.8 ± 3.3[a]
	24	1.3 ± 0.4[a]	1.3 ± 0.4[a]	15.7 ± 3.3	82.7 ± 6.1[a]	6.9 ± 2.7[a]
Alcohol	0.5	3.1 ± 0.3	3.1 ± 0.3	8.8 ± 2.5	27.7 ± 5.9	2.4 ± 1.3
	1	3.0 ± 0.6	3.0 ± 0.6	11.8 ± 3.7	33.2 ± 6.3	2.5 ± 0.7
	6	2.5 ± 0.4	2.5 ± 0.4	20.9 ± 4.3	54.3 ± 6.5	7.0 ± 2.3
	12	2.4 ± 0.4	2.4 ± 0.4	23.5 ± 4.3	79.1 ± 6.2	22.4 ± 4.3
	24	2.1 ± 0.5	2.1 ± 0.5	17.7 ± 3.9	106.2 ± 7.1	16.0 ± 3.2
NS	0.5	3.3 ± 0.4	3.3 ± 0.4	9.4 ± 2.8	29.5 ± 6.3	2.8 ± 0.8
	1	2.9 ± 0.5	2.9 ± 0.5	11.6 ± 3.3	34.1 ± 5.4	2.6 ± 1.3
	6	2.6 ± 0.4	2.6 ± 0.4	21.4 ± 5.0	53.8 ± 6.1	6.8 ± 2.2
	12	2.5 ± 0.4	2.5 ± 0.4	24.4 ± 4.9	83.3 ± 5.2	21.4 ± 5.4
	24	2.2 ± 0.4	2.2 ± 0.4	16.3 ± 3.6	108.5 ± 9.8	14.3 ± 2.8

[a]$P < 0.05$ *vs* alcohol solvent and NS control groups.

