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FIELD OF VISION

Neonatal colon perforation due to anorectal malformations: Can it be avoided?

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Abstract

Anorectal malformations (ARM) are common anomalies in neonates. Diagnostic and therapeutic delays in the management of ARM may lead to colonic perforation, and even death. Physical examination of the perineum is often sufficient to diagnose ARM in neonates. Notwithstanding, delayed diagnosis of ARM has become increasingly familiar to surgeons, as evidenced by the number of recent publications on this topic in the literature. In this commentary, we discuss spontaneous colonic perforation due to delayed diagnosis of ARM in neonates, and highlight the importance of early diagnosis in assuring good outcomes with surgical management. At this point, a thorough examination of the perineum during the initial newborn assessment is mandatory, particularly in those patients presenting with abdominal signs or symptoms.

Key words: Anorectal malformation; Imperforate anus; Bowel perforation; Colon

Core tip: Anorectal malformations (ARM) are common anomalies observed in neonates. The delay in diagnosing a neonate with ARM results in significant complications, occasionally life-threatening morbidity, such as colon perforations. However, delayed diagnosis of ARM seems not the unique factor leading to colonic perforation, deficiency of musculature in the gut wall may also contribute. Colonic perforation due to ARM may not be avoided completely; however, early diagnosis is essential in assuring better outcomes with surgical management.

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COMMENTARY ON HOT TOPICS

We have read with great interest the recent article by Kapadnis *et al* describing a 2.5 kg neonate presenting after 72 h with sigmoid colon perforation due to anorectal malformation (ARM). Delayed diagnosis of ARM has become increasingly familiar to surgeons, as evidenced by the number of recent publications on this topic in the literature[1,2]. Despite the recommendations for peri-natal assessment[3], the overall incidence of a delayed diagnosis has recently been reported to be as high as 21.2%[2]. The delay in diagnosing a neonate with ARM results in significant complications, occasionally life-threatening morbidity, such as colon perforations. Spontaneous perforation of the colon is estimated to occur in 2% of neonates

Table 1 Classification of intestinal perforations complicated with anorectal malformations (n = 25)

Type of perforation	Frequency[1]	Description	Recommended management
Type 1		Perforation occurring before relief of obstruction	
Type 1a	16%	Involving cecum or proximal ascending colon	Cecostomy + distal colostomy
Type 1b	8%	Involving transverse colon including the 2 flexures	Exteriorization of perforation (as colostomy)
Type 1c	60%	Involving distal sigmoid or rectum	Closure of perforation + proximal colostomy
Type 1d	4%	Other sites such as vagina in cloaca	Closure of perforation + proximal colostomy
Type 2	12%	Perforation occurring in the postoperative period	Exteriorization of the perforation site

[1]Calculated by combining the 17 cases reported in the literature and the authors' series.

with ARM, and the incidence rises to 9.5% when the diagnosis is delayed[2]. Thus, it seems crucial to diagnose and treat ARM early to avoid colon perforation.

ARMs are common anomalies observed in neonates[4]. The reported incidence ranges between 1:3300 and 1:5000 live births. In Western countries , there is a male preponderance with 55%-70% of the patients in larger series being males[6]. They vary in severity from mild anal stenosis to complete caudal regression. These disorders usually require surgical intervention in the neonatal period and postoperative follow-up to obtain and maintain fecal and urinary continence. Diagnostic and therapeutic delays in the management of ARM may lead to complications such as sepsis, aspiration, abdominal distension, colonic perforation, respiratory embarrassment, electrolyte imbalance, and even death. The diagnosis of ARM is usually made at birth or shortly thereafter physical examination. Standardized national and international guidelines recommend a routine physical examination of all newborns within the first 48 h of life[3,5]. It has been reported that the median age at diagnosis of perforation in ARM cases was 48 h[6]. Generally, delayed diagnosis of ARM is defined as a diagnosis made after the first 48 h[2]. Undoubtedly, the necessity to diagnose ARM in a timely manner is reliant on a comprehensive neonatal examination performed by a pediatrician or pediatric trainee with sufficient experience. Furthermore, neonatal examination of all newborns should be made within the first 48 h of life. Increasing the awareness among pediatricians of the challenges and complications due to delayed ARM diagnosis may be the important first step. Additional training to adequately diagnose ARM, or change current guidelines to explicitly rule out ARM is also required. Some researchers believe that a higher incidence of associated anomalies may promote earlier diagnosis of the ARM[2], whereas others failed to confirm this hypothesis[7]. Wilson *et al*[7] believed that the only significant predictor of delayed diagnosis of ARM was a failure to receive a comprehensive neonatal examination within 48 h, reiterating that timely diagnosis of ARM is best achieved by adequate clinical examination.

However, colonic perforations cannot be simply attributed to the delayed diagnosis or treatment of ARM, because there are a few case reports of bowel rupture occurring during intrauterine life[8]. Based on their research and review of the literature, Raveenthiran[6] summarized two distinct patterns of perforations involving four different sites and recommended management (Table 1). Approximately 88% of perforations are of type 1, whereas only 12% are of type 2. Among the type 1 perforations, 60% occur in the rectum and sigmoid colon[6]. This difference suggests that the mechanism of perforation could be different for the two types. A higher ratio of rectosigmoid perforation in ARM implies an embryologic origin. As ARM is a developmental field defect, the tail end of the gut can be expected to have deficiency of musculature. The downstream obstruction leads to increased intraluminal pressure, and this, along with the muscular deficiency, is probably responsible for more frequent rupture of the rectum in ARM. Mathur *et al*[9] reported five perforations (6.5%) among 77 cases of ARM with congenital pouch colon (CPC). A high incidence of bowel perforation in CPC also favors the muscular deficiency theory. At this point, delayed diagnosis of ARM seems not the unique factor leading to colonic perforation.

Despite the fact that not all colonic perforations are the result of delayed diagnosis of ARM, the majority are, and early diagnosis is essential so that surgical management can commence to achieve better outcomes. At this point, a thorough examination of the perineum during the initial newborn assessment is mandatory, particularly in those patients presenting with abdominal signs or symptoms.

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REVIEW

Caustic injury of the upper gastrointestinal tract: A comprehensive review

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Abstract

Prevention has a paramount role in reducing the incidence of corrosive ingestion especially in children, yet this goal is far from being reached in developing countries, where such injuries are largely unreported and their true prevalence simply cannot be extrapolated from random articles or personal experience. The specific pathophysiologic mechanisms are becoming better understood and may have a role in the future management and prevention of long-term consequences, such as esophageal strictures. Whereas the mainstay of diagnosis is considered upper gastrointestinal endoscopy, computed tomography and ultrasound are gaining a more significant role, especially in addressing the need for emergency surgery, whose morbidity and mortality remains high even in the best hands. The need to perform emergency surgery has a persistent long-term negative impact both on survival and functional outcome. Medical or endoscopic prevention of stricture is debatable, yet esophageal stents, absorbable or not, show promising data. Dilatation is the first therapeutic option for strictures and bougies should be considered especially for long, multiple and tortuous narrowing. It is crucial to avoid malnutrition, especially in developing countries where management strategies are influenced by malnutrition and poor clinical conditions. Late reconstructive surgery, mainly using colon transposition, offers the best results in referral centers, either in children or adults, but such a difficult surgical procedure is often unavailable in developing countries. Possible late development of esophageal cancer, though probably overemphasized, entails careful and long-term endoscopic screening.

Key words: Caustic ingestion; Corrosive stricture; Developing countries; Surgical management; Endoscopic management

Core tip: The incidence of corrosive ingestion is high and largely unreported in developing countries, where prevention is lacking. Computed tomography and endoscopic ultrasound are gaining a more meaningful role in addressing the need for emergency surgery. The need to perform emergency surgery has a persistent long-term negative impact both on survival and functional outcome. Prevention of stricture is still a debatable issue, yet esophageal stents may offer promising outcomes. It is crucial to avoid malnutrition, especially in developing countries where management strategies are conditioned by poor clinical conditions. Late reconstructive surgery is often unavailable in developing countries.

Contini S, Scarpignato C. Caustic injury of the upper gastrointestinal tract: A comprehensive review. *World J Gastroenterol* 2013; 19(25): 3918-3930 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/3918.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.3918

INTRODUCTION

Ingestion of corrosive substances remain an important

public health issue in Western countries despite education and regulatory efforts to reduce its occurrence. These injuries are still increasing in developing countries[1,2], related to the social, economic, and educational variables and mainly to a lack of prevention[3,4]. The problem is largely unreported in these settings and its true prevalence simply cannot be extrapolated from the scarce papers or personal experience. Data available are heavily skewed towards well-resourced centers and do not mirror the full reality of the condition. Moreover, in industrialized and developing countries, the therapeutic approach and management strategies appear to be different, likely because of technology and endoscopic expertise.

Two independent MEDLINE and EMBASE searches from 1990-2012 were performed to identify relevant articles. The following medical subject headings terms were used in the searches: caustic ingestion, caustic lesions, corrosive injuries, esophagus, esophageal dilatation. Bibliographies of retrieved studies were reviewed and general medical and major gastroenterology journals manually searched over the previous 5 years.

Table 1 Most commonly ingested caustic substances

Caustic substance	Type	Commercially available form
Acids	Sulfuric	Batteries
		Industrial cleaning agents
		Metal plating
	Oxalic	Paint thinners, strippers
		Metal cleaners
	Hydrochloric	Solvents
		Metal cleaners
		Toilet and drain cleaners
		Antirust compounds
	Phosphoric	Toilet cleaners
Alkali	Sodium hydroxide	Drain cleaners
		Home soap manufacturing
	Potassium hydroxide	Oven cleaners
		Washing powders
	Sodium carbonate	Soap manufacturing
		Fruit drying on farms
Ammonia	Commercial ammonia	Household cleaners
	Ammonium hydroxide	Household cleaners
Detergents, bleach	Sodium hypochlorite	Household bleach, cleaners
	Sodium polyphosphate	Industrial detergents
Condy's crystals	Potassium permanganate	Disinfectants, hair dyes

EPIDEMIOLOGY AND PATHOPHYSIOLOGY

Worldwide, children represent 80% of the ingestion injury population globally[5], primarily due to accidental ingestion[6]. In contrast, ingestion in adults is more often suicidal in intent, and is frequently life-threatening.

Traditionally, ingested corrosive substances are either alkalis or acids (Table 1). Alkaline material accounts for most caustic ingestions in Western countries whereas injuries from acid are more common in some developing countries, like India, where hydrochloric acid and sulfuric acid are easily accessible[7]. Acids and alkalis produce different types of tissue damage. Acids cause coagulation necrosis, with eschar formation that may limit substance penetration and injury depth[8]. Conversely, alkalis combine with tissue proteins and cause liquefactive necrosis and saponification, and penetrate deeper into tissues, helped by a higher viscosity and a longer contact time through the esophagus. Additionally, alkali absorption leads to thrombosis in blood vessels, impeding blood flow to already damaged tissue[9]. Injury occurs quickly, depending on the agent's concentration and time of exposure (Figure 1)[10], with a 30% solution of sodium hydroxide being able to produce full thickness injury in 1 s[11]. Accordingly, alkali ingestion may lead to more serious injury and complications, but this distinction is probably not clinically relevant in the setting of strong acid or base ingestion, both being able to penetrate tissues rapidly, potentially leading to full-thickness damage of the esophageal/gastric wall. The conventional acceptance that acids preferentially damage the stomach, due to the protective esophageal eschar, has recently been questioned, with observation of extensive esophageal damage and perforations after acid ingestion[12]. Likewise, compared with alkali, ingestion of a strong acid may be associated with a higher incidence of systemic complications, such as renal failure, liver dysfunction, disseminated intravascular coagulation and hemolysis[13].

Esophageal injury begins within minutes and may persist for hours. Initially, tissue injury is marked by eosinophilic necrosis with swelling and hemorrhagic congestion[9]. Experimental findings suggest that arteriolar and venular thrombosis with consequent ischemia may be more important than inflammation in the pathogenesis of acute corrosive injury[10]. Four to 7 d after ingestion, mucosal sloughing and bacterial invasion are the main findings. At this time granulation tissue appears, and ulcers become covered by fibrin. Perforation may occur during this period if ulceration exceeds the muscle plane. Fibroblasts appear at the injury site around day 4, and around day 5, an "esophageal mold" is formed, consisting of dead cells and secretions. Esophageal repair usually begins on the 10th day after ingestion, whereas esophageal ulcerations begin to epithelialize approximately 1 mo after exposure. The tensile strength of the healing tissue is low during the first 3 wk since collagen deposition may not begin until the second week. Hence, endoscopy (and of course dilatation) is preferably avoided 5-15 d after ingestion[14]. Scar retraction begins by the third week and may continue for several months, resulting in stricture formation and shortening of the involved segment of the gastrointestinal tract. Additionally, lower esophageal sphincter pressure becomes impaired, leading to increased gastroesophageal reflux (GER), which in turn accelerates stricture formation[15]. GER is indeed a likely significant factor in persistent strictures not responding to sequential esophageal dilatations. Esophageal motility studies report low amplitude and nonperistaltic contractions, with a significantly higher exposure to pH below 4, compared with control groups[16]. Therefore, all caustic esophageal burn patients should be screened for GER

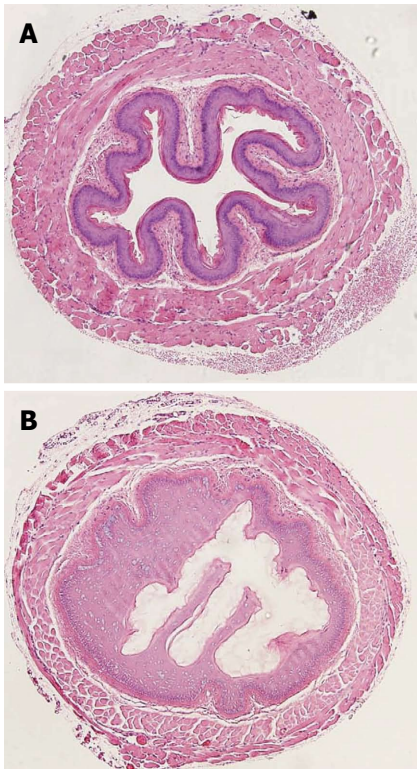

Figure 1 Murine esophagus exposed for 10 min to control (A) and 10% NaOH (B). Reproduced from Osman *et al*[10].

periodically, and GER should be controlled aggressively.

Reactive oxygen species generation with subsequent lipid peroxidation may contribute either to the initial esophageal injury, or to the subsequent stricture formation. Malondialdehyde, an end-product of lipid peroxidation, was found at significantly higher levels than normal in esophageal tissue exposed to sodium hydroxide, signifying the presence of reactive oxygen species at 24 h post exposure. These concentrations remained high for 72 h after exposure compared with no injured controls. Furthermore, significantly lower glutathione concentrations, a known endogenous free-radical scavenger, were found in the same tissues compared with controls, further supporting the presence of reactive oxygen species and free-radical damage[17].

CLINICAL PRESENTATION

Clinical features depend on the type of the substance, amount, physical form and time of presentation (early or delayed). Crystals or solid particles may adhere to the mucosa of the mouth, making them difficult to swallow and thereby diminishing the injury produced to the esophagus, but potentially increasing the damage to the upper airway and pharynx. Conversely, liquids are easily swallowed and are most likely to damage the esophagus and stomach, the extent of injury correlating directly with mortality and late sequelae[18,19]. Patients with oropharyngeal burns do not have significant damage to the esophagus in up to 70%, hence their presence is not a reliable index of esophageal damage[20]. Hoarseness and stridor suggest laryngeal or epiglottic involvement; dysphagia and odynophagia imply esophageal damage while epigastric pain and bleeding are more common in stomach involvement. The absence of pain does not preclude significant gastrointestinal damage. Later changes, such as appearance or worsening of abdominal or chest pain, should be carefully monitored and promptly investigated, since esophageal or gastric perforations can occur at any time during the first 2 wk after ingestion[5].

The relationship between symptoms and severity of injury is uncertain[21]. Stridor and drooling were considered 100% specific for significant esophageal injury[22,23], but no single symptom or symptom cluster can predict the degree of esophageal damage[20,24,25].

The incidence of coexistent gastric injury in the literature ranges from 20.0% to as high as 62.5%[26,27], extending from simple hyperemia/erosions to diffuse transmural necrosis. Delayed gastric emptying with consequent accumulation of food in the stomach (likely due to the contraction of the antropyloric region) may affect the severity of injuries. The most common presentation of an acute corrosive gastric burn is abdominal pain, vomiting, and hematemesis. Rarely, a full thickness burn can cause an immediate gastric perforation, which tends to present a few days after ingestion. Gastric perforation, early or delayed, carries a significant mortality[28], and is more rarely reported in children. Clinical examination and a careful follow-up with a computed tomography (CT) scan are likely more useful than endoscopy in assessing threatened or existing perforation[29]. Bleeding following corrosive ingestion is usually self-limiting: though massive hemorrhage from the stomach or duodenum has been reported a short time after corrosive ingestion[30], severe bleeding typically occurs at 2 wk after ingestion[29].

Respiratory complications from caustic ingestion may result in laryngeal injury and upper airway edema, which ultimately may require tracheotomy[31] and is usually coupled with extensive esophageal damage. Laryngeal injuries were diagnosed by flexible fiberoptic or rigid laryngoscopy in 38% of patients after caustic ingestion, but only few (8%) required immediate intubation and mechanical ventilation for respiratory distress on admission[11]. This low rate of lower airway and pulmonary complications suggests that the protective pharyngeal-glottic mechanism is highly efficient in preventing the caustic substance to reach the lower airway.