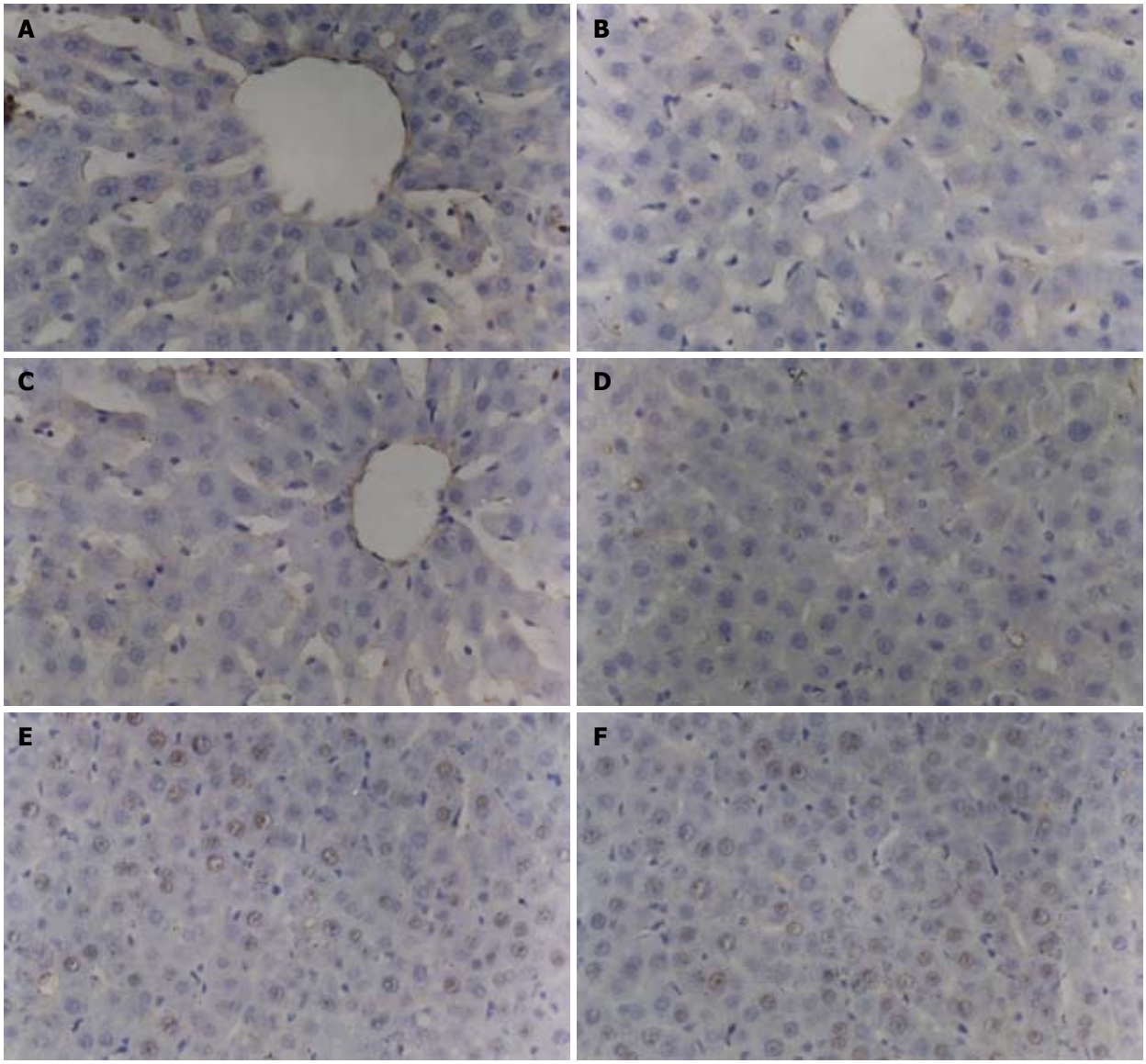

Figure 1 Positive cells are mainly hepatic parenchymal cells located near the sinus hepaticus and sinusoid endothelial cells of liver in melatonin exposure group (A, D), alcohol solvent control group (B, E), NS control group (C, F). The positive cell rate of two control groups increased gradually and reached its peak 12 h after reperfusion, then decreased. This condition also occurred in melatonin exposure group, but the extent was much lower than that in the control groups.