EVALUATION AND ASSESSMENT

Laboratory studies

Correlation between laboratory values and the severity/outcome of injury is poor. A high white blood cell count (> 20000 cells/mm^3), elevated serum C-reactive protein, age and the presence of an esophageal ulcer have been considered predictors of mortality in adults[32]; an arterial pH less than 7.22 or a base excess lower than -12 have been considered indication of severe esophageal injury

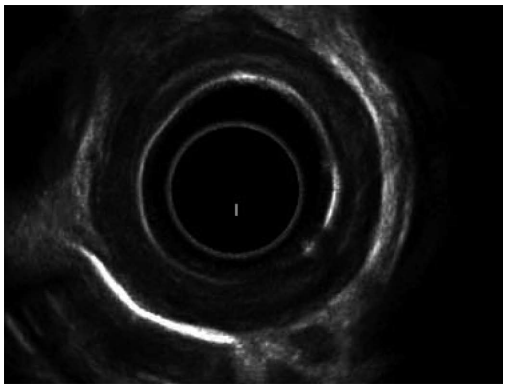

Figure 2 Endoscopic ultrasound showing involvement of the muscularis propria of esophageal wall. Reproduced from Kamijo *et al*[37].

and of emergency surgery[33]. Essentially, laboratory studies are more useful in monitoring and guiding patient management than in predicting morbidity or mortality[34].

Traditional radiology

Shortly after ingestion, a plain chest radiograph may reveal air in the mediastinum suggesting esophageal perforation, as well as free air under the diaphragm, indicating gastric perforation. If it is felt necessary to confirm a clinically suspected perforation, a water-soluble agent, such as Hypaque™ or Gastrografin™, and less irritant than barium sulphate, should probably be used, though both can be equally irritant[35]. Conversely, barium sulfate should be the preferred contrast agent in late barium swallowing, providing greater radiographic details than water-soluble contrast agents[22].

Ultrasounds

Evaluation of esophageal wall caustic damage by endoscopic ultrasound (EUS) using a miniprobe seems safe, though prolongs examination time without showing any difference with endoscopy in predicting early complications[36]. The destruction of the muscular layers of the esophagus observed at EUS seems a reliable sign of future stricture formation[37]; furthermore, ultrasound examination with a radial probe may predict the response to dilatation, which usually requires more sessions when the *muscolaris propria* is involved at EUS, as in Figure 2[38]. In spite of these encouraging reports, the role of US examination in caustic injuries is still under evaluation.

CT scan

A CT scan likely offers a more detailed evaluation than early endoscopy about the transmural damage of esophageal and gastric walls and the extent of necrosis[39]. It is more valuable than endoscopy in assessing threatened or established stomach perforation[29], and a CT grading system (Table 2 and Figure 3) has been proposed to predict esophageal stricture[40,41]. With the advantage of not being invasive, CT scan has a promising role in the early evaluation of caustic injury damage.

Table 2 Computed tomography grading system for caustic lesions

Grade	Features
Grade 1	No definite swelling of esophageal wall
Grade 2	Edematous wall thickening without periesophageal soft tissue involvement
Grade 3	Edematous wall thickening with periesophageal soft tissue infiltration plus well-demarcated tissue interface
Grade 4	Edematous wall thickening with periesophageal soft tissue infiltration plus blurring of tissue interface or localized fluid collection around the esophagus or descending aorta

Reproduced from Ryu *et al*[40].

Endoscopy

Esophagogastroduodenoscopy is considered crucial and usually recommended in the first 12-48 h after caustic ingestion, though it is safe and reliable up to 96 h after the injury[13,42]; gentle insufflation and great caution are mandatory during the procedure. Endoscopy and even dilatation have been performed without consequences from 5 to 15 d after corrosive ingestion[43], though potentially hazardous due to tissue softening and friability during the healing period. Adequate sedation (general anesthesia in children) is compulsory, yet endotracheal intubation is strictly required only for patients in respiratory distress. The constraint to stop the endoscope in the presence of a circumferential second or third degree esophageal burn is not mandatory[44,45].

When lip and oropharyngeal injuries are the main clinical findings, esophageal or gastric injuries are generally no greater than grade 1[46]. Although severe esophageal injuries have been reported in 12.0%[47] and 19.3%[48] of asymptomatic children, significant lesions at endoscopy are not usually observed when symptoms are absent after unintentional ingestion of less aggressive substances[24,49], thus making routine post-ingestion endoscopy questionable in this group of patients. All adult patients must undergo endoscopy after suicidal ingestion, because of the larger amount of more corrosive agents swallowed compared with unintentional injuries, where early esophagoscopy has been questioned[50]. Ultimately, though endoscopy is considered by most a cornerstone in the diagnosis of corrosive ingestions, which patients would clearly benefit from it is still debated. Considering that 10%-30% of caustic ingestions globally do not show any upper gastrointestinal injury[22,51], the indication for early endoscopy should be made on a case-by-case basis, with consideration of symptoms, otorhinolaryngeal injuries, and the amount and nature of the ingested substance.

Contraindications to endoscopy are a radiologic suspicion of perforation or supraglottic or epiglottic burns with edema, which may be a harbinger of airway obstruction, therefore indicating endotracheal intubation or tracheostomy. A third degree burn of the hypopharynx is a further contraindication for endoscopy[22].

Endoscopic classification[8] is important for prognosis and management (Table 3). Generally, grade 0 and 1 le-

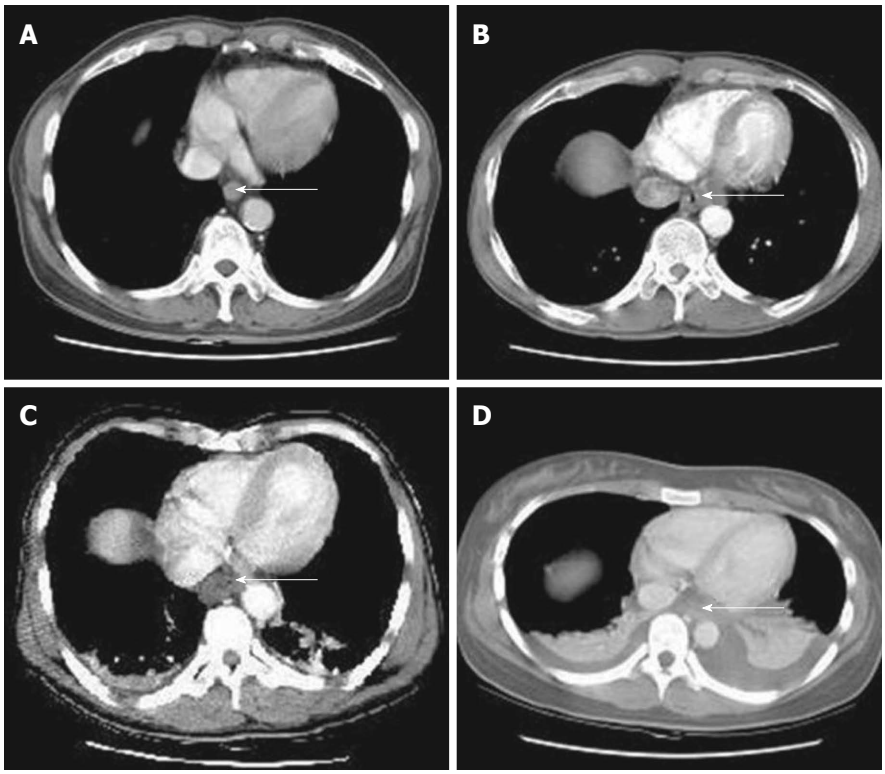

Figure 3 Computed tomography grading of esophageal caustic injuries. A: Grade 1; B: Grade 2; C: Grade 3; D: Grade 4. Reproduced from Ryu *et al*[40]. Arrows show the esophageal wall.

Table 3 Endoscopic classification of caustic injuries

Grade	Features
Grade 0	Normal
Grade 1	Superficial mucosal edema and erythema
Grade 2	Mucosal and submucosal ulcerations
Grade 2A	Superficial ulcerations, erosions, exudates
Grade 2B	Deep discrete or circumferential ulcerations
Grade 3	Transmural ulcerations with necrosis
Grade 3A	Focal necrosis
Grade 3B	Extensive necrosis
Grade 4	Perforations

Reproduced from Zargar *et al*[14].

sions do not develop delayed sequels, such as esophageal strictures or gastric outlet obstruction, whose incidence increases with the severity of the lesion. Additionally, the degree of esophageal injury at endoscopy is an accurate predictor of systemic complications and death, with each increased injury grade correlated with a 9-fold increase in morbidity and mortality[14]. Emergency surgery can be planned according to the endoscopic degree of burn, though an isolated black eschar does not always indicate full-thickness injury and the need for immediate surgical treatment: such patients may deserve further evaluation and careful observation. Recently, some concerns have been raised about the correlation between endoscopic findings and the extent of necrosis[39]: gastrectomy was considered unnecessary at laparotomy in 12% of patients with gastric injuries staged 3b at endoscopy, while the decision to perform esophagectomy based exclusively on endoscopic findings led to unnecessary esophagectomy in 15% of cases[52], suggesting the need for better criteria to improve patient selection for emergency surgery.

MANAGEMENT

Acute management

Immediate treatment is usually conservative, as the definitive extent of the injury is determined within minutes after ingestion. Hemodynamic stabilization and adequacy of the patient's airway are priorities. If the airway is unstable, fiberoptic laryngoscopy allows intubation under direct visualization, avoiding "blind" intubation with the risk of bleeding and additional injuries. In challenging patients, a surgical airway may be required. Gastric lavage and induced emesis are contraindicated for the risk of re-exposure to the corrosive agent and additional injury to the esophagus. The effectiveness of milk and water either as antidotes or to dilute the corrosive agents has never been proven. pH neutralization, with either a weak acid or base, is not recommended for fear of an exothermic reaction, which may increase the damage. Milk and activated charcoal are contraindicated because may obscure subsequent endoscopy. Nasogastric tubes may be applied to prevent vomiting and as stent in severe circumferential burns, but their validity has never be proven. In any case they should not be placed blindly because of the risk of esophageal perforation[53].

To date, the efficacy of proton-pump inhibitors and H_2 blockers in minimizing esophageal injury by suppressing acid reflux has not been proven, though an impressive endoscopic healing after *iv* omeprazole infusion has been observed in a small prospective study[54].

The utility of corticosteroid is controversial. A meta-analysis of studies between 1991 and 2004, and an ad-

ditional analysis of the literature over a longer period from 1956 to 2006 did not find any benefit of steroid administration in terms of stricture prevention. Steroids are usually reserved for patients with symptoms involving the airway[55,56].

The administration of broad-spectrum antibiotics is usually advised mainly if corticosteroids are initiated, as well as if lung involvement is identified[53,57].

Patients whose injuries are graded 1 and 2A are permitted oral intake and discharged within days with antacid therapy. In more severe cases (grade 2 or 3), observation in an intensive care unit and adequate nutritional support is required.

Early surgery

Patients with clinical or radiological evidence of perforation require immediate laparotomy, usually followed by esophagectomy, cervical esophagostomy, frequently concomitant gastrectomy and even more extensive resections, and jejunostomy feeding[58-60]. Some patients without features of perforation at admission may later develop necrosis, perforation and massive bleeding with disastrous results. Indications for emergency surgery rely more often on clinical grounds than on radiological findings; in the presence of doubtful clinical features a decision to perform laparotomy is likely more advantageous for patients than a conservative attitude especially in patients who ingested large amounts of corrosive substances[60].

Laboratory and endoscopic criteria for emergency surgery have been suggested, including disseminated intravascular coagulation, renal failure, acidosis and third degree esophageal burns[58,61]. Unfortunately, these are often late findings and surgery may improve mortality and morbidity in grade 3A injuries only[14].

Severe injuries of the stomach at endoscopy require careful monitoring with a low threshold for laparotomy. At surgery, a gastrotomy allows an accurate evaluation of the extent of damage, since mucosal (and transmural) necrosis may be more extensive than what is apparent from the serosal side. There is no role for procedures such as closure of a perforation. Conservative management of severe gastric injuries at laparotomy, with partial or total conservation of the stomach, has been recently advocated by some in the absence of clinical and biological signs of severity[62].

The need to perform surgery for caustic injuries has a persistent long-term negative impact both on survival and functional outcome. Moreover, esophageal resection *per se*, is an independent negative predictor of survival after emergency surgery[52].

Laparoscopy has been proposed when gastric perforation is highly suspected[63], but the mini-invasive approach has two caveats: unless in very expert hands, it is not a substitute for a comprehensive abdominal exploration, particularly in the posterior aspects of the stomach and duodenum, and it can extend the operative time excessively in a situation where time is a major determinant of outcome. However, it might be considered a useful tool when the stomach cannot be evaluated by endoscopy. Some authors have proposed routine laparoscopic examination in all injuries of second degree or greater[63,64] but the experience is still limited and laparoscopy may be neither feasible nor helpful in such dramatic circumstances.

All injured organs must be resected, if possible, during the first operation. Minimal resection followed by a planned second-look procedure is not recommended. However, secondary extension of caustic burns is unpredictable and re-exploration is indicated when in doubt. An extended resection to adjacent abdominal organs, even the pancreas, does not necessarily carry a prohibitive risk of death in referral centers[60], but an extensive colon resection may compromise future reconstruction and require vascular surgery for atypical transplants. A massive intestinal necrotic injury represents a reasonable limit for resection.

Emergency surgery may be required in the case of severe, uncontrolled late gastric bleeding, usually 1-2 wk after ingestion. Total gastrectomy may be necessary. In duodenal hemorrhages, under-running of the bleeding vessel through a duodenotomy is advised[29].

Acute surgery is quite exceptional in the pediatric population and most authors recommend exhausting all resources to try to preserve the child's native esophagus[25].

Late sequelae

Following a grade 2B and a grade 3 esophageal burn, stricture incidence may be 71%[14] and 100%, respectively[45,53]. Strictures usually develop within 8 wk after the ingestion in 80% of patients, but it can happen as early as after 3 wk or as late as after 1 year. Obviously, ingestion of powerful caustic substances (*e.g.*, sodium hydroxide) is followed by severe, long-standing strictures and dramatically altered esophageal motility[65].

Late sequelae of corrosive gastric injury include intractable pain, gastric outlet obstruction, late achlorhydria, protein-losing gastroenteropathy, mucosal metaplasia and development of carcinoma[66]. Gastric outlet obstruction has an incidence of 5%[67], mainly in the prepyloric area, where prolonged contact with the antral mucosa due to pyloric spasms and to resulting pooling of the caustic agent in this region[55] usually results in stricture in more than 60% of patients[68]. When the volume of the corrosive substance ingested is large, the entire stomach is scarred leading to a diffusely contracted stomach.

Stricture prevention

Steroids: Systemic administration of steroids seems ineffective in preventing strictures[55,56], especially in patients with 3rd degree esophageal burns. Intralesional triamcinolone injections have been proposed to prevent strictures[69], but optimal dose, frequency, and best application techniques are still to be defined[70].

Antibiotics: Though an old study reports a marked decrease in stricture formation with the use of antibiotics[71],

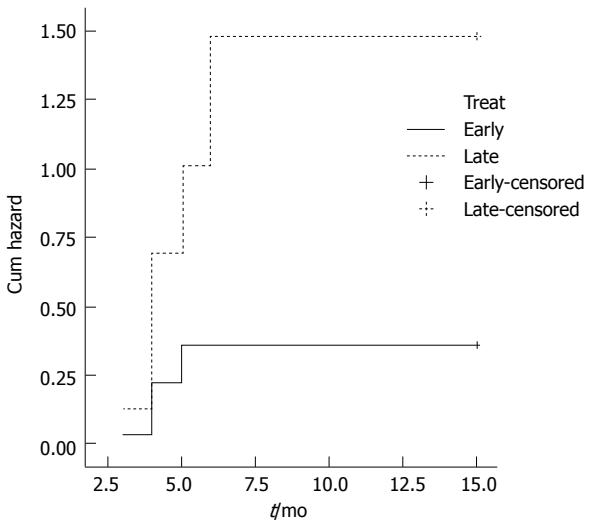

Figure 4 Significantly higher hazard of re-dilatation in patients submitted to late dilatation. P = 0.0008. Reproduced from Contini *et al*[97].

no prospective trial evaluated their utility, and their value in the setting of caustic ingestion, in the absence of concomitant infection, is unknown[18]. There is a consensus that patients treated with steroids should also be treated with antibiotics, but prophylactic antibiotics to prevent strictures, in the absence of steroid therapy, has not been advocated[72].

Nasogastric tube: Though a nasogastric tube may be helpful to ensure patency of the esophageal lumen, the tube itself can contribute to the development of long strictures and routine use is not uniformly recommended[22]. Any esophageal catheterization may be a nidus for infection and nasogastric placement may worsen gastroesophageal reflux in this patient population, with a consequent delay in mucosal healing. However, enteral nutrition through a nasogastric tube has been demonstrated to be as effective as jejunostomy feeding in maintaining nutrition in such patients, with a similar rate of stricture development[73]. Moreover, positioning a nasogastric tube has the advantage of providing a lumen for dilatation should a tight stricture develops. Therefore, after caustic injuries the placement of a nasogastric tube may be considered, but the decision should be made with caution and done on a case-by-case basis.

Mitomycin C: Mitomycin C, a chemotherapeutic agent with DNA crosslinking activity, when injected or applied topically to the esophageal mucosa, may be valuable in preventing strictures, but this drug has deleterious adverse effects, especially if systemic absorption occurs across the intact mucosa[74]. A recent systematic review indicated encouraging results in the long term[75], but prospective studies are clearly mandatory to determine the most effective concentration, duration and frequency of application[76]. The theoretical risk of secondary long-term malignancy should also be taken into account[77].

Intraluminal stent: Specially designed silicone rubber[78] or, more recently, polyflex stents[79] have been found helpful in preventing stricture formation but the efficacy is less than 50%, with a high migration rate (25%). Patient selection remains a challenge and the development of hyperplastic tissue is a concern. Home-made polytetrafluoroethylene stents have shown promising results with a 72% efficacy[80] at 9-14 mo, similar to home-made silicone stents positioned by endoscopy[81] or through laparotomy[82] for 4-6 mo. Biodegradable stents (poly-*L*-lactide or polydioxanone) are under evaluation for benign strictures[83,84], with a 45% success rate at 53 mo in a patient population with only two caustic strictures, a migration rate of around 10%, and a significant hyperplastic tissue response. Experimentally, biodegradable stents were not able to prevent strictures in pigs after circumferential submucosal resection[85]. Moreover, cost and minimal experience in caustic strictures make the use of biodegradable devices questionable, especially in developing countries.

Other modalities for stricture prevention under evaluation: Intraperitoneal injection of 5-fluorouracil has been effective in preventing strictures experimentally[86]. Anti-oxidant treatment (vitamin E, H_1 blocker, mast cell stabilizer, methylprednisolone) and phosphatidylcoline[87,88] inhibit collagen production and stricture formation by decreasing tissue hydroxyproline, the ultimate product of collagen degradation, but no human study is available. Octreotide and interferon-alfa-2b have been shown in animals to depress the fibrotic activity in the second phase of wound healing of the esophageal wall after a corrosive burn[89]. Cytokines have also been used experimentally with success to prevent stricture formation[90]. Until now, none of the above approaches, albeit appealing, has been tested in humans.

Stricture management

Endoscopic dilatation: Timely evaluation and dilatation of the stricture play a central role in achieving a good outcome[91]. Late management is usually associated with marked esophageal wall fibrosis and collagen deposition[5], which makes dilatation more complex. Maximal esophageal wall thickness, observed at CT scan, was associated with a higher number of sessions required for adequate dilatation[92], and recurrent strictures were significantly more frequent after delayed dilatation (Figure 4)[93-95]. Moreover, delayed presentation and treatment have been found to be strong predictors of future esophageal replacement[96]. This issue, which may entail different management strategies[3] for early or late patients, may be crucial in developing countries, where late presentations are more than 50%[2,97,98].

Dilatation can be carried out with balloon or bougies (usually Savary) without a clear advantage for each method[70]. However, the failure rate after pneumatic dilatation is higher in caustic ingestion-related strictures than in other benign strictures[99]; Savary bougies are considered more reliable than balloon dilators in consoli-

dated and fibrotic strictures such as old caustic stenosis or in long, tortuous strictures[100,101], and may offer the operator the advantage of feeling the dilatation occurring under his hands[102]. Dilatation should be avoided from 7 to 21 d after ingestion for the risk of perforation, though early, prophylactic dilatation with bougienage has been reported to be safe and effective even in this period[43]. The perforation rate after dilatation of benign esophageal strictures varies between 0.1% and 0.4%[70], but for caustic strictures it fluctuates from 0.4% to 32.0%, dropping from 17.6% to 4.5% with increased experience[103]. The 5%-8% perforation rate after balloon dilatation[104] may be as high as 32% in caustic strictures[105]. Indeed, radiological intramural and well-contained transmural esophageal ruptures were observed in 30% of balloon dilatation procedures[106]. In addition, balloon inflation may cause either extrinsic mechanical compression of the trachea or obstruction at the endotracheal tube tip[107]. Therefore, the use of the balloon catheter in children entails careful intraoperative monitoring and likely requires greater endoscopic skill and experience than for Savary bougies. If these requirements are not met, as is often the case in developing countries, pneumatic dilatations will carry a considerable risk and then require extra caution, so that bougie dilatation is preferred.

The interval between dilatations varies from less than 1 to 2-3 wk and usually 3-4 sessions are considered sufficient for durable results, although the number of dilatations required may be unpredictable and quite high[103]. In challenging strictures, a nylon thread left between the nose and the gastrostomy maintains luminal access and facilitates further dilatations when an expert endoscopist is not available[108,109]. A cut-off value for unsuccessful dilatation treatment may be difficult to define, especially in developing countries, where alternative surgical options are not widely available.

A good nutritional state is crucial for a successful outcome, especially in children, and both an improvement in nutritional status and sustained esophageal patency should be considered reference points for a successful dilatation[3]. Changes in feeding practices may be required in order to maintain an adequate nutritional status[110]. In developing countries, delayed presentation and severity of strictures due to the more corrosive substances usually ingested, together with poor nursing and surgical care make this target quite challenging. In such a scenario, feeding by nasogastric tube for long periods may be tolerated with difficulty and a gastrostomy is more effective and often necessary to attain an acceptable nutritional state. Moreover, gastrostomy allows a retrograde approach for dilatation, which is usually easier and safer[111,112].

RISK OF CANCER

Esophageal neoplasms (both adenocarcinoma and squamous cell carcinoma) may develop as a late complication of caustic injury at a rate 1000-3000 times higher than expected in patients of a similar age[113] and have actually been reported only 1 year after ingestion[114]. The reported incidence ranges from 2% to 30%, with an interval from 1 to 3 decades after ingestion[53]. Cancer is most commonly observed at the areas of anatomic narrowing, and may be related to increased exposure to the caustic substance. Esophageal bypass surgery does not prevent the development of esophageal cancer following caustic ingestion[53]. The problem may be overestimated, in accordance with the low number of esophageal cancer reported in a large series with long-term follow-up[9,115,116], yet endoscopic screening is still recommended for patients following caustic ingestion. Moreover, the role of other confounding factors, such as alcohol abuse or smoking habit, should be considered[39].

DISMOTILITY

Orocecal transit time is prolonged mainly in patients with lower third esophageal involvement of the burn[65], probably related to autovagotomy due to vagal entrapment in the cicatrization process involving the lower third of the esophagus. Moreover, impaired vagal cholinergic transmission, possibly due to the same mechanism[117] can explain the increased fasting gallbladder volume and decreased gallbladder emptying found in patients after lower esophageal damage.

Gastric emptying time of liquids after caustic ingestion, was found to be significantly prolonged in patients with lower esophageal strictures, but not in upper-middle esophageal strictures, even in the absence of symptoms suggestive of gastric outlet obstruction or gastroparesis[118].

Late surgery

Surgery for non-responding esophageal strictures: When esophageal dilatation is not possible or fails to provide an adequate esophageal caliber in the long-term, esophageal replacement by retrosternal stomach or, preferably, right colonic interposition should be considered. Mortality and morbidity are low in expert hands[119,120]. The more demanding pharyngoesophageal strictures may be treated with acceptable results, provided considerable expertise is available[121]. The native esophagus can be left or removed. Though resection of the scarred esophagus may be performed without a substantial increase in morbidity and mortality compared to by-pass[120], a 13% incidence of esophageal cancer after by-pass[93], the risk of infected esophageal mucocele in 50% of the patients after 5 years[94], and the impossibility of endoscopic follow-up for cancer are all arguments favoring esophageal resection. Removal of the native esophagus seems advisable in children because of the risk of cancer in a long life period. Conversely, the doubled mortality rate (11.0% *vs* 5.9%) of resection *vs* by-pass[122], the possible damage to the trachea and laryngeal nerve, and the low reported incidence (3.2%) of esophageal malignancy, could support a conservative strategy. In children, reconstruction with gastroplasty seems easier, and more functional failures can be expected with coloplasty[123-125]. In developing

countries, experienced pediatric surgical centers are not widely available and this should be considered before abandoning the conservative approach of dilatation.

Surgery for stomach injuries: The timing and type of elective surgery for gastric outlet obstruction is still controversial. Early surgery has been advised to decrease mortality and morbidity[67,126]. Conversely, elective surgery earlier than 3 mo has been considered risky because of poor nutritional state and the presence of adhesions and the edematous gastric wall[27]. Moreover, assessment of the limits of the gastric resection may be difficult, due to ongoing fibrosis. Endoscopic balloon dilatation and/or intralesional steroid injection have been proposed as alternatives[127,128]. However, endoscopic gastric dilatation should be considered a temporary substitute for surgical resection because gastric wall fibrosis usually diminishes the long-term functional result[129,130]. Moreover, although dilatation averts surgery in less than 50% of patients[127], perforation can occur in strictures longer than 15 mm[131]. Pyloroplasty has been recommended for moderate strictures[67], but progressive fibrosis causing recurrent stricture occurs frequently. Gastrojejunostomy is a safer alternative to gastric resection in the presence of extensive perigastric adhesion, an unhealthy duodenum, and poor general condition; marginal ulceration is rarely reported[27,132] possibly due to physiologic antrectomy resulting from mucosal damage[66]. Partial gastric resection is preferred by many[133,134] for the long-term risk of malignant transformation, though the need for gastric resection as prophylaxis against future malignancy has been overstated in the literature[29]. Previous reports of gastric carcinomas after acid ingestion are usually old and limited[135,136]. Regular follow-up and surveillance endoscopy is a more reliable approach.

Late reconstructive surgery after emergency esophagectomy: When the stomach has been removed or shows chronic injuries, the use of a gastric tube for esophageal reconstruction is obviously precluded. Reconstruction is probably advisable at the end of the evolving scarring process, usually after 6 mo, although the optimal timing of reconstruction has been reported from 2 mo to years[94,137,138]. The functional success rate after colon reconstruction at 5 years is 77% and the severity of the initial insult or a delay more than 6 mo, may strongly influence the outcome[119]. Coloplasty dysfunction is responsible for half of the failures, with an overall 70% success rate after revision surgery in expert hands. An emergency tracheostomy may have an adverse impact on the outcome of a colopharyngoplasty[139]. Secondary esophagocoloplasty should be considered with good results if intraoperative colon necrosis occurs at the time of primary reconstruction[140].

CONCLUSION

Ingestion of corrosive substances is increasingly reported in developing countries, due to lack of education and prevention. The relationship between symptoms and severity of injury may be vague, and patients should be carefully monitored, since esophageal or gastric perforations can occur at any time during the first 2 wk after ingestion. Endoscopy is considered a cornerstone in the diagnosis of corrosive ingestions, yet the indication for early endoscopy should likely be made on a case-by-case basis. Reported discrepancies between endoscopic findings and the extent of necrosis found at surgery suggest the need for better criteria to improve patient selection for emergency surgery. A CT scan may offer a promising role in assessing the evolution of the injury and impending perforations. In suicide attempts, mortality is still high and the need to perform emergency surgery for caustic injuries has a persistent long-term negative impact both on survival and functional outcome. However, timely and early surgery may be the only hope for patients with severe injuries, and a rather aggressive attitude should be considered in such patients.

Main late sequelae include esophageal strictures, often accompanied by undernourishment, especially in developing countries. The likelihood of a gastric outlet obstruction should always be kept in mind. The presence of severe GER and of esophageal dysmotility may worsen the prognosis. Stricture prevention by stents seems promising but the experience is still limited. Systemic corticosteroids offer no role. Endoscopic dilatation is usually successful in achieving a patent esophageal lumen, but in complex strictures several attempts must be carried out, and in such patients bougies may be preferred to balloon dilatation. A cut-off value for unsuccessful dilatation treatment may be difficult to define, especially in developing countries, where alternative surgical options are not widely available. Both an improvement in nutritional status and a sustained esophageal patency should be considered reference points for a successful dilatation. Gastrostomy may be lifesaving in this perspective. Mortality and morbidity of esophageal replacement in patients not responding to dilatation are low in expert hands. The preservation of the native esophagus is still debated. When late reconstructive surgery is carried out after early emergency surgical treatment, the outcome is strongly influenced by coloplasty dysfunction, responsible for half of the failures. Risk of esophageal cancer after caustic ingestion might be overestimated, yet endoscopic screening is still recommended.

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REVIEW

Why interleukin-10 supplementation does not work in Crohn's disease patients

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Abstract

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD) or ulcerative colitis are chronic intestinal disorders, which are on the increase in "Westernised" countries. IBD can be caused by both genetic and environmental factors. Interleukin-10 (IL-10) is an immunoregulatory cytokine that has been identified as being involved in several diseases including IBD. Studies have shown that polymorphisms in the promoter region reduce serum levels of IL-10 and this reduction has been associated with some forms of IBD. Mouse models have shown promising results with IL-10 supplementation, as such IL-10 supplementation has been touted as being a possible alternative treatment for CD in humans. Clinical trials have shown that recombinant human IL-10 is safe and well tolerated up to a dose of 8 μg/kg. However, to date, the results of the clinical trials have been disappointing. Although CD activity was reduced as measured by the CD activity index, IL-10 supplementation did not result in significantly reduced remission rates or clinical improvements when compared to placebo. This review discusses why IL-10 supplementation is not effective in CD patients currently and what can be addressed to potentially make IL-10 supplementation a more viable treatment option in the future. Based on the current research we conclude that IL-10 supplementation is not a one size fits all treatment and if the correct population of patients is chosen then IL-10 supplementation could be of benefit.

Key words: Inflammatory bowel disease; Crohn's disease; Interleukin-10; Recombinant human interleukin-10

Core tip: Inflammatory bowel disease (IBD) is a chronic condition with no known cure. This review addresses the current available treatments for IBD before discussing a potential new treatment strategy using the immunoregulatory cytokine interleukin-10 (IL-10). To date clinical trial results have been disappointing. We highlight the limitations of current IL-10 supplementation treatment and suggest how, with changes to IL-10 delivery and the correct choice of patient, IL-10 supplementation could become a viable treatment option.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic intestinal disorders that are typified by ulcerative colitis (UC) and Crohn's disease (CD). They are considered to be caused by an aberrant intestinal immune response to commensal microbiota in genetically susceptible individuals[1-3]. IBD

affects over 1.4 million people in the United States and over 2.2 million in Europe and is on the increase[4-7]. In New Zealand CD affects 16 per 100000 and UC 7 per 100000. Clinical symptoms include pain, diarrhoea, rectal bleeding and weight loss, which can have a debilitating effect on sufferers[8]. There are both environmental and genetic factors that have a role in the development and progression of IBD. IBD is more prevalent in "Westernised" countries, believed to be a result of diet and lifestyle and also an effect of improved sanitation[9-11].

Genome-wide association studies (GWAS) have highlighted the complexity of IBD. To date, 163 IBD susceptibility loci have been identified[12], 30 associated with CD, 23 with UC and 110 with both[10,12-14]. Some susceptibility genes have been identified, covering genes involved in autophagy (*ATG16L1* and *IRGM*), pattern recognition receptors, intestinal epithelium maintenance and immune response[4].

The anti-inflammatory cytokine interleukin-10 (IL-10) has been identified as being involved in IBD[15]. Studies[16-18] have shown that polymorphisms in the *IL-10* promoter alter IL-10 serum levels and have been linked to IBD. IL-10 supplementation has been tested as a potential therapy for CD[19-28]. This review will focus on the use of IL-10 supplementation explaining why it is currently ineffective at treating patients with CD and showing how that effectiveness could be improved.

IL-10

Functions of IL-10

IL-10 was first identified as a cytokine secreted by $CD4^+$ Th2-cells that inhibits cytokine production in antigen presenting cells[29], and was described as a cytokine synthesis inhibitory factor. The gene for human *IL-10* is located in the 1q32 band on chromosome 1 and encodes for 5 exons. The encoded protein is a homodimer with a mass of 37 kDa consisting of 160 amino acid monomers[16,19,30]. The structure of IL-10 resembles interferon gamma (IFN-γ) and both IL-10 receptor (IL-10R) subunits are members of the interferon receptor family[31,32].

IL-10 is a pluripotent cytokine and could be considered the most important anti-inflammatory cytokine found in the human immune response[4,19]. IL-10 is produced by different cell types including B- and T-lymphocytes, macrophages, monocytes, dendritic cells and mast cells[16,33]. IL-10 has the ability to differentially affect the function of different subsets of immune cells, affecting both the innate immune system and the adaptive immune system, and is therefore considered to have a broad effect in immunoregulation and host defense[34]. Broadly speaking, IL-10 inhibits pro-inflammatory mediator production while increasing the production of anti-inflammatory mediators[19,34].

Many of the pro-inflammatory cytokines suppressed by IL-10 are known to be regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Dysregulation of NF-κB has been implicated in the pathogenesis of chronic inflammatory disease including IBD[35,36]. It has been shown that IL-10 can block IKK activation and directly inhibit the nuclear localisation ofthe NF-κB p65/p50 heterodimer[37,38]. It has also been shown that IL-10 can selectively induce nuclear translocation and DNA-binding of p50 homodimer, which has been shown to inhibit transcription[39].

IL-10 down-regulates major histocompatibility complex Ⅱ (MHC class Ⅱ) expression[40] and the expression of the co-stimulatory ligands CD80/CD86 (B7-1, B7-2) in monocytes[41], macrophages[42,43] and dendritic cells[44,45]. While both MHC class Ⅱ and co-stimulatory ligands are needed to effectively activate $CD4^+$ Th2 cells by antigen presentation, this results in decreased macrophage and T cell derived cytokine synthesis, *e.g.*, IL-1, IL-6, IL-8, IFN-α, tumor necrosis factor-α[25,46-48]. However IL-10 also has immunostimulatory effects, by up-regulating MHC class Ⅱ expression on B lymphocytes[49] and increasing the synthesis of several antibody isotypes *e.g.,* immunoglobulins (IgM, IgA and IgG)[50].