while AST level gradually increased with the time and maintained at its original level in the melatonin exposure group ($P < 0.05$, Table 1). Hepatic injury occurred at the early stage of pan-intestinal IR SMA was blocked for 30 min. The possible reason is that indirect clipping SMA reduced the portal blood flow. The accrescence of ALT indicates the hepatic injury, but the ischemic time is too short to lead to prominent AST changes. After the block of SMA was released, the hepatic injury due to reduced blood flow was ameliorated, but a great amount of active substances, such as free radicals, TNF-α, ICAM-1, *etc*, entered hepatic tissues *via* superior mesenteric and portal veins leading to secondary hepatic injuries, which can explain the variable curves of ALT and AST in the other two control groups. Compared with the other two control groups, exogenous melatonin could ameliorate the hepatic function after intestinal IR injury at the early or late phase. Recent studies about the protective effect of melatonin on IR injury in various organs showed that melatonin can eliminate free radicals, inhibit release of inflammatory media and apoptosis[8-10]. On the other hand, many factors can result in IR injury, such as free radicals[11], overload of calcium[12], inflammation of white blood cells and vascular endothelial cells[13] and apoptosis, *etc*[14]. Therefore, we detected serum TNF-α, MDA and ICAM-1-stained cells in liver tissues in this study. TNF-α gradually increased during the first 12 h after reperfusion and then decreased when it reached its peak between 12 and 24 h in the three groups. However, the TNF-α level was lower in the melatonin exposure group than in the other two control groups 12 and 24 h after reperfusion ($P < 0.05$), suggesting that TNF-α increases in systemic circulation after pan-intestinal IR, which can be effectively inhibited by exogenous melatonin[15-17]. Furthermore, TNF-α is generally accepted as an important inflammatory medium which has the same position as the interleukin family, and participates in almost all inflammatory reactions[18,19]. According to the curves of TNF-α and AST in the first 12 h systemic circulation after reperfusion, the secondary hepatic injury after pan-intestinal reperfusion is related to the concentration of TNF-α in systemic circulation. MDA is a final product of lipid peroxide, and its concentration in tissues can directly reflect the extent of lipid peroxide injury[20]. The concentration of MDA in each group gradually increased after reperfusion, which is generally considered the result of generous release of free radicals and accumulation of lipid peroxide products after pan-intestinal IR[21]. However, the concentration of MDA was obviously lower in the melatonin exposure group than in the other two control groups 6, 12 and 24 h after reperfusion, indicating that melatonin can relieve the IR injury by eliminating free radicals[22]. It was reported that ICAM-1 is directly correlated with the inflammation of white blood cells and vascular endothelial cells[23]. In this study, ICAM-1 gradually increased after reperfusion and decreased after reaching its peak between 12 and 24 h, which was in accordance with the concentration of TNF-α in systemic circulation. However, ICAM-1 was obviously lower in the melatonin exposure group than in the other two control groups. Analysis of the data showed that after clipping SMA for 30 min, (1) the first hepatic injury was relatively mild and reversible while the secondary injury was permanent and severe; (2) a large number of free radicals induced the expression of inflammatory factors such as TNF-α and ICAM-1 leading to hepatic injury through the portal vein, and free radicals directly caused peroxide injury after reperfusion; (3) exogenous melatonin protected liver from intestinal IR injury by inhibiting the production of free radicals, reducing the concentration of TNF-α in systemic circulation, and suppressing the expression of ICAM-1 in liver, *etc*. It has been recently shown that liver is the final metabolic place of melatonin and melatonin protects liver from intestinal IR injury[24-26]. Studies have shown that melatonin has no side effect when a large dose is used[27,28]. Intestine can absorb nutrients and has immune functions, and its vascular anatomy is specifically related with liver, the biggest digestive gland in human body. Since the blood in intestinal vein goes through the portal vein to the liver, intestinal IR inevitably affects the normal physical function of liver. During IR, a great amount of xanthic dehydrogenase in intestinal mucosa would change into xanthic oxydase, and xanthic oxydase can induce the production of free radicals which enhance the expression of adherence factors such as ICAM-1. These adherence factors are the main etiological agents of the secondary hepatic injury[29]. Up to now, rejecting reaction, graft-*versus*-host disease (GVHD) and infection are the restrained factors for intestine transplantation[30].

In conclusion, IR is the key mechanism underlying liver injuries at the early stage of organ transplantation.

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COMMENTS

Background

Common intestinal ischemic diseases, intestinal resection caused by various etiologies, intestinal transplantation due to short bowel syndrome and long-term intravenous nutrition induce various hepatic injuries. On the other hand, combined liver and intestine transplantation has some immune dominance compared with simple intestinal transplantation, and acute or chronic rejecting reaction occurred in combined liver and intestine transplantation obviously decreases. Thus, it is necessary to study the mechanism of hepatic injury caused by intestine ischemia-reperfusion (IR) and discuss the injury relieving therapies.

Research frontiers

Melatonin is an effective free radical scavenger in liver, kidney, pancreas, digestive tract, *etc*. It is generally accepted that melatonin plays a role in protecting liver from intestinal IR injury by eliminating free radicals in cytoplasm. Thus, melatonin secreted by conarium is an endocrine hormone and has a very complicated mechanism of action and its receptors are distributed in almost all human organs. At present, researchers are paying their attention to the modulating function of melatonin in various inflammatory media.

Innovations and breakthroughs

Melatonin interferes with hepatic injury after intestinal reperfusion. The possible protective mechanism of melatonin by producing and releasing inflammatory agents was discussed. Melatonin was found to be able to reduce the concentration of serum TNF-α and inhibit the expression of ICAM-1.

Applications

Exogenous melatonin can protect liver from intestinal IR injury after intestinal

ischemic-reperfusion by eliminating free radicals, reducing the production and release of inflammatory media, *etc*. As an effective and safe clinical medicine, it can be extensively used in surgery.

Terminology

Melatonin is an endocrine hormone secreted by conarium. Endogenous melatonin plays an important role in organic biorhythm, developing cycle, internal environmental stabilization and anti-caducity. TNF-α is an important inflammatory medium and participates in almost all inflammatory reactions. ICAM-1 is a member of the adhesive molecule family and is closely related to adhesion of neutrophile granulocytes and vascular endothelial cells in inflammatory reaction.

Peer review

This is a well written and interesting manuscript. The authors demonstrated that melatonin can protect liver from intestinal IR injury by abating the concentration of serum TNF-α and inhibiting the expression of ICAM-1 in liver cells.

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CASE REPORT

Peutz-Jeghers syndrome with small intestinal malignancy and cervical carcinoma

Lian-Jie Li, Zhi-Qing Wang, Bao-Ping Wu

Lian-Jie Li, Zhi-Qing Wang, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China
Bao-Ping Wu, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China
Author contributions: Wu BP performed the endoscopic examination and the polypectomy; Li LJ and Wang ZQ collected the data; Li LJ wrote the paper.
Correspondence to: Bao-Ping Wu, MD, Huiqiao Building, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China. bpwu@263.net
Telephone: +86-20-61642261 Fax: +86-20-61642494
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Abstract

We report a case of 30-year-old woman with Peutz-Jeghers syndrome (PJS). Because of small intestinal obstruction, she received the small intestinal polypectomy in 2001, and the pathological diagnosis was Peutz-Jeghers polyp canceration (mucinous adenocarcinoma, infiltrating full-thickness of the intestine). The patient did not feel uncomfortable after 6 mo of chemotherapy and other management. We kept a follow-up study on her and found that she suffered from cervical cancer in 2007, with a pathological diagnosis of cervical adenosquamous carcinoma.The patient presented with typical features of PJS, but without a family history. The PJS accompanied with both small intestinal and cervical malignancies has not been reported so far in the world.

Key words: Peutz-Jeghers syndrome; Polypectomy; Small intestine malignancy; Cervix cancer; Multiple organ neoplasms

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INTRODUCTION

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disease characterized by hamartomatous polyps in the gastrointestinal tract and mucocutaneous melanin pigmentation. Patients with PJS are at increased risk for common and unusual types of gastrointestinal and extra-gastrointestinal tumors[1].This report describes the clinicopathological characteristics of PJS complicating multiple organ neoplasms. It provides evidence not only for the risk of malignancy in this disorder, but also for a hamartoma-adenoma-carcinoma sequence.

CASE REPORT

We report here a case of a 30-year old woman who suffered from multiple organ neoplasms with PJS. The melanin pigmentation around lips appeared when she was 3 mo old, after that, melanin pigmentation increased and also appeared on the palm and planta. She presented with iterative abdominal pain and vomit in 1997, and was found having multiple polyps in colon by colonoscopy. The polyps had been resected several times, but the symptoms still existed.

The patient presented to us in April 2001. Physical examination revealed the pigmentation on the oral lips, the buccal mucosa (Figure 1A), and the hands and feet (Figure 1B), about 1-3 mm in diameter. Endoscopy and gastrointestinal tract contrast examination revealed multiple polyps in sinus ventriculi, small intestine and colon. Most polyps were resected and demonstrated hamartomatous polyps, some of which showed adenomatous changes (Figure 2A), and the one in small intestine revealed carcinomatous changes (mucinous adenocarcinoma, infiltrating full-thickness of the intestine) (Figure 2B). The patient received FOLFOX4 chemotherapy after surgery. Tumor markers and routine blood tests were mainly normal during the treatment. The patient gradually recovered, then we kept follow-up visit on her.