Despite unanswered questions, our current knowledge credits IL-10 with having a significant critical role in regulating intestinal immune homeostasis, this is highlighted by the fact that impaired IL-10 signalling contributes to IBD[4,51,52]. Rare homozygous mutations in *IL10RA* and *IL10RB*, resulting in defective IL-10 signalling were identified in children with early-onset IBD[52] thereby confirming IL-10's critical role in maintaining intestinal homeostasis.

IL-10 signalling

During IL-10 signalling the IL-10 homodimer binds to the tetrameric receptor IL-10R complex, consisting of 2 molecules of IL-10R α-chain (IL-10R1) and two molecules of the IL-10R β-chain (IL-10R2)[53-55]. This binding activates Janus Kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), which self-phosphorylate and subsequently phosphorylate IL-10R1 at tyrosine residues, 446 and 496, which recruits signal transducer and activator of transcription 3 (STAT3) *via* its SH2-domain. STAT3 is phosphorylated by JAK1 and Tyk2, causing dimerisation and translocation to the nucleus, where target genes are induced[2,4,20,53] (Figure 1).

There is contradictory evidence regarding the role of STAT3 in IBD[56], with studies showing that it can play both a pathogenic[57-60] or a regulatory[61-64] role in IBD depending on the specific activator and cell type[65]. STAT3 mediates mucosa-protective functions in epithelial and myeloid cells but can also contribute to inflammation if active in other cell types[66]. It has been shown that STAT3 is essential for all known functions of IL-10 and that STAT3 acts as a transcription factor for other genes within the anti-inflammatory response[67,68]. STAT3 is primarily recruited and activated in macrophages, and this activation is transient[69], which avoids the inflammation associated with an increase of activated STAT3 in IBD[70-72].

Genetic variants in *IL-10*, the IL-10 receptor and *STAT3* genes are associated with IBD, highlighting the

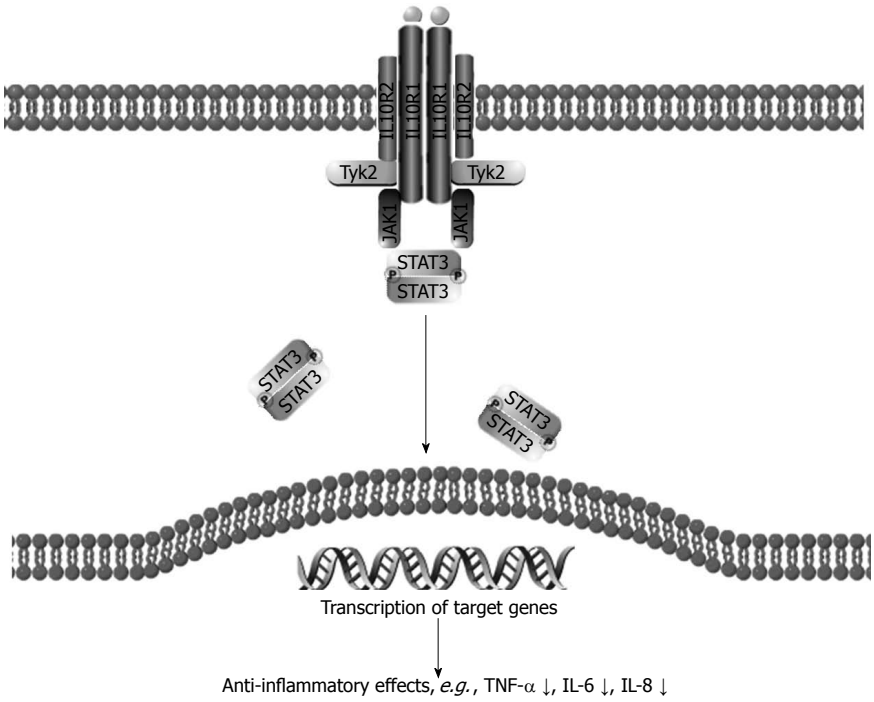

Figure 1 Interleukin-10 signalling pathway. Interleukin-10 (IL-10) binds to the tetrameric receptor IL-10 receptor (IL-10R) complex, this activates Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), which self-phosphorylate resulting in the binding and phosphorylation of signal transducer and activator of transcription 3 (STAT3). STAT3 dimerises and translocates to the nucleus, inducing target genes. TNF-α: Tumor necrosis factor-α.

involvement of the IL-10 signalling cascade in the pathogenesis of CD and UC, further supporting the hypothesis that defective anti-inflammatory mechanisms may be key to IBD development[2,11,15,52,73-75].

IL-10 AND IBD

How IL-10 relates to IBD

The first evidence of a role of IL-10 in IBD, came from a GWAS study by Franke *et al*[15] that showed a significant ($P = 1.35 \times 10^{-12}$) association between a single nucleotide polymorphism (SNP) rs3024505 near the three-prime untranslated regions of the *IL-10* gene and UC, there was modest association with CD.

IL-10 knockout mice develop chronic enterocolitis, which is similar to human CD, if they are not kept in germ-free conditions. Administration of IL-10 ameliorates inflammation in both animal and *in vitro* models[76], indicating a potential role for IL-10 in the down-regulation of Th1-mediated mucosal inflammation[16,77].

Because IL-10 mediated immune responses are so important in maintaining intestinal homeostasis and commensal flora tolerance, it has been hypothesized that a defect in IL-10 production may be involved in the pathogenesis of CD[78]. In fact impaired IL-10 production has been found in severe cases of both UC[79] and CD[78]. Studies show that CD patients have normal[80,81] or high IL-10 levels[18,82]. However, low IL-10 production in intestinal mucosa has been shown to be associated with increased postoperative recurrence[22,83] and it has been shown that administration of recombinant human IL-10 in low IL-10 producers significantly reduced recurrence after surgery[22].

IL-10 mucosal levels

It has been shown that intestinal epithelial cells from healthy and inflamed colonic tissue express IL-10 mRNA and protein to the same extent. However during inflammation and also in patients with CD, there are significantly increased numbers of mononuclear cells producing IL-10[20,25]. Circulating levels of IL-10, as determined by serum levels of IL-10[82] and mRNA levels[84], have been shown to correlate with disease activity.

IL-10 serum levels

It is believed that circulating levels of IL-10 are critical in immune regulation. Basal levels of IL-10 modulate production of other cytokines and thus minor changes can affect the cytokine network, which in turn affects inflammation.

Studies have been inconsistent regarding serum levels of IL-10 in IBD, as stated earlier some studies show higher IL-10 levels in CD, Wang *et al*[18] found that CD patients had significantly higher levels of IL-10 compared to controls. Kucharzik *et al*[82] reported increased serum IL-10 concentrations in patients with active CD or UC compared to controls. Mitsuyama *et al*[85] showed an increase in serum IL-10 in active UC patients but not CD. In contrast, Nielsen *et al*[81] reported that serum IL-10 concentrations did not differ among UC, CD and healthy control subjects. These inconsistencies could be the result of variations, *e.g.,* age, severity of disease and ethnicity in the studied populations or in different methodological designs.

As IL-10 is an anti-inflammatory cytokine, we expect that high serum levels of IL-10 are likely to be good for patients with chronic inflammatory disease. In fact low IL-10 levels are known to increase disease severity in CD patients compared to high IL-10 levels[85,86]. From steroid treatment it has been shown that steroid non-responders have low IL-10 levels while steroid responders have sus-

tainable high IL-10 levels during and after treatment[87]. Sufficient IL-10 levels seem to be required for recovery but do not offer a cure.

We can hypothesize that IL-10 has an optimal level to be beneficial to reduce chronic inflammatory diseases, and may prove detrimental at too high or too low levels. Diseases associated with IL-10 SNPs such as psoriasis and rheumatoid arthritis are known to have high IL-10 serum levels[18,88,89], while in other diseases like UC, IL-10 levels vary between individuals and studies, with a trend toward increased IL-10 production, though the big studies are lacking[90-92].

IL-10 serum level and disease severity is not restricted to IBD, other diseases including autoimmune diseases, such as systemic lupus erythematosis, Behçets, type 1 diabetes mellitus[14], psoriasis[93], atherosclerosis[94] and rheumatoid arthritis[89] have all been shown to be associated with *IL-10* SNPs. Susceptibility to several cancers including prostate[95], breast[96], cervical[97] and more recently gastric[98,99] have been associated with *IL-10* promoter polymorphisms.

IL-10 promoter polymorphisms

SNPs are the most common form of genetic variation in humans. A SNP occurs at a location where more than one possible nucleotide occurs naturally within a population at a frequency >1%[100]. SNPs can be in both coding and non-coding regions of DNA. Due to the degeneracy of the genetic code. Even if the SNP is in a gene it may not change the amino acid and so has no effect on the protein (synonymous SNP), however non-synonymous SNPs do change the protein and are more commonly associated with disease. It is these variations that are most interesting to researchers as these can account for whether/how a person develops a disease, the severity of disease and how they respond to treatment.

Important variability in IL-10 secretion has been reported and is associated with SNPs in the *IL-10* promoter at 3 locations -592, -819, -1082[78,101,102]. The *IL-10* promoter polymorphisms C-592A (rs1800872), C-819T (rs1800871) and G-1082A (rs1800896) have been extensively studied. The most recent studies of Franke *et al*[15], Amre *et al*[17], Wang *et al*[18], Andersen *et al*[73], Fowler *et al*[103], Fernandez *et al*[104] and Tedde *et al*[105] reported a significant association between IL-10 rs1800896 and IBD.

The "A" allele of rs1800896 was found to be more common in IBD patients, especially in UC patients, individuals with the A/A genotype have lower IL-10 production than the G/G genotype[106], Koss *et al*[107] found that the -1082 AA is associated with decreased IL-10 production in both CD patients and controls. Wildtype -1082 (rs1800896) "G" and -592 (rs1800872) "C" are known to be associated with increased IL-10 levels; therefore we expect the GCC haplotype to show the highest IL-10 expression and ATA the lowest. This hypothesis was studied by Reuss *et al*[108] who showed in THP-1 monocyte cells that IL-10 expression was highest in the GCC haplotype compared to ACC and ATA (P = 0.042 and P = 0.0026). In the twin-study which followed, the haplotype showed no correlation with IL-10 serum levels. Wang *et al*[18] showed a significant (P = 0.001) increase in IL-10 production for TAT haplotype in healthy controls. The -592A allele was also shown to be associated with reduced transcription and decreased IL-10 secretion[16,102,109].

CURRENT IBD TREATMENTS

The current treatment options available for IBD, include: surgery, aminosalicylates, *e.g.,* 5-aminosalicylic acid, corticosteroids, *e.g.,* prednisone, immunosuppressants, *e.g.,* azathioprine, cyclosporine or biologicals, *e.g.,* infliximab[110,111]. The choice of treatment is dependent on phenotype, disease activity, characteristics of the drug and the patient. The choice should look to balance effectiveness with side effects and long term complications. As with any drug treatment there are side effects, these range from the usually well-tolerated upset stomach, nausea and headache to the more severe bone marrow and liver problems. As well as the associated side effects these treatments only work for some cases and can also result in a loss of response. Thus stronger treatments are required which have more severe side effects and long term consequences, and so alternative therapies are being investigated.

ALTERNATIVE TREATMENTS

Environmental and dietary factors are thought to play a role in the development of CD[112,113] and so changes to diet and lifestyle can have beneficial effects. Studies[114-118] have shown that specific foods are associated with IBD and that avoiding certain foods can reduce both the severity and frequency of symptoms. There are several classes of new drugs being developed: monoclonal antibodies, small molecules, fusion proteins and recombinant growth factors, as well as stem cell based therapies; one of these new therapies is IL-10 supplementation.

IL-10 supplementation for CD

Studies have suggested that IL-10 has huge therapeutic potential in intestinal inflammation, and that it should inhibit the up-regulated pro-inflammatory cytokines in CD and UC[25]. In most studies to date, Tenovil (Schering-Plough, Kenilworth, NJ, United States), has been used, which is the brand name of rhuIL-10. It is produced by a genetically engineered *Escherichia coli* strain, that expresses a 161 amino acid protein identical to human IL-10 with an additional amino-terminal methionine residue[119,120].

Why doesn't it work?

Based on the success of animal models[121-126] of intestinal inflammation, IL-10 therapy was heralded as a potential anti-inflammatory treatment in CD and several human trials have been undertaken. The first trial conducted by van Deventer *et al*[28] showed that IL-10 supplementation

Table 1 Summary of key findings from interleukin-10 trials in human and animal studies

Ref.	Model	Intervention	Outcome
	Human		
Colombel *et al*[22]	65 patients having recently undergone intestinal resection surgery	4 μg/kg daily or 8 μg/kg twice weekly for 12 wk	No clear evidence of effect
Fedorak *et al*[23]	95 mild to moderately active CD (CDAI 200-350)	1, 5, 10 or 20 μg/kg of daily for 4, 20 wk follow up	Improved clinical response (based on CDAI score) and improved endoscopic appearance
Schreiber *et al*[26]	329 therapy-refractory chronic active CD (CDAI 200-400)	1, 4, 8 or 20 μg/kg of Tenovil subcutaneously for 28 d	Non-significant clinical improvements
van Deventer *et al*[28]	46 patients with active steroid-resistant CD (CDAI 200-350)	0.5, 1, 5, 10 or 25 μg/kg daily for 1, 3 wk follow up	Reduction in the average score of CDAI
Braat *et al*[128]	10 patients with moderate to severe CD	10 enteric-coated capsules containing 10^{10} cfu of LL-Thy12 twice daily for 7 d	Clinical benefit observed in 8 of 10 patients, including 5 showing complete remission
	Animal		
Barbara *et al*[121]	DNB induced colitis Spf Sprague-Dawley rats	Ad5IL-10 (5×10^8-1×10^{10} pfu)	Improved colitis macroscopically and histologically and decreased MPO activity and LTB4 levels
Grool *et al*[123]	40 male NZ white rabbits formalin-immune complex induced colitis	100 or 500 μg/kg single Ⅳ infusion of rIL-10	Anti-inflammatory response as measured by decreased mucosal damage, leukocyte recruitment, MPO and LTB4
Ribbons *et al*[124]	TNBS induced colitis in 74 Sprague-Dawley rats	0.5, 5, 50, 500 μg/kg rIL-10 subcutaneous injection twice daily for 5 d	Mild anti-inflammatory effects Significant reduction in MPO
Sasaki *et al*[125]	3% DSS induced C57B6 mice	Intra-peritoneal administration of adIL-10	Significantly reduced disease activity and weight loss and completely prevented histopathologic injury to the colon
Tomoyose *et al*[126]	4% DSS induced colitis BALB/c mice	Recombinant mouse rIL-10 (1, 100, 1000 unit/mL)	Marked improvement in intestinal inflammation Inhibition of tissue damage and production of pro-inflammatory cytokines
Steidler *et al*[127]	DSS induced and spontaneous IL10$^{-/-}$ mouse models of colitis	Daily intragastric inocula of 2×10^7 or 10^9 LL-mIL10	Reduced histological score by 50% in DSS and prevented onset of colitis in IL-10$^{-/-}$ mice

CD: Crohn's disease; CDAI: Crohn's disease activity index; cfu: Colony forming units; MPO: Myeloperoxidase; LTB4: Leukotriene B4; TNBS: 2, 4, 6 trinitrobenzenesulfonic acid; DSS: Dextran sodium sulphate; adIL-10: Adenoviral IL-10; DNB: Dinitrobenzene sulphonic acid; Spf: Specific pathogen free; Ad5IL-10: Human type 5 adenovirus + murine IL-10; pfu: Plaque forming units; LL-mIL10: *Lactococcus lactis* secreting murine IL-10.

was safe and well tolerated. This was confirmed by subsequent studies[22,23,26]. van Deventer *et al*[28] showed a reduction in the average score of CD activity index (CDAI), but this was not significant. Fedorak *et al*[23] showed that 5 μg/kg of Tenovil given subcutaneously for 28 d to patients with mild to moderate CD activity resulted in improved clinical response (based on CDAI score) and improved endoscopic appearance of the disease. Schreiber *et al*[26] showed that 8 μg/kg of Tenovil given subcutaneously for 28 d to patients with mild to moderate CD activity resulted in a non-significant clinical improvement. However, Colombel *et al*[22] found no evidence that treatment with Tenovil for 12 wk in CD patients after intestinal resection prevented recurrence of CD. The key findings of these studies are summarised in Table 1.

These data show that IL-10 treatment did not result in significantly reduced remission rates or clinical improvements when compared to placebo[21,24]. In fact a Cochrane review in 2010[21] concluded that "Interleukin 10 does not appear to provide any treatment of active Crohn's disease. ...interleukin 10 does not increase the number of remissions (complete or clinical), but increases the rate of withdrawal due to adverse events relative to placebo." This review only included three of the studies mentioned above[23,26,28] and although more patients receiving IL-10 withdrew from studies there was no significant difference in the number of patients reporting adverse reactions between treatment and control.