In November 2007, the patient complained of abnormal vaginal discharge for 2 mo, without vaginal

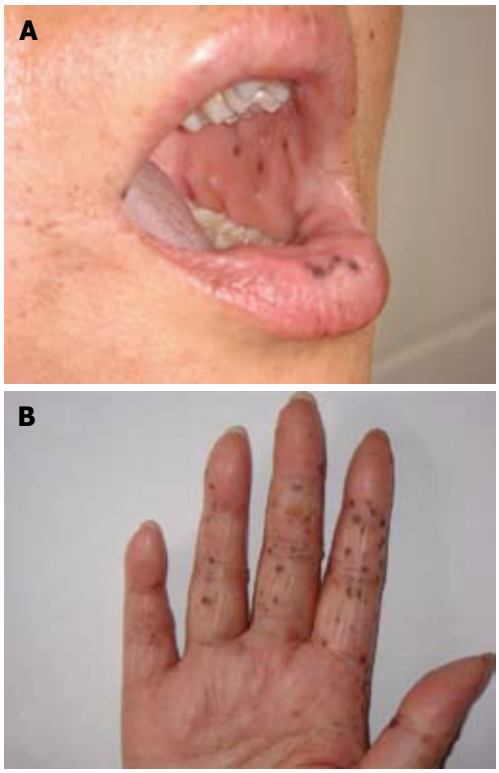

Figure 1 Melanin pigmentation. A: oral lips and buccal mucosa; B: Palm.

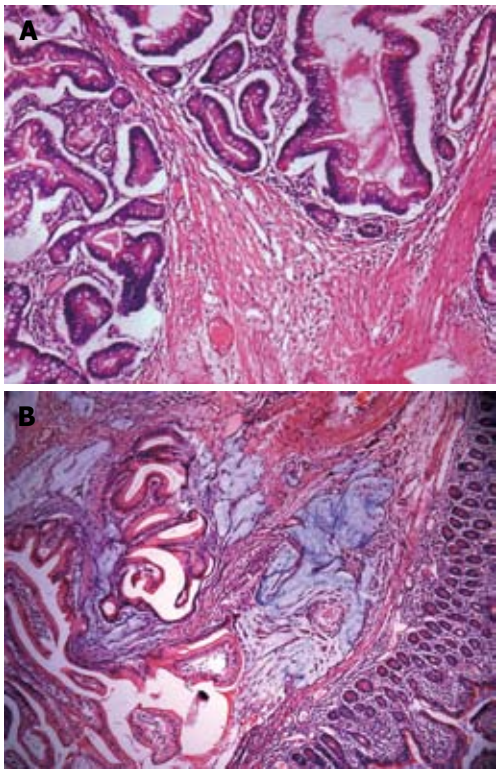

Figure 2 Polyp (HE × 100). A: Adenomatous changes in Peutz-Jeghers polyp; B: Focal mucinous adenocarcinoma in the small intestinal polyp.

bleeding and abdominal pain. Gynecological B-mode ultrasonography found occupying lesion located between inferior segment of cervix and vagina. Gynecologic examination revealed thick cervix, and cervical scraping

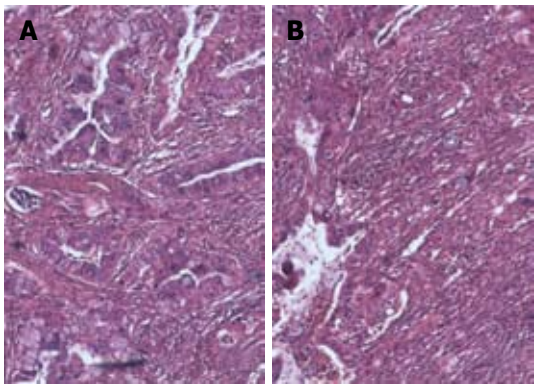

Figure 3 Cervical adenosquamous carcinoma. A: Moderately differentiated adencarcinoma; B: Well-differentiated squamous carcinoma (HE × 100).

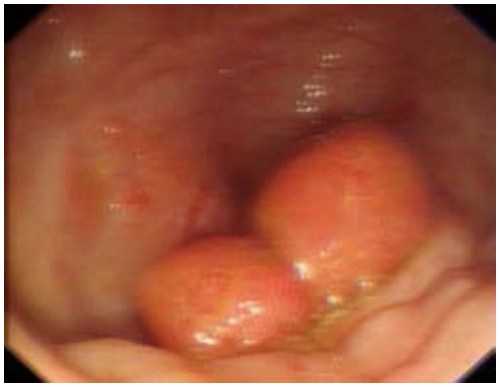

Figure 4 Endoscopic view of multiple polyps in sigmoid colon (12 mm in diameter).

smear confirmed cervical adencarcinoma. After neoadjuvant chemotherapy (Paclitaxel 120 mg/dL, Carboplatin 350 mg/dL, one day per wk for 6 wk), total hysterectomy was performed in February 2008. Final pathological diagnosis was cervical adenosquamous carcinoma (Figure 3), and cancerometastasis of lymph nodes in left external iliac (1/1). The patient also received pelvic radiotherapy after surgery (50 GY in total).

The tumor markers (CA125, CA19-9, CEA) were normal when the patient paid a return visit in May 2008. She was found to have multiple polyps in her stomach, terminal ileum and sigmoid colon by endoscopy (Figure 4), polyps were resected and pathology still confirmed hamartomatous polyps. In addition, none of her lineal relations has the symptoms and physical signs of PJS, and no tumor patient was found in her family history.

DISCUSSION

PJS patients have an increased risk for several malignancies including small intestine, stomach, pancreas, colon, esophagus, ovary, uterus, lung, and breast cancer. PJS is associated with a markedly increased risk of malignancy that is not confined to the gastrointestinal tract. A metaanalysis found that,

compared with the general population, patients with PJS have a relative risk (RR) of 15 times higher than for developing many kinds of cancer. And the cumulative risk for all cancers was 93% from age 15 to 64. Very high RRs for the development of cancer were observed in the small intestine (520), stomach (213), pancreas (132), colon (84) and esophagus (57), and RRs are greater than 10 for the development of breast, lung and ovarian cancer[2]. Several gonadal malignancies occur in PJS patients. In female patients, sex cord tumors with annular tubes (SCTAT) are found in the ovaries of many individuals examined. Patients with these tumors can present with menoxenia, hyperestrogenism or sexual precocity[3]. Minimal deviation of cervical adenocarcinoma has been reported in PJS patients. Presenting symptoms include abnormal vaginal bleeding or a mucoid vaginal discharge. It is an extremely well differentiated adenocarcinoma of the cervix. It usually shows poor malignant behavior and poor prognosis with mucinous type of adenocarcinoma[4].