However this is not the whole story, as other studies not included in the Cochrane analysis have shown that patients respond differently to IL-10 supplementation. Colombel *et al*[22] reported that endoscopic recurrence in patients with low IL-10 levels at time of surgery reduced to 47% with Tenovil treatment compared to 80% in the placebo group. Schreiber *et al*[26] found that patients responded differently to IL-10 treatment, with patients suffering from high disease activity having a greater rate of clinical improvement. These data suggest that IL-10 levels and disease activity are factors in how a patient responds to IL-10 supplementation. Also, as previously stated, some CD patients already have raised levels of IL-10[18,82]. These patients will not benefit from IL-10 supplementation and may suffer detrimental effects as high doses of systemically administered IL-10 induce the pro-inflammatory cytokine IFN-γ[27].

The different response to IL-10 supplementation is not surprising given the heterogenous nature of CD. A therapy that targets one step within a complex immunological pathway may only benefit a small proportion of patients but if you select the correct sub-population of patients who under-produce IL-10, for example those that have a penetrating phenotype who have a greater deficiency in IL-10[78], you may see a significant beneficial response to IL-10 therapy[25].

There are five potential explanations as to why IL-10 treatment has not been effective as a therapeutic strategy: (1) the administered dose of IL-10 results in an intestinal concentration of IL-10 that is too low to elicit a response; (2) there are differences among individuals depending upon disease phenotype/severity; (3) IL-10 is only successful at preventing and not treating an established disease; (4) IL-10 alone fails to suppress all the pro-inflammatory mediators involved in chronic inflammation; or (5) IL-10's immunostimulatory effects counterbalance its immunosuppressive properties.

Can IL-10 supplementation work?

Most of the potential explanations as to why IL-10 supplementation currently doesn't work can be overcome.

The modest therapeutic benefits[23,26] and adverse effects can potentially be attributed to limited mucosal bioavailability of IL-10 and the fact that the trials so far have not separated patients by genotype or disease phenotype/severity. To address the low bioavailability of mucosal IL-10 without resorting to the detrimental high levels of IL-10 systemic administration, *Lactococcus lactis* (*L. lactis*) was engineered to secrete IL-10, and this was used to successfully prevent the onset of colitis in the IL-10 KO model and caused a 50% reduction in inflammation in the DSS mouse model[127]. This study was followed up with a small phase 1 human trial using *L. lactis* modified to contain the human IL-10 sequence (LL-Thy12)[128]. 10 capsules containing 1×10^{10} cfu of LL-Thy12 were given to 10 patients with moderate to severe CD twice daily for 7 d. The results showed this approach is both safe and biologically contained, avoiding the side effects associated with high systemic doses while still retaining the ability to reduce disease activity. This was a small trial in a controlled environment without a control comparison and so further studies are needed to confirm the effectiveness of this treatment. However based on these initial results this form of IL-10 supplementation is showing promise as a treatment for patients with chronic intestinal inflammation.

Alternative ways to improve local delivery of IL-10 include gene therapy using replication-deficient adenoviral vectors delivered directly to the gastrointestinal epithelial cells. This approach has proven successful in two mouse studies[121,129] showing an effect on colitis without the associated side-effects of systemic administration. Gelatine microspheres containing IL-10 (GM-IL-10) were developed by Nakase *et al*[130] to deliver sustained IL-10 release locally without losing bioactivity. Colonic inflammation in mice treated with GM-IL-10 was reduced compared to mice treated with IL-10 alone.

The second point of selecting patients based on disease phenotype and/or severity can be easily addressed based on clinical diagnosis. Selecting patients based on genotype is slightly more complicated and would require that potential candidates for treatment be screened. Genotyping has become relatively quick and easy to perform and the cost is reducing as the technology advances. However who actually performs the genotyping service, who pays for the service and gaining patient consent may be problematic. This could potentially be overcome by having the patients enrol onto a research study. However IL-10 serum levels are determined 50% by genetics and 50% by environment[108] and so just because a person has the low IL-10 producing SNP doesn't necessarily mean they will have low IL-10 levels. Therefore a better measure to determine potential benefit of IL-10 supplementation would be to measure the serum level of IL-10, which can be done using commercially available ELISA kits. This should prove to be easier to conduct and in gaining patient consent.

As mouse models proved[121,131] IL-10 administration was only successful when administered prior to initiation of colitis and was unable to treat any established inflammation. Therefore IL-10 supplementation could be used to prevent relapses rather than to treat active inflammation.

If locally delivered IL-10 fails to have an effect, then it may be due to the fact IL-10 alone is unable to suppress all the pro-inflammatory mediators involved in chronic inflammation.Therefore it would be necessary to develop a combination treatment containing IL-10. However the evidence suggests IL-10 alone should have an effect and so it may be that IL-10 supplementation is not a suitable treatment for that disease phenotype.

CONCLUSION

Based on this knowledge, it is our opinion that a subpopulation of CD patients, who have lower expression of IL-10, and who have active disease could benefit from targeted IL-10 supplementation therapy. However further studies are needed to determine the exact population of patients who would benefit the most from this treatment and to determine if there are any long term detrimental effects of this treatment. Given that current treatments of IBD may not be beneficial to a patient or have severe side effects, we believe it is worth exploring this potential treatment avenue.

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REVIEW

Treatment options of inflammatory appendiceal masses in adults

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Abstract

At present, the treatment of choice for uncomplicated acute appendicitis in adults continues to be surgical. The inflammation in acute appendicitis may sometimes be enclosed by the patient's own defense mechanisms, by the formation of an inflammatory phlegmon or a circumscribed abscess. The management of these patients is controversial. Immediate appendectomy may be technically demanding. The exploration often ends up in an ileocecal resection or a right-sided hemicolectomy. Recently, the conditions for conservative management of these patients have changed due to the development of computed tomography and ultrasound, which has improved the diagnosis of enclosed inflammation and made drainage of intra-abdominal abscesses easier. New efficient antibiotics have also given new opportunities for nonsurgical treatment of complicated appendicitis. The traditional management of these patients is nonsurgical treatment followed by interval appendectomy to prevent recurrence. The need for interval appendectomy after successful nonsurgical treatment has recently been questioned because the risk of recurrence is relatively small. After successful nonsurgical treatment of an appendiceal mass, the true diagnosis is uncertain in some cases and an underlying diagnosis of cancer or Crohn's disease may be delayed. This report aims at reviewing the treatment options of patients with enclosed appendiceal inflammation, with emphasis on the success rate of nonsurgical treatment, the need for drainage of abscesses, the risk of undetected serious disease, and the need for interval appendectomy to prevent recurrence.

Key words: Appendicitis; Phlegmon; Abscess; Computed tomography; Antibiotics; Percutaneous drainage; Surgery

Core tip: The management of adult patients with inflammatory appendiceal masses is controversial. This report aims at reviewing the treatment options of these patients, with emphasis on the success rate of nonsurgical treatment, the need for drainage of abscesses, the risk of undetected serious disease, and the need for interval appendectomy to prevent recurrence. The debate arises over the importance of the complication rate of interval appendicectomy. Moreover, if appendicectomy is not performed, consideration needs to be given to what investigations should be undertaken and in which patients. It is also worth recalling that the appendix is used in reconstructive surgery.

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INTRODUCTION

Acute appendicitis is one of the most common causes of acute abdomen and can be classified into uncomplicated

and complicated. The life-time risk of appendicitis is 7%-8%, with the highest incidence in the second decade. The inflammation in acute appendicitis may sometimes be enclosed by the patients own defense mechanisms, by the formation of an inflammatory phlegmon or a circumscribed abscess. The management of these patients is controversial. Immediate appendectomy may be technically demanding because of the distorted anatomy and the difficulties to close the appendiceal stump because of the inflamed tissues. The exploration often ends in ileocecal resection or a right-sided hemicolectomy due to the technical problems or a suspicion of malignancy because of the distorted tissues[1-9]. Recently, the conditions for conservative management of these patients has changed due to the development of computed tomography (CT) and ultrasound (US), which has improved the diagnosis of enclosed inflammation and made drainage of intra-abdominal abscesses easier[10-15]. New efficient antibiotics have also given new opportunities for nonsurgical treatment of appendicitis[16-21]. The traditional management of these patients is nonsurgical treatment followed by interval appendectomy to prevent recurrence. The need for interval appendectomy after successful nonsurgical treatment has recently been questioned because the risk of recurrence is relatively small[22-27]. After successful nonsurgical treatment of an appendiceal mass, the true diagnosis is uncertain in some cases and an underlying diagnosis of cancer or Crohn's disease (CD) may be delayed[27].

This report reviews the treatment options of patients with enclosed appendiceal inflammation, with emphasis on the success rate of nonsurgical treatment, the need for drainage of abscesses, the risk of undetected serious disease, and the need for interval appendectomy to prevent recurrence. The debate arises over the importance and level of the complication rate of interval appendicectomy. Moreover, if appendicectomy is not performed, consideration needs to be given to what investigations should be undertaken and in which patients. It is also worth recalling that the appendix is occasionally used in reconstructive surgery[26,28].

DEFINITIONS

Acute appendicitis is inflammation of the vermiform appendix and remains the most common cause of the acute abdomen in young adults. The term complicated appendicitis is often used to describe a palpable appendiceal mass, an appendiceal phlegmon, or a localized abscess without distinction. A phlegmon is an inflammatory tumor consisting of the inflamed appendix, its adjacent viscera and the greater omentum, whereas an abscess is a pus-containing appendiceal mass[27-31]. The diagnosis of enclosed inflammation is made by finding a palpable mass at clinical examination before or after anesthesia, or by finding an inflammatory mass or a circumscribed abscess by CT, US or at surgical exploration of the abdomen. We consider that nonsurgical treatment has failed when the patient undergoes appendectomy during the same hospital stay after attempted nonsurgical treatment. The patients treated with drainage are those who had drainage (without appendectomy) of an abscess either percutaneously or by surgical exploration. Morbidity includes postoperative infectious complications, intestinal fistula, small bowel obstruction, and recurrence after initially successful nonsurgical management[27].

TREATMENT OPTIONS OF NONCOMPLICATED ACUTE APPENDICITIS

Although the etiology of acute appendicitis is poorly understood, it is probably caused by luminal obstruction in the majority of cases. Luminal obstruction can be caused by fecaliths, lymphoid hyperplasia, foreign bodies, parasites and both primary (carcinoid, adenocarcinoma, Kaposi sarcoma and lymphoma) and metastatic (breast and colon) tumors. Once appendiceal obstruction occurs, the continued secretion of mucus results in elevated intraluminal pressure and luminal distention. This eventually exceeds capillary perfusion pressure, which leads to venous engorgement, arterial compression, and tissue ischemia. As the epithelial mucosal barrier becomes compromised, luminal bacteria multiply and invade the appendiceal wall, which causes transluminal inflammation. The most common bacteria that can cause acute appendicitis are intestinal bacteria including *Escherichia coli* and bacteria belonging to the *Bacteroides fragilis* group. Continued ischemia results in appendiceal infarction and perforation[29-31]. However, the observation of spontaneous resolution of acute appendicitis cases and some reports of a good outcome in patients treated with antibiotics suggest that not all cases of acute appendicitis are caused by mechanical obstruction and progression to complicated disease. Some researchers have suggested that uncomplicated and complicated forms of appendicitis are two distinct diseases, with different etiologies. As in other intra-abdominal infections, such as salpingitis, diverticulitis and enterocolitis, which are often treated only with antibiotics, the infectious etiology of acute appendicitis is advocated by some scholars. Conservative treatment is most effective when administered within 12 h of symptom onset, ideally within the first 6 h[16-21,29-33]. Antibiotic therapy is associated with a 68%-84% success rate and a trend toward decreased risk of complications without prolonging hospital stay. The authors have described a low morbidity and mortality rate, and a recurrence rate between 5% and 15%[25-33].

At present, the treatment of choice for uncomplicated acute appendicitis in adults continues to be surgical (open or laparoscopy) and it is the gold standard. The most common operative complications are wound infection, intra-abdominal abscess, and ileus caused by intra-abdominal adhesions (Dindo *et al*[34] classification), which vary in frequency between open and laparoscopic appendectomy. The overall complication rates for open and laparoscopic appendectomy are respectively 11.1% and 8.7%, with a mortality rate < 0.5%[35-41]. The exclusive treatment with antibiotics cannot be routinely recommended in current

medical practice and should only be considered in selected patients or conditions in which surgery is contraindicated or in the context of clinical studies[18,19,31,32].

PROPORTION OF PATIENTS WITH APPENDICITIS WHO DEVELOP ENCLOSED APPENDICEAL INFLAMMATION AND CLINICAL PRESENTATION

Circumscribed appendiceal inflammation is common and often undiagnosed preoperatively. The proportion of all patients with appendicitis treated for enclosed inflammation is 3.8%-5.0%. The risk of perforation is negligible within the first 12 h of untreated symptoms, but then increases to 8.0% within the first 24 h. It then decreases to 1.3%-2.0% during 36-48 h, and subsequently increases again to 5.8%-7.6% for each ensuing 24-h period[42-47].

The diagnosis is suspected in patients with a palpable mass or with symptom duration > 3 d and is more common in children, especially in those aged < 5 years. Delay in presentation, age > 55 years, and elevated temperature (> 38.8 ℃) on admission are predictors of perforated appendicitis. Additionally, patients older than 55 years of age have a 29% prevalence of perforated appendicitis in the first 36 h from symptom onset. Patients with hyperbilirubinemia and clinical symptoms of appendicitis should be identified as having a higher probability of appendiceal perforation than those with normal bilirubin levels[48,49].

Enclosed inflammation is found more often in studies in which the diagnosis is based on CT or US than in those based on clinical diagnosis (14.2% *vs* 5.1%). It is also more common in children than in adults as shown by the trend of 8.8% in children, 6.5% in patients of all ages, and 4.8% in adults. There is an early risk of perforation even within the first 36 h of symptom onset, which may be higher in men than women. This suggests that diagnostic imaging should be used more frequently in children, in patients with a long duration of symptoms, and in patients with a palpable mass. Appendectomy should be performed without delay in adults, especially men and those aged > 55 years once diagnosis is confirmed[42-47].

RADIOLOGICAL DIAGNOSIS

There is continued debate about the relative merits of US and CT[10-15,50-59]; the latest meta-analysis has concluded that CT[60-69] is significantly more sensitive than US for the diagnosis of appendicitis, but that US should be considered in children. Sonography has high sensitivity (86%-100%), specificity (88%-95%), and accuracy (91%-92%) in diagnosing acute appendicitis. CT is comparable to sonography with respect to sensitivity, specificity, and accuracy for adults (90%-97%, 93%-100%, and 94%-99%, respectively) and children (95%-97%, 91%-99%, and 96%, respectively) with appendiceal diameter > 6 mm, although some studies have revealed lower diagnostic rates in children than in adults. The major area of debate is regarding which patients suspected of having acute appendicitis should have a CT scan before appendectomy. There are several articles in the literature that argue against routine preoperative imaging of patients with suspected acute appendicitis. In these articles, the routine use of imaging has not been shown to decrease the rate of negative appendectomy, and may actually delay the diagnosis and appropriate intervention in cases of acute appendicitis. Other studies have shown a benefit from preoperative imaging in suspected acute appendicitis, and the development of guidelines for CT in patients with an equivocal presentation has decreased the rate of negative appendectomy from 25% to 6%. A review of a large, prospectively gathered database of general surgical procedures in Washington state has found the negative appendectomy rate to be 9.8% in patients with no preoperative imaging and only 4.5% in those who had a preoperative CT scan. This difference was statistically significant. Based on these findings, CT scans seem to have significant benefit in the evaluation of patients with suspected acute appendicitis, to exclude other pathology, in selected patients such as elderly people[52,70].

Various CT techniques have been described for diagnosing acute appendicitis, including enhanced CT with rectally administered colon contrast medium, enhanced focused CT with thin collimation (3-5 mm), nonfocused technique with oral and intravenous contrast material, focused technique with oral contrast medium, and focused helical CT with colonic contrast medium, and have a high diagnostic accuracy. CT provides a rapid complete diagnostic evaluation of the right lower quadrant, with reported accuracy rates in the diagnosis of appendicitis of up to 95%-100%[11,52,66]. The obvious disadvantages of CT include exposure to ionizing radiation and the potential for contrast medium reactions. Those who benefit most from preoperative imaging are those with an atypical presentation and women of childbearing age. However, it is recognized that this is not without increased cost, radiation exposure and a potential delay in diagnosis. The use of US is particularly important in children and can be of use in premenopausal women[50-52,58]. Institution of a clinical pathway using CT can lead to a substantial decrease in the number of negative appendectomies from 16% to 4%. CT has greater potential than US to reveal alternative diagnoses and complications, such as perforation and abscess formation. US has lower sensitivity than CT in the setting of appendiceal perforation. The appendix is significantly larger in diameter in perforated appendicitis than in appendicitis with no perforation (15 mm *vs* 11 mm). Direct CT signs (*i.e.*, phlegmon, abscess, and extraluminal air) are more specific for perforated appendicitis. Indirect signs (bowel wall thickening, ascites, ileal wall enhancement, intraluminal air, and combined intraluminal air and appendicolith) are also found with higher incidence in appendiceal perforation[13,53,54,61,63]. Intraluminal appendiceal air in the setting of acute appendicitis is a marker of perforated or necrotic appendicitis.

Recognition of this finding in otherwise uncomplicated appendicitis at imaging should raise suspicion for image-occult perforation or necrosis[56]. Defect in the enhancing appendiceal wall allows excellent sensitivity (94.9%) and specificity (94.5%) for the diagnosis of perforated appendicitis when evaluated in a group of patients with known appendicitis. A defect in the enhancing appendiceal wall has the highest sensitivity (64.3%) of any individual finding[53]. Detecting a defect in the enhancing appendiceal wall by using cine mode display of transverse thin-section CT images allows 96.1% accuracy for diagnosing appendiceal perforation[55]. In one series, appendicolith, free fluid, a focal defect in the enhancing appendiceal wall, and enlarged abdominal lymph nodes were not sensitive or specific for the presence of perforation. That study has concluded that unless abscess or extraluminal gas is present multidetector CT cannot establish the diagnosis of perforation[63].

The range of diagnoses that can mimic appendicitis is wide and includes right ureteric calculus, epiploic appendagitis, torsion of Meckel's diverticulum, mesenteric adenitis, inflammatory bowel disease, colitis, gynecological disorders, and right-sided diverticulitis. CT is useful in differentiating between these disorders[63].

Magnetic resonance imaging (MRI) has had little role in the evaluation of acute abdominal pain. However, increasing concerns over the potentially hazardous effects of ionizing radiation associated with CT have made MRI the study of choice to evaluate pregnant women and children with symptoms of appendicitis and equivocal US findings. MRI is highly accurate with a sensitivity of 100%, specificity of 98%, positive predictive value of 98%, and negative predictive value of 100%. Although MRI may be used in any patient with suspected acute appendicitis, there is a special role for MRI in pregnant women with new-onset abdominal pain. MRI has many advantages. It is valuable in the imaging of pregnant women and children because there is no exposure to ionizing radiation. Although MRI is safe during pregnancy, no intravenous contrast should be used during pregnancy because gadolinium is a category C drug and potentially teratogenic. However, noncontrast MRI provides detailed images, which usually provide the correct diagnosis. MRI is operator independent and the results are highly reproducible. MRI is more useful than US in obese patients and in patients with a retrocecal appendix, which is difficult to visualize on US. Drawbacks of MRI are that it is more expensive than other imaging modalities and not as widely available. The examination itself takes longer to perform and may be degraded by motion artifact. There are concerns that, with the exception of trained radiologists, other health care providers are not comfortable interpreting MRI findings[52,70-73].

IMMEDIATE SURGICAL TREATMENT *VS* NONSURGICAL TREATMENT

Emergency appendectomies are still considered the primary means of treating acute appendicitis, with mortality rates of 0.5%-2.4% and 0.07%-0.7% for patients with and without perforation, respectively. Overall, post-appendectomy complication rates are typically 10%-19% for acute appendicitis without perforation and reach 12%-30% for perforated acute appendicitis[19]. Perforation increases the mortality rate of acute appendicitis from 0.0002% to 3% and increases the morbidity from 3% to 47%[52]. Perforated appendicitis may be treated first by conservative treatment or percutaneous abscess drainage with great improvement of the clinical symptoms[74-80]. This is in contrast to nonperforated appendicitis, which requires operation as early as possible in order to reduce morbidity. Immediate surgical treatment of enclosed appendiceal inflammation is associated with a > 3-fold increase in morbidity compared with conservative management, and may result in an unnecessary ileocecal resection or right-sided hemicolectomy for technical reasons or suspicion of malignancy in about 3% of patients[9,27]. Nonsurgical treatment is successful in about 93% of patients, but may need percutaneous drainage of abscesses in about 20%. Most perforated appendicitis give way to generalized peritonitis and cannot be drained. Indications of drainage are absence of generalized peritonitis and presence of percutaneously or surgically drainable abscess[75-78]. Nonsurgical treatment is associated with lower morbidity and shorter hospital stay compared with immediate appendectomy. The results of immediate surgery compared with those of nonsurgical treatment, eventually followed by interval appendectomy, have been reported in 19 retrospective studies[27]. Right-sided hemicolectomy for suspicion of a malignant disease or for technical reasons, but where only inflammatory changes could be found at histopathological examination, has been reported in 17 of 493 adult patients. In all but three of the studies, the authors have concluded that nonsurgical treatment is to be recommended. Conservative treatment is associated with significantly fewer overall complications, wound infection, abdominal/pelvic abscess, ileus/bowel obstruction, and reoperation. No significant difference has been found in the duration of first hospitalization, overall duration of hospital stay, and duration of intravenous antibiotics[79]. Immediate surgery is associated with morbidity in 35.6% of patients compared with 13.5% in nonsurgical treatment and an additional 11.0% after interval appendectomy. The majority of the studies have practiced elective interval appendectomy after successful nonsurgical treatment.

PRIMARY NONSURGICAL TREATMENT FOLLOWED BY DELAYED OR INTERVAL APPENDECTOMY OR WITHOUT APPENDECTOMY

The results of primary nonsurgical treatment followed by delayed appendectomy during the same hospital stay have been compared with those of interval appendec-

tomy and with or without surgical intervention 6-12 wk later (interval appendectomy)[80-88]. Delayed appendectomy[89-93] is associated with morbidity in 18.2% compared with 12.4% after interval appendectomy. The return to work takes longer for patients treated with interval appendectomy, mainly because the patients want to have the planned interval appendectomy done before they are willing to return to work. One prospective study[7] has randomized patients to primary nonsurgical treatment followed by delayed or interval or no appendectomy. The group with nonsurgical treatment without appendectomy had the lowest morbidity and the shortest length of stay. In patients with an appendiceal mass, the authors have concluded that conservative treatment without interval appendicectomy is the best treatment.

FAILURE RATE OF NONSURGICAL TREATMENT AND NEED FOR ABSCESS DRAINAGE

All studies have reported a low failure rate for nonsurgical treatment without appendectomy; some of them even without giving antibiotics[75-80]. The failure rate for all the studies was 7.2%. Failure was associated generally with abscess diameter > 4-5 cm[77-79]. The proportion of patients in need of abscess drainage is strongly related to how the diagnosis is made, with 100% in studies of patients selected because of a drained abscess, 47.5% in patients with a palpable mass or preoperatively found abscess, 27.6% in patients with an abscess or phlegmon diagnosed by CT or US, 9.5% in patients with a palpable mass, and no need for drainage in studies of patients with a phlegmon diagnosed by CT or US. There is no association between the need for drainage and patient age.

COMPLICATIONS FOLLOWING INTERVAL APPENDICECTOMY

The morbidity of interval appendectomy has been reported in a few studies with a pooled value of 11.0%[94-97]. The age of the included patients had no influence on the results. The complication rate following interval appendicectomy is a consideration to be balanced against the recurrence rate. The complication rate varies from 8% to 23%. True surgical complications include wound infection (15.0%), pelvic abscess (5.0%), and aspiration pneumonia (1.5%). Another retrospective study reported a complication rate of 13%, but a prolonged fever, which others may not have cited as a true complication, accounted for almost half of these complications and only one wound infection occurred in 38 interval appendicectomies. An 8% complication rate was reported in a review of 50 interval appendicectomies, but about 25% of these were prolonged fever, about 50% cecal damage, and the remainder subcutaneous abscesses. Laparoscopic interval appendicectomy may decrease the complication rate and length of hospital stay[36,92]. A small retrospective study of 10 patients undergoing laparoscopic interval appendicectomy reported no complications and all patients were discharged on the day after surgery. A prospective study of open and laparoscopic appendicectomy for acute appendicitis in 65 patients showed a significantly lower wound infection rate in the laparoscopic group; however, it is not possible to extrapolate directly this finding to interval appendicectomy, even though one would expect a lower wound infection rate. In one study, the morbidity rates, particularly for intra-abdominal abscesses and wound infection, were lower for laparoscopic appendectomy in complicated appendicitis than those reported in the literature for open appendectomy, whereas operating times and hospital stays were similar[88].

RISK OF RECURRENCE

The recurrence rate of appendiceal pathology if appendicectomy is not performed is central to the debate over the use of routine interval appendicectomy. For some authors, the risk of recurrence after successful nonsurgical treatment was about 10% (3%-25% in the literature) and was often associated with an appendicolith. The majority of recurrences occur within 6 mo after initial hospital stay. Recurrence is characterized by a milder course than the primary attack in most cases. Elective interval appendectomy is associated with morbidity in about 11% (0%-23%) of patients. These results do not motivate routine elective interval appendectomy after successful nonsurgical treatment[16,20,27,98]. The literature review shows that at least 75%-90% of routine interval appendicectomies in adults are unnecessary. It would be reasonable and perhaps safer, as malignancy can be missed at appendicectomy, to replace routine interval appendicectomy with adequate follow-up of symptoms, performing appendicectomy only if symptoms recur or persist. Appropriate investigation should be done if the appendix is not removed, provided the patient has access to surgical care should symptoms recur[27].

HISTOLOGY

Several studies have examined the microscopic changes in the interval appendicectomy specimen. Many specimens show chronic inflammatory changes (52%)[5] and acute inflammation (50%)[3,8]. However, this may be of little clinical importance in the asymptomatic patient. The real concern is whether leaving the appendix *in situ* will prevent the detection of a cecal carcinoma or an ileal or appendicular malignancy[27].

RISK OF MISSING OTHER DIAGNOSES

Nonsurgical treatment is associated with a risk of missing or delaying an underlying cancer diagnosis or CD in about 2% of patients. The concern of failing to diagnose a rare case of appendiceal malignancy without interval appendicectomy may persist even with colonic investi-

gation, although it is likely that these patients will have recurrent symptoms[99-101]. Most of the cancer cases occur in patients aged > 40 years. The risk of missing an important alternative diagnosis is probably lower if imaging is used for the diagnosis of enclosed appendiceal inflammation. This underlines the need of follow-up after nonsurgical treatment, especially in patients aged > 40 years. By tradition, this follow-up consists of colonoscopy or a barium study of the colon, but a virtual colonoscopy, CT scan, or US is probably more accurate to detect malignant conditions outside the colon or CD. Malignant disease was detected during follow-up in 1.2% of patients. This risk was related to age at diagnosis with 0.2% in children, 1.8% in studies of all ages, and 1.4% in adults. There was no difference in relation to how the diagnosis was done. CD was detected in 0.7% during follow-up after nonsurgical treatment. This risk was related to age with 0.1% in children, 0.8% in all ages, and 1.5% in adults. There was no difference in relation to how the diagnosis was done. Appendicular malignancy is rare and may be missed if appendicectomy is not performed; however, it is likely that such patients will have either a nonresolving mass or early recurrence. Colonic malignancy is a more common concern, but interval appendicectomy is not a reliable method of detecting a cecal tumor. Imaging is needed when cecal malignancy is possible. Colonic investigation should be a consideration regardless of whether interval appendicectomy is performed[27].

CONCLUSION

In patients with suspicion of contained appendiceal inflammation, based on a palpable mass or long duration of symptoms, the diagnosis should be confirmed by imaging techniques, especially CT scan. The patient should receive primary nonsurgical treatment with antibiotics and abscess drainage as needed. After successful nonsurgical treatment, no interval appendectomy is indicated in some cases, but the patient should be informed about the risk of recurrence especially in the presence of appendicolith. The risk of missing another underlying condition (cancer or CD) is low, but motivates a follow-up with a colon examination and/or a CT scan or US, especially in patients above the age of 40 years.

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MINIREVIEWS

Exposure to ambient air particulate matter and non-alcoholic fatty liver disease

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Abstract

The present study was designed to alert the public opinion and policy makers on the supposed enhancing effects of exposure to ambient air particulate matter with aerodynamic diameters < 2.5 mm ($PM_{2.5}$) on non-alcoholic fatty liver disease (NAFLD), the most common chronic liver disease in Western countries. For far too long literature data have been fixated on pulmonary diseases and/or cardiovascular disease, as consequence of particulate exposure, ignoring the link between the explosion of obesity with related syndromes such as NAFLD and air pollution, the worst characteristics of nowadays civilization. In order to delineate a clear picture of this major health problem, further studies should investigate whether and at what extent cigarette smoking and exposure to ambient air $PM_{2.5}$ impact the natural history of patients with obesity-related NAFLD, *i.e.*, development of non alcoholic steatohepatitis, disease characterized by a worse prognosis due its progression towards fibrosis and hepatocarcinoma.

Key words: Non-alcoholic fatty liver disease; Particulate matter with aerodynamic diameters < 2.5 μm; Cytochrome P-450; Reactive oxygen species

Core tip: Important arguments Diesel exhaust particles are known to be major constituents of atmospheric particulate matter (PM) in metropolitan areas. Exposure to PM is positively associated with increases in the morbidity and daily mortality. Obesity-related health complications include cardiovascular disease, type 2 diabetes, hyperlipidemia, hypertension and non-alcoholic fatty liver disease (NAFLD). Exposure to ambient air PM may induce/worsen NAFLD.

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INTRODUCTION

The rising incidence of obesity in today's environment is associated with many obesity-related health complications, including cardiovascular disease, type 2 diabetes, hyperlipidemia, hypertension, and non-alcoholic fatty liver disease (NAFLD)[1-4]. This constellation is also recognized as the metabolic syndrome and is characterized by underlying insulin resistance. NAFLD or generally speaking hepatic steatosis is defined as the accumulation of lipid, primarily in the form of triacylglycerols in individuals who do not consume significant amounts of alcohol

(< 20 g ethanol/d) and in whom other known causes of steatosis, such as certain drugs and toxins, have been excluded[5]. The spectrum of NAFLD includes simple fatty liver, non alcoholic steatohepatitis (NASH) characterized by inflammation, apoptosis, ballooning degeneration, Mallory hyaline, fibrosis, cirrhosis post NASH, hepatocellular carcinoma and advanced liver disease, which leads to liver-related death[5-10].

Some epidemiological studies, deeply informed and full of insights, have demonstrated that exposure to ambient particulate matter (PM) is positively associated with increases in the morbidity and daily mortality caused by diseases, including ischemic heart disease[11,12] and chronic obstructive pulmonary disease[13,14], which are closely related to life habits. Diabetes mellitus and its complications are the other typical diseases related to life habits. Over the past several decades, prevalence of type 2 diabetes mellitus has reached epidemic levels in Western countries[15], which is a significant public health interest. The prognosis of patients with diabetes mellitus is worsened generally by a variety of complications including macro- or micro-angiopathy[16], fatty liver[17-20], nephropathy and infection in the presence or absence of overweight/obesity. Some epidemiological studies have reported a positive association between mortality in patients with diabetes mellitus and ambient levels of PM[21,22].

Air pollutants expelled from diesel engine-powered automobiles include diesel exhaust particles (DEP), which are known to be major constituents of atmospheric PM in metropolitan areas. DEP generate reactive oxygen species (ROS)[23], through a non enzymatic process[24], or enzymatic reactions catalyzed by cytochrome P-450 (Cyp)[25]. Furthermore, DEP enhance the gene expression for Cyp enzymes[25]. DEP induce a variety of biological damage at least partly through oxidative stress[25].

The present study was designed to alert the public opinion, international media and policy makers on the negative effects of exposure to ambient air particulate matter with aerodynamic diameters < 2.5 mm on NAFLD, a most common chronic liver disease in Western countries, which represents the first indication of liver transplantation.

SMOKING AND NAFLD

A growing body of evidence supports the potential effects of exposure to some environmental factors on liver diseases. Environmental exposure related to toxic waste sites was associated with an increased prevalence of autoimmune liver disease[26,27]. Therefore, increasing attention is being given to the effects of environmental factors on liver diseases, including NAFLD. Several recent studies have too reported the association of smoking with the incidence of and acceleration of disease progression in NAFLD, as well as with advanced fibrosis in this process[28-32].

Cigarette smoke exposure, whether passive or active, carries a high disease burden worldwide[33] and is considered a worldwide major cause of preventable morbidity and mortality[34].

Yuan *et al*[35] provide novel evidence demonstrating that tobacco smoke exposure may accelerate the development of experimental NAFLD. The study extends an earlier report from the group showing that in apo B transgenic mice, chronic environmental (second-hand) smoke exposure is associated to features of atherosclerotic plaque initiation[36]. Using the same model, the former authors now show that exposure to second-hand smoke potentiates steatogenesis elicited by a high-fat diet, as assessed by red oil staining and hepatic triglyceride quantification[35]. Since increased hepatic lipogenesis has been shown to account for about 30% of triglyceride accumulation in steatotic livers[37], the investigators subsequently review the impact of second-hand smoke on liver lipogenic pathways. Interestingly, cultured hepatocyte cell lines exposed to second-hand smoke display enhanced accumulation of triglycerides and increased expression of acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS), two key enzymes governing hepatic synthesis of fatty acids. These data therefore indicate that the steatogenic properties of tobacco smoke are at least partly explained by a direct effect on hepatocytes.

In deciphering molecular determinants underlying tobacco-dependent activation of lipogenesis, the research focus on two key regulators of lipid metabolism, Sterol regulatory element binding protein-1c (SREBP-1c) and AMP-activated protein kinase (AMP kinase). SREBPs are a family of basic-helix-loop-helix-leucine zipper transcription factors synthesized as inactive precursors embedded in the endoplasmic reticulum[38]. Activation of SREBPs requires proteolytic cleavage, thereby allowing nuclear translocation and transcriptional activation of target lipogenic genes[39]. Whereas SREBP-2 governs synthesis of cholesterol, SREBP-1c promotes biosynthesis of fatty acids by upregulating enzymes such as ACC and FAS. The serine/threonine protein kinase AMP kinase is an energy sensor that acts as a metabolic master switch[40]. The phosphorylated active form of the enzyme simultaneously inhibits energy-consuming biosynthetic pathways such as lipogenesis and activates ATP-producing catabolic pathways such as fatty acid oxidation[40]. It has been shown that AMP kinase inhibits fatty acid synthesis both by phosphorylating target lipogenic enzymes and downregulating expression of transcription factors such as SREBP-1c[41-43]. In accordance with these data, Yuan *et al*[35] demonstrated that second-hand smoke exposure inhibits phosphorylation and activation of AMP kinase, thereby resulting in increased SREBP-1 activity and enhancement of fatty acid synthesis. Zein *et al*[44] showed that cigarette smoking were associated with increased fibrosis severity in human NALFD, suggesting it may accelerate disease progression.