STK11/LKB1 was identified strongly relative to the PJS, which is a tumor-suppressor involved in intracellular signal transduction and cellular polarity[5]. Some studies provided molecular evidences of a hamartoma-adenoma-carcinoma sequence in PJS. The second hit in *LKB1* causing loss of heterozygosity (LOH) in adenomatous and carcinomatous lesions in PJS polyps was noted by several investigators[6,7]. In addition, LOH of *p53*, *K-Ras* and β-*catenin* mutations were found in adenomas developing in hamartomatous polyps, indicating that molecular alterations in these genes drive carcinogenesis in PJS as well[8]. However, the precise frequency of LOH of *LKB1* in PJS polyps in human remains unclear, and studies in mice showed that loss of the wild-type *LKB1* allele is not a prerequisite for the formation of hamartomatous polyps[9]. Therefore, the need for the second-hit in *LKB1* during polyp development in PJS, and the role of *LKB1* as a typical 'Knudson' two-hit tumor-suppressor gene, is questioned. One theory suggests mucosal prolapse as a pathogenic mechanism underlying the development of typical hamartomatous polyps in PJS. In this hypothesis, PJS hamartomatous polyps represent an epiphenomenon to the cancer-prone condition and the hamartoma-adenoma-carcinoma sequence as such does not exist[10]. The important role of *LKB1* in cellular polarity may provide new insights into the molecular mechanism of polyp and carcinoma development in PJS. Loss of polarity function may also affect asymmetric stem cell division in PJS and lead to expansion of the stem cell pool[11]. It could contribute to polyp formation and explain the increased cancer risk as well. A recent study found *STK11*-deficient mesenchymal cells produced less TGF-β, and defective TGF-β signaling to epithelial cells coincided with epithelial proliferation. TGF-β signaling defects in polyps of individuals with PJS, suggesting that the identified stromal-derived mechanism of tumor suppression is also relevant to PJS[12].

We report the unique case of a patient with the Peutz-Jeghers syndrome who developed intestinal malignancy and cervical cancer in 7 years. As far as we know, no similar case has been reported to date. The pathological changes of polyps would support the development of hamartoma-adenoma-carcinoma; and the carcinomatous change of cervix would add the risk of extra-gastrointestinal tumors in this disorder. However, the pathogenic mechanism of these changes is still unknown. It should be studied progressively on whether germline mutation of *STK11/LKB1* exists or other factors participate in the process of malignant changes. We also suggest that the patient and her family members should be followed up with endoscopy.

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Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
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Deaprtment of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Dr. Simon S Campbell, MD
Department of Gastroenterology, Manchester Royal Infirmary, Oxford Road, Manchester, M12 9WL, United Kingdom

Ravi S Chari, MD, Associate Professor
Division of Hepatobiliary Surgery and Liver Transplantation, Departments of Surgery and Cancer Biology, 1313 21st Avenue South Suite 801 Oxford House, Vanderbilt University Medical Center, Nashville, TN 37232-4753, United States

Dr. Wang-Xue Chen
Institute for Biological Sciences, National Research Concil Canada, 100 Sussex Drive, Room 3100, Ottawa, Ontario K1A 0R6, Canada

Kazuma Fujimoto, Professor
Department of Internal Medicine, Saga Medical School, Nabeshima, Saga, Saga 849-8501, Japan

Khek-Yu Ho, Professor
Department of Medicine, National University Hospital, 119074, Singapore

Richard A Kozarek, MD
Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

Ton Lisman, PhD
Thrombosis and Haemostasis Laboratory, Department of Haematology G.03.550, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Natalia A Osna
Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Avenue, Omaha NE 68105, United States

Maurizio Parola, Professor
Department Medicina e Oncologia Sperimentale University of Torino Corso Raffaello 30, 10125 Torino, Italy

Tomohiko Shimatani, Assistant Professor
Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

Gisela Sparmann, MD
Division of Gastroenterology, Department of Internal Medicine, University of Rostock, Ernst-Heydemann-Str. 6, Rostock D-18057, Germany

Gyongyi Szabo, MD, PhD, Director of Hepatology and Liver Center
University of Massachusetts Medical School, 364 Plantation Street, NRB Floor 2, Room 215, Worcester, Massachusetts 01605-2324, United States

Alastair John Watson, Professor
The Henry Wellcome Laboratory, Nuffield Building, University of Liverpool, Crown St., Liverpool, L69 3GE, United Kingdom

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www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
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June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
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www.czech-hepatology.cz/phm2008

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October 18-22, Vienna, Austria
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November 12, Santiago de Chile, Chile
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May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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