Moreover, Yuan *et al*[35] extends this assumption to NAFLD and provides compelling evidence indicating that tobacco smoke might alter the regulatory effect of AMP kinase on lipid metabolism. Future studies should

closely investigate the clinical relevance of these findings. Nevertheless, in the meantime, tobacco cessation might be considered in the management of patients with NAFLD.

NAFLD AND AIR POLLUTION

The harmful effects of air pollutants on atherosclerotic cardiovascular diseases are well-documented[31]. These effects might be mediated through oxidative stress and insulin resistance[45], which are also known to have pivotal roles in the pathogenesis of fatty liver[46]. Therefore, it can be assumed that such environmental factors might be too associated with NAFLD. It is well-documented that DEP, which are major constituents of atmospheric PM in urban areas, generate ROS[47]. The ROS are generated *via* enzymatic reactions catalyzed by Cyp[48], or by a non-enzymatic route[49].

Folkmann *et al*[50] assessed the effects of oxidative stress elicited by DEP in the aorta, liver, and lungs of dyslipidemic ApoE(-/-) mice, at the age when visual plaques appeared in the aorta. Vascular effects secondary to pulmonary inflammation were omitted by injecting DEP into the peritoneum. Six hours later, the expression of inducible nitric oxide synthase mRNA increased in the liver. Injection of DEP did not induce inflammation or oxidative damage to DNA in the lungs and aorta. Therefore, the study proposed a direct effect of DEP on inflammation and oxidative damage to DNA in the liver of dyslipidemic mice[50].

Another study[51] evaluated the effects of following exposure of male C57BL/6 mice fed high fat chow to concentrated air particulate matter or filtered air for 6 wk, progression of NAFLD was evaluated by standardized histological assessment of hepatic inflammation and fibrosis. Progression of NAFLD was evaluated by histology cal examination of hepatic inflammation and fibrosis. Tan *et al*[51] indicated that ambient PM that reaches the liver has the potential to induce Kupffer cell cytokine secretion. Circulating fine PM may then accumulate in both atherosclerotic plaques and hepatic Kupffer cells[51]. The activation of cytokine release by Kupffer cells may then trigger inflammation and hepatic stellate cell collagen synthesis[51]. It is extraordinary that interleukin-6, the concentration of which increased up to 7-fold in the above-mentioned study, is too significantly abundant in cases of human NAFLD[52]. Some human studies confirmed the harmful effects of environmental toxins on liver diseases.

Cave *et al*[53] has showed that non-obese chemical workers highly exposed to vinyl chloride may develop insulin resistance and toxicant-associated steatohepatitis. Limited data exists on the potential role of environmental pollution on liver disease in the general population. Another study was conducted, by Cave *et al*[54] always, on 4582 adult participants without viral hepatitis, hemochromatosis, or alcoholic liver disease, from the National Health and Nutrition Examination Survey in 2003-2004, to investigate whether environmental pollutants are associated with an elevation in serum alanine aminotransferase (ALT) and suspected NAFLD. The ORs for ALT elevation were estabilished across exposure quartiles for 17 pollutants, after adjustments for age, race/ethnicity, sex, body mass index, poverty income ratio, and insulin resistance[54]. It showed that exposure to polychlorinated biphenyls in addition heavy metals, evident lead and mercury, was correlated with unexplained ALT elevation, and increased adjusted ORs for ALT elevation in a dose-dependent form[54].

Therefore, a growing number of studies suggest that air pollution can aggravate the adverse effects of obesity and insulin resistance[29,55,56]. Similarly, some other studies have documented the association of exposure to air pollutants with metabolic syndrome, as well as predisposition to diabetes mellitus and aggravation of its complications[57-59]. Given the inflammatory and oxidative properties of air pollutants, in addition their association with insulin resistance and metabolic syndrome, and considering the interaction of the latter conditions with fatty changes in liver, more studies about the effects of environmental factors, notably air pollution, on NAFLD are warranted. The high susceptibility of the young age group to the harmful effects of air pollutants, especially pertaining to early stages of chronic diseases[13,60-64], further stresses that more attention should be given to preventing late-onset effects of air pollutants.

FUTURE DIRECTIONS

It has been reported that ambient PM containing elementary carbon, sulfate, heavy metals, and organic compounds can cause and enhance cardiopulmonary diseases[65,66]. DEP form a large constituent of ambient urban PM. Inhalation or intratracheal instillation of DEP or the components of DEP has been shown to enhance lung inflammation and asthma[67,68], and to deteriorate biological cardiovascular functions[69]. On the other hand, cardiovascular disorders are critical participants in life habit diseases. Diabetes mellitus is another typical life habit disease and is characterized by complicated cardiovascular risk factors[70-72]. In epidemiological studies, individuals with diabetes mellitus have higher risk for death from exposure to polluted ambient air[73,74]. However, few experimental studies have elucidated the association between ambient air pollution and NAFLD.

In fact, Zein *et al*[44] showed that smoking may accelerate the progression of human NALFD and these observations may support a recommendation of smoking cessation in patients with NAFLD. This recommendation is added to the general recommendations of dietetic or lifestyle approach right for NAFLD patients[75].

As reported by Tan *et al*[51], exposure to ambient air particulate matter with aerodynamic diameters < 2.5 μm ($PM_{2.5}$) may be a significant risk factor for NAFLD progression. In other words, could air pollution be the so-called "second hit", according to the well-known theory?

A better understanding of the impact of ambient PM

exposure on NAFLD progression may require studies utilizing a variety of ambient PM sources[51].

CONCLUSION

So far it has been known that $PM_{2.5}$ result from fuel combustion (motor vehicles, power generation, industrial facilities), residential fireplaces and wood stoves. $PM_{2.5}$ are usually selected as indicators of air pollution since those particles cause morbidity[76]. In fact, $PM_{2.5}$ alone exposure could cause inflammation *via* tumor necrosis factor alpha[77], endothelial function and autonomic nervous system injuries, ozone potentiating these effects[78].

Further studies should investigate the effects of long-lasting exposures to cigarette smoking and to ambient air $PM_{2.5}$ on specific pathways of the hepatic metabolism, better delineating the cellular and molecular mechanisms involved. Importantly, very informative reports should clarify whether cigarette smoking, habit started at very young age, and early exposure to ambient air $PM_{2.5}$ impact the obesity, also the adolescents' one, and the obesity-related NAFLD, favouring development of NASH, disease characterized by a worse prognosis due its progression towards fibrosis, liver cirrhosis and hepatocarcinoma.

By modifying the natural history of patients with NAFLD, air pollution adds a new argument in the debate of regulating the toxic emissions.

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ORIGINAL ARTICLE

Differential mucin phenotypes and their significance in a variation of colorectal carcinoma

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Abstract

AIM: To investigate mucin expression profiles in colorectal carcinoma (CRC) histological subtypes with regard to clinicopathologic variables and prognosis.

METHODS: Mucin (MUC)2 and MUC5AC expressions were assessed by immunohistochemistry for a total of 250 CRC cases that underwent surgical resection. CRCs included 63 well-to-moderately differentiated adenocarcinomas (WMDAs), 91 poorly differentiated adenocarcinomas (PDAs), 81 mucinous adenocarcinoma (MUAs), and 15 signet-ring cell carcinomas (SRCCs). MUC2 and MUC5AC were scored as positive when ⩾ 25% and ⩾ 1% of cancer cells were stained positive, respectively. The human mutL homolog 1 and human mutS homolog 2 expressions were assessed by immunohistochemistry in PDAs to investigate mismatch-repair (MMR) status. Tumors that did not express either of these two were considered MMR-deficient. Results were analyzed for associations with clinicopathologic variables and the prognosis in individual histological CRC subtypes.

RESULTS: MUC2-positive and MUC5AC-positive WMDA percentages were 49.2% and 30.2%, respectively. In contrast, MUC2-positive and MUC5AC-positive PDA percentages were 9.5% and 51.6%, respectively. MUC2 levels tended to decrease and MUC5AC levels tended to increase from WMDA to PDA. In 21 tumors comprising both adenoma and adenocarcinoma components in a single tumor (4 WMDAs, 7 PDAs, and 10 MUAs), MUC2 was significantly downregulated in PDA and MUC5AC was downregulated in PDA and MUA in the adenoma-carcinoma sequence. These results suggested that MUC2 levels might be associated with malignant potential and that MUC5AC expression was an early event in tumorigenesis. Despite worse prognoses than WMDA, high MUC2 expression levels were maintained in MUA (95.1%) and SRCC (71.5%), which suggested a pathogenesis for these subtypes distinct from that of WMDA. No significant associations were found between MUC2 expression and any clinicopathologic variables in any histological subtype. MUC5AC expression in PDA was closely associated with right-sided location (P = 0.017), absence of nodal metastasis (P = 0.010), low tumor node metastasis stage (P = 0.010), and MMR deficiency (P = 0.003). MUC2 expression in WMDA was a marginal prognostic factor for recurrence/metastasis-free survival (RFS) by univariate Cox analysis (P = 0.077) but not by multivariate Cox analysis (P = 0.161). MUC5AC expression in PDA was a significant prognostic factor for RFS by univariate Cox analysis (P = 0.007) but not by multivariate Cox analysis (P = 0.104). Kaplan-Meier curves and log-rank tests revealed that MUC2 expression was marginally associated with a better WMDA prognosis [P = 0.064 for RFS and P = 0.172 for overall survival (OS)] but not for PDA. In contrast, MUC5AC expression was significantly and marginally associated with a better PDA prognosis in terms of RFS and OS, respectively

(P = 0.004 for RFS and P = 0.100 for OS), but not for WMDA and MUA.

CONCLUSION: Mucin core protein expression profiles and clinical significance differ according to histological CRC subtypes. This may reflect different pathogeneses for these tumors.

Key words: Mucin 2; Mucin 5AC; Microsatellite instability; Mismatch repair; Colorectal carcinoma; Poorly differentiated adenocarcinoma; Pathogenesis; Adenoma-carcinoma sequence; Prognosis

Core tip: Altered mucin expression may be correlated with biological behavior and possibly with the prognosis of colorectal carcinoma (CRC). However, many contradictory results make it difficult to interpret its clinical significance, possibly because of CRC variations. Therefore, we examined mucin (MUC)2 and MUC5AC expressions in different pathological CRC subtypes by immunohistochemistry to determine their true clinical significance. Our results suggest that the expression profiles and the clinical significance of these mucin core proteins are different according to histological subtypes. This may reflect different pathogeneses for these tumors.

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INTRODUCTION

Mucins are a diverse family of high-molecular-weight glycoproteins that are widely expressed in epithelial tissues and are characterized by the presence of tandem repeat sequences that are rich in highly *O*-glycosylated serine and threonine residues[1]. Mucins can be classified as either membrane-associated or secretory glycoproteins. To date, a total of 20 human mucins have been identified. Secreted mucins can be gel-forming or non-gel-forming and include mucin (MUC)2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9, and MUC19. Transmembrane mucins include MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, and MUC21; these are anchored to the plasma membranes of various cells through a transmembrane domain. These mucin proteins are encoded by various *MUC* genes[2]. The genes for gel-forming mucins MUC2 and MUC5AC are found in a cluster on chromosome 11p15.5[3]. The *MUC2* gene codes for a typical secretory mucin, which is predominantly found in colorectal goblet cells, and the *MUC5AC* gene is mainly expressed in gastric and tracheal-bronchial mucosa.

Altered expressions of MUC2 and MUC5AC may be significantly correlated with the biological behavior of and, possibly, the prognosis for colorectal carcinoma (CRC). However, many contradictory results make it difficult to interpret their clinical significance. For example, MUC2 expression is significantly decreased according to CRC disease progression[4-6]. MUC2-positive CRC shows a relatively good prognosis or a low incidence of liver and nodal metastasis[7,8]. Suppressing the *MUC2* gene expression in colon carcinoma cell lines in vitro was associated with methylation of its promoter region[7]. In contrast, other studies reported that MUC2 expression was not a significant marker of tumor invasion depth, liver metastasis, or overall survival[9,10]. However, the absence of MUC5AC expression can be a prognostic indicator of a more aggressive colorectal tumor. Highly villous adenoma with severe dysplasia expressed a less MUC5AC than larger adenomas of moderate villous histology and dysplasia[11]. Carcinomas with low grade atypia exhibited a higher incidence of MUC5AC expression as compared with carcinomas showing high grade atypia[6]. Consistently, MUC5AC expression analysis combined with survival analysis has demonstrated that those patients with MUC5AC-negative CRC had lower rates for disease-free status and of overall survival[12].

Most studies analyzed CRC without detailed classifications. However, in the World Health Organization (WHO) classification, CRC consists of various histological subtypes, such as conventional adenocarcinoma, mucinous adenocarcinoma (MUA), signet-ring cell carcinoma (SRCC), squamous cell carcinoma, adenosquamous carcinoma, medullary carcinoma, undifferentiated carcinoma, and other very rare variants[13]. Conventional adenocarcinoma is further sub-classified into well-to-moderately differentiated adenocarcinoma (WMDA) and poorly differentiated adenocarcinoma (PDA) based on the percentage of the area showing a gland-like structure[13]. Most CRCs encountered in the clinic are WMDA and poorly differentiated/undifferentiated carcinomas are rare, accounting for up to 16% of all CRCs in the United States[14,15]. The purpose of this study was to assess MUC2 and MUC5AC expressions in different pathological CRC subtypes by immunohistochemistry and to determine their true clinical significance.

MATERIALS AND METHODS

Patients and tumor samples

For this study, WMDA included all consecutive cases that were surgically resected in Tokyo Kosei Nenkin Hospital from April 1998 to March 2000, but excluded 10 other histological CRC subtypes. These cases included 63 tumors from 63 patients. In addition, a total of 187 histological CRC subtypes other than WMDA were collected from all CRC cases resected in Dokkyo Medical University Koshigaya Hospital between 1990 and 2011 and Tokyo Kosei Nenkin Hospital between 1991 and 2010.

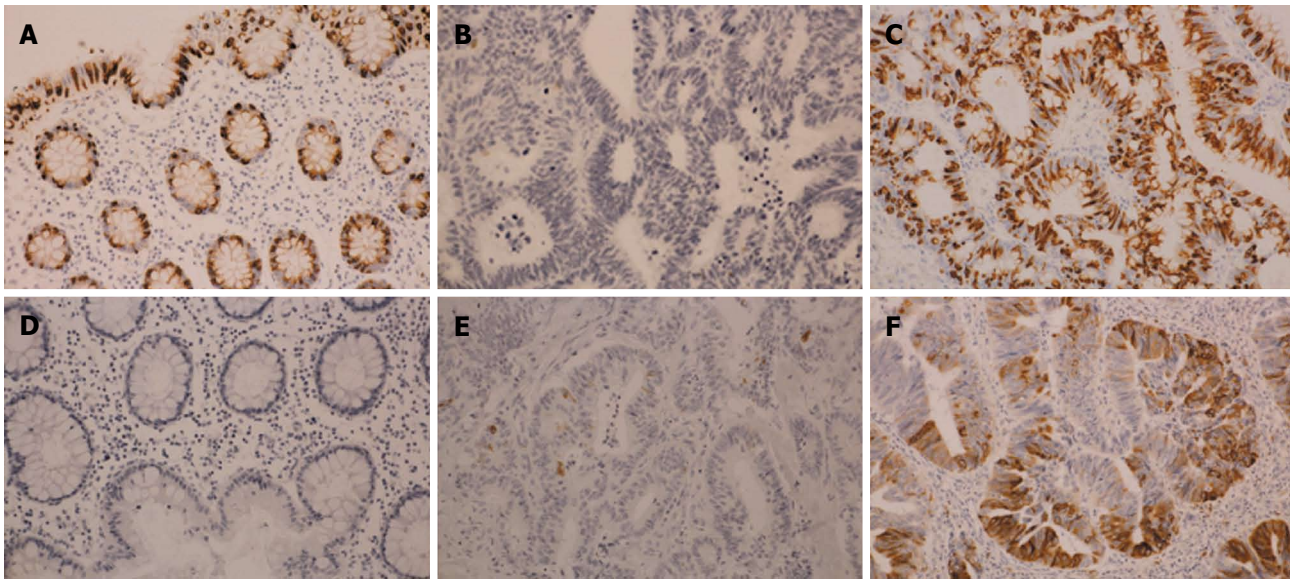


Figure 1 Expression of mucin 2 and mucin 5AC in colorectal carcinomas. A: Mucin (MUC)2 expression in normal colonic mucosa; B: MUC2 expression in cancer: level 0; C: MUC2 expression in cancer: level 4; D: MUC5AC expression in normal colonic mucosa; E: MUC5AC expression in cancer: level 1; F: MUC5AC expression in cancer: level 4 (immunohistochemical staining, × 10).

Formalin-fixed, paraffin-embedded tissue blocks were obtained from the archival material stored in the pathology departments of the both hospitals. A sufficient number of samples to provide for complete investigations were available for all these cases. Patients whose medical records were sufficiently complete were included in survival analysis. Patients with invasive cancers originating from other sites were excluded from the analysis. Clinicopathologic classifications and stage groupings were based on the WHO classification of colorectal tumors and the tumor node metastasis (TNM) staging by the American Joint Committee on Cancer[13,16]. Our study protocol was approved by the ethical review boards of the participating hospitals.

Immunohistochemistry

Tumor specimens were fixed in 10% neutral-buffered formalin for 48 h, embedded in paraffin, and cut into 4-μm-thick sections, and then mounted on silane-coated glass slides. Antigen-retrieval was done by autoclaving (121 ℃) for 5 min in pH 9 Antigen Retrieval Liquid (Nichirei, Tokyo, Japan) for MUC2, MUC5AC, and hMSH2, and by microwave irradiation for 10 min in pH 9 Antigen Retrieval Liquid for human mutL homolog 1 (hMLH1). Primary antibodies used were the mouse monoclonal antibody for MUC2 (1:100 dilution; clone Ccp58, Novocastra, Newcastle Upon Tyne, United Kingdom), the mouse monoclonal antibody for MUC5AC (1:100 dilution; CLH2, Novocastra), the rabbit anti-MLH1 monoclonal antibody (1:400 dilution; EPR3894, GeneTex, San Antonio, TX, United States), and the rabbit anti-MSH2 polyclonal antibody (1:200 dilution; 15520-1-AP, Proteintec, Chicago, IL, United States). Samples were treated overnight with each primary antibody at 4 ℃. Immunostaining was performed blindly by an investigator (Fukuda K) who was unaware of the clinical information using an N-Histofine Simple Stain MAX-PO kit (Nichirei).

The immunostaining results for mucin core proteins were assessed semi-quantitatively: 0, no staining; 1, < 5% of cells; 2, 5% to < 25% of cells; 3, 25% to < 50% of cells; 4, ⩾ 50% of cells (Figure 1). The immunostaining results for mismatch repair (MMR) proteins were either completely negative (negative) or nearly 100% positive (positive). Immunoreactivity was independently evaluated by two investigators (Fukuda K and Imai Y), and discrepancies were resolved by discussion.

In light of their expression levels in normal colonic mucosa, levels 3-4 for MUC2 and levels 1-4 for MUC5AC were evaluated as positive.

Statistical analysis

Comparisons of two cohorts with or without a specific clinicopathologic variable were made by a χ^2 test with/without a Yates' correction or Fisher's exact probability test based on the expected values in a contingency table. Age was compared with Mann-Whitney *U* test. Comparisons of the mucin expression levels between adenoma and adenocarcinoma components in a single tumor were made by Wilcoxon signed-rank test for sample numbers of ⩾ 6. Univariate analysis by Cox regression analysis was used to identify possible prognostic predictors. Variables for which *P* values were < 0.10 were entered into multivariate regression analysis (forced entry method). Survival curves were generated using the Kaplan-Meier method, and curves were compared by log-rank test. *P* value < 0.05 was considered significant. Statistical analysis was performed using IBM SPSS Statistics 20 (IBM, Armonk, NY, United States).

Table 1 Clinicopathologic characteristics in colorectal carcinoma *n* (%)

Variables		WMDA (*n* = 63)	PDA (*n* = 91)	MUA (*n* = 81)	SRCC (*n* = 15)
Gender	Male	39 (61.9)	45 (49.5)	46 (56.8)	6 (40.0)
	Female	24 (38.1)	46 (50.5)	35 (43.2)	9 (60.0)
Age (yr)	Range	32-87	35-92	26-90	30-82
	Median	65	64	71	70
Family history of CRC	Yes	5 (7.9)	5 (6.1)	5 (6.3)	2 (13.3)
	No	58 (92.1)	77 (93.9)	75 (93.7)	13 (86.7)
	Unknown		9	1	
Location	Left-sided	43 (68.3)	38 (41.8)	40 (49.4)	6 (40.0)
	Right-sided	20 (31.7)	53 (58.2)	41 (50.6)	9 (60.0)
Depth	Up tp MP	10 (15.9)	4 (4.4)	5 (6.2)	1 (6.7)
	Beyond MP	53 (84.1)	87 (95.6)	76 (93.8)	14 (93.3)
Venous invasion	Yes	48 (76.2)	77 (85.6)	41 (50.6)	14 (93.3)
	No	15 (23.8)	13 (14.4)	40 (49.4)	1 (6.7)
	Unknown		1		
Lymphatic invasion	Yes	35 (55.6)	82 (91.1)	54 (66.7)	12 (80.0)
	No	28 (44.4)	8 (8.9)	27 (33.3)	3 (20.0)
	Unknown		1		
Nodal metastasis	Yes	34 (54.0)	69 (78.4)	37 (46.8)	9 (64.3)
	No	29 (46.0)	19 (21.6)	42 (53.2)	5 (35.7)
	Unknown		3	2	1
Chemotherapy	Yes	17 (27.0)	42 (53.8)	31 (41.9)	7 (46.7)
	No	46 (73.0)	36 (46.2)	43 (58.1)	8 (53.3)
	Unknown		13	7	
Irradiation	Yes	4 (6.3)	2 (2.6)	3 (4.1)	1 (7.1)
	No	59 (93.7)	76 (97.4)	71 (95.9)	14 (92.9)
	Unknown		13	7	
TNM stage	Ⅰ/Ⅱ	29 (46.0)	17 (18.9)	40 (50.0)	4 (26.7)
	Ⅲ/Ⅳ	34 (54.0)	73 (81.1)	40 (50.0)	11 (73.3)
	Unknown		1	1	0

CRC: Colorectal carcinoma; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma; MP: Muscularis propria; TNM: Tumor node metastasis.

RESULTS

Clinicopathologic characteristics

WMDA cases included 63 tumors from 63 patients. Five of these patients (5 tumors) had a family history of CRC. Additional chemotherapy and irradiation were administered for 17 tumors in 17 patients and 4 tumors in 4 patients, respectively. CRCs other than WMDA included a total of 187 tumors: 91 PDAs (90 patients); 81 MUAs (81 patients); and 15 SRCCs (15 patients). One patient had triple cancers (No. 148: two PDAs and one MUA) and one patient had double cancers (No. 170: one PDA and one MUA). Ten of these patients (12 tumors) had a family history of CRC, one of which was proven to be a hereditary non-polyposis colorectal cancer pedigree (No. 148). Predominant occurrence in females, right-sided location, depth of tumors beyond muscularis propria, lymphatic invasion, nodal involvement (except for MUA), TNM stage Ⅲ/Ⅳ (except for MUA) were more frequent in CRCs other than WMDA as compared with WMDA (Table 1). In addition to surgery, chemotherapy and irradiation were administered for 80 tumors in 80 patients and 6 tumors in 6 patients, respectively.

The clinicopathologic characteristics of each CRC subtype are summarized in Table 1. CRC patient prognosis was significantly associated with these histological subtypes (Figure 2).

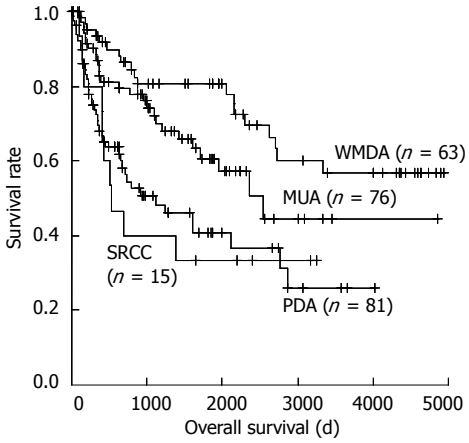


Figure 2 Colorectal carcinoma overall survival curves generated by the Kaplan-Meier method. WMDA: Well-to-moderately differentiated adeno-carcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma.

Expression of MUC2 and MUC5AC in CRCs

Expression of the mucin core proteins was assessed individually in different pathological subtypes. MUC2 was expressed in the perinuclear cytoplasm of goblet cells in normal colonic mucosa and diffusely in the cytoplasm of cancer cells. MUC5AC was not expressed in normal colonic mucosa but was occasionally expressed in pericancerous normally appearing colonic mucosa. About half of WMDA cases (49.2%) were positive for MUC2 (levels 3-4), and 30.2% of these cases were positive for MUC5AC (levels 1-4). In contrast, only one tenth of PDA cases (9.5%) were positive for MUC2 and 51.6% of these cases were positive for MUC5AC. PDA was more frequently negative for MUC2 and positive for MUC 5AC than was WMDA. Nearly all MUA cases (95.1%) were positive for MUC2, and over half of these cases (54.3%) aberrantly expressed MUC5AC. Although small in number, 71.5% of SRCC cases were positive for MUC2 and nearly half (46.7%) expressed MUC5AC. These results are summarized in Figure 3.

Expression of the mucin core proteins in the adenoma-carcinoma sequence

Among our study subjects, 21 tumors in 20 patients had an adenoma component indicative of originating from the adenoma-carcinoma sequence. We investigated the sequential expression status of the mucin core proteins. MUC2 expression was found in the adenoma component in all cases, except for one PDA case. MUC2 expression was relatively well maintained in the carcinoma component of WMDA and mildly decreased in some MUA cases. MUC2 expression significantly decreased in the carcinoma component of PDA. MUC5AC was aberrantly expressed in the tubular/tubulovillous adenoma components in all but three cases, and there was a signifi-

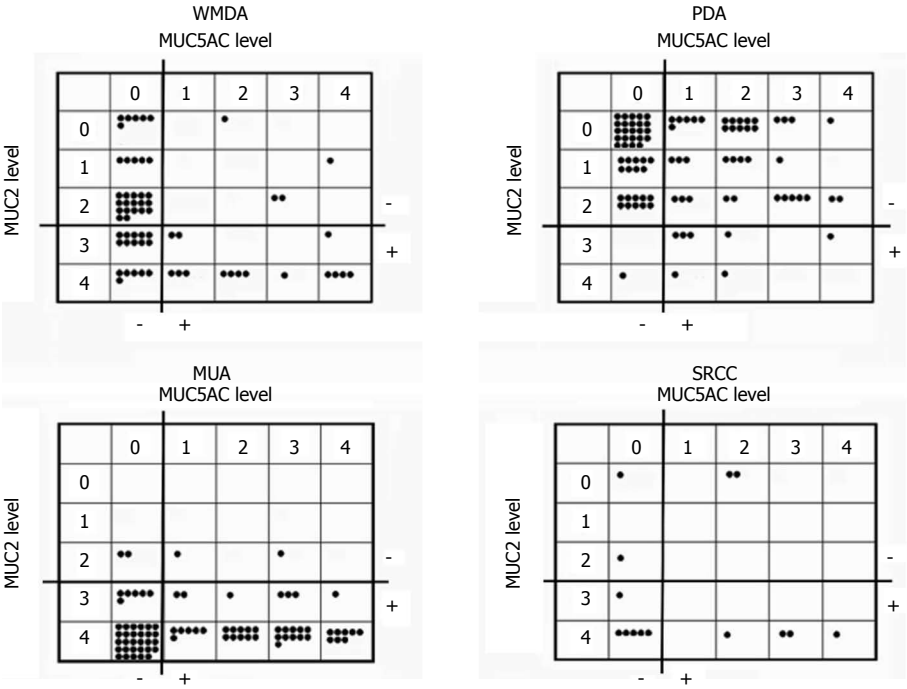


Figure 3 Expression profiles of mucin 2 and mucin 5AC in each colorectal carcinoma histological subtype. Each closed circle indicates one tumor. MUC: Mucin; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma.

Table 2 Expression levels of the mucin core proteins in the adenoma carcinoma sequence

	Histology		MUC2			MUC5AC		
No. of patient	Adenoma	Carcinoma	Adenoma	Carcinoma	*P* value[1]	Adenoma	Carcinoma	*P* value[1]
208	TV	WMDA	4	3	ND	4	1	ND
225	T	WMDA	4	4		3	2	
237	TV	WMDA	4	4		2	4	
243	TV	WMDA	4	4		4	4	
84	TV	PDA	4	2	0.016	4	0	0.042
86	TV	PDA	4	0		1	0	
87	TV	PDA	4	0		0	0	
102	TV	PDA	4	0		4	0	
116	T	PDA	2	0		2	0	
138	T	PDA	4	0		3	0	
148	T	PDA	3	0		0	0	
15	TV	MUA	4	4	0.063	3	0	0.016
36	TV	MUA	4	4		3	3	
37	TV	MUA	4	4		0	0	
51	TV	MUA	4	4		3	3	
131	T	MUA	4	2		3	1	
148	TV	MUA	4	2		4	0	
151	TV	MUA	4	3		3	1	
155	TV	MUA	4	4		4	2	
175	TV	MUA	4	4		4	2	
191	TV	MUA	4	3		3	0	

[1]Wilcoxon signed-rank test. MUC: Mucin; T: Tubular adenoma; TV: Tubulovillous adenoma; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; ND: Not determined.

cant decrease in MUC5AC expression in the carcinoma components as compared with the adenoma components in PDA and MUA (Table 2).

Expression of the mucin core proteins and clinicopathologic variables

Expression status of the mucin core proteins was analyzed in association with clinicopathologic variables in each histological CRC subtype that had sufficient numbers for statistical analysis. In a contingency table analysis, no statistically significant associations were found between MUC2 expression and any clinicopathologic variables in any of the histological subtypes. In contrast, MUC5AC expression was significantly associated with right-

Table 3 Expression of the mucin core proteins and clinicopathologic variables *n* (%)

		WMDA			PDA			MUA		
		MUC2			MUC2			MUC2		
		-	+	*P* value	-	+	*P* value	-	+	*P* value
Median age (range), yr		64 (32-82)	64 (34-87)	0.783	64 (35-92)	66.5 (55-86)	0.633	65 (52-88)	71 (26-90)	0.842
Gender	Male	21 (33.3)	18 (28.6)	0.537	41 (45.1)	4 (4.4)	1.000	1 (1.2)	45 (55.6)	0.311
	Female	11 (17.5)	13 (20.6)		42 (46.1)	4 (4.4)		3 (3.7)	32 (39.5)	
Locus	Right-sided	10 (15.9)	10 (15.9)	0.932	47 (51.6)	6 (6.6)	0.461	1 (1.2)	40 (49.4)	0.359
	Left-sided	22 (34.9)	21 (33.3)		36 (39.6)	2 (2.2)		3 (3.7)	37 (45.7)	
Venous invasion	Yes	24 (38.1)	24 (38.1)	1.000	71 (79.0)	6 (6.6)	0.325	3 (3.7)	38 (46.9)	0.616
	No	8 (12.7)	7 (11.1)		11 (12.2)	2 (2.2)		1 (1.2)	39 (48.1)	
Lymphatic invasion	Yes	21 (33.3)	14 (22.2)	0.102	76 (84.4)	6 (6.7)	0.148	1 (1.2)	51 (63.0)	1.000
	No	11 (17.5)	17 (27.0)		6 (6.7)	2 (2.2)		3 (3.7)	26 (32.1)	
Nodal metastasis	Yes	20 (31.7)	14 (22.2)	0.167	64 (72.7)	5 (5.7)	0.362	2 (2.5)	35 (44.3)	1.000
	No	12 (19.0)	17 (27.0)		16 (18.2)	3 (3.4)		2 (2.5)	40 (50.6)	
TNM stage	Ⅰ/Ⅱ	12 (19.0)	17 (27.0)	0.167	14 (15.6)	3 (3.3)	0.171	2 (2.5)	38 (48.1)	1.000
	Ⅲ/Ⅳ	20 (31.7)	14 (22.2)		68 (75.5)	5 (5.6)		2 (2.5)	37 (46.8)	
dMMR	Yes	ND			16 (17.6)	3 (3.3)	0.356	ND		
	No				67 (73.6)	5 (5.5)				
		MUC5AC			MUC5AC			MUC5AC		
		-	+	*P* value	-	+	*P* value	-	+	*P* value
Median age (range), yr		64 (32-83)	69 (47-87)	0.099	63 (36-87)	70 (35-92)	0.096	67 (34-87)	72 (26-90)	0.061
Gender	Male	26 (41.3)	13 (20.6)	0.677	22 (24.2)	23 (25.3)	0.919	23 (28.4)	23 (28.4)	0.371
	Female	18 (28.6)	6 (9.5)		22 (24.2)	24 (26.3)		14 (17.3)	21 (25.9)	
Locus	Right-sided	10 (15.9)	10 (15.9)	0.410	20 (22.0)	33 (36.3)	0.017	12 (14.8)	29 (35.8)	0.003
	Left-sided	34 (54.0)	9 (14.3)		24 (26.4)	14 (15.4)		25 (30.9)	15 (18.5)	
Venous invasion	Yes	34 (54.0)	14 (22.2)	0.757	38 (42.2)	39 (43.3)	1.000	16 (19.8)	25 (30.9)	0.224
	No	10 (15.9)	5 (7.9)		6 (6.7)	7 (7.8)		21 (25.9)	19 (23.5)	
Lymphatic invasion	Yes	26 (41.3)	9 (14.3)	0.560	41 (45.6)	41 (45.6)	0.714	25 (30.9)	29 (35.8)	0.875
	No	18 (28.6)	10 (15.9)		3 (3.3)	5 (5.6)		12 (14.8)	15 (18.5)	
Nodal metastasis	Yes	23 (36.5)	11 (17.5)	0.892	40 (45.5)	29 (33.0)	0.010	17 (21.5)	20 (25.3)	0.950
	No	21 (33.3)	8 (12.7)		4 (4.5)	15 (17.0)		19 (24.1)	23 (29.1)	
TNM stage	Ⅰ/Ⅱ	21 (33.3)	8 (12.7)	0.892	3 (3.3)	14 (15.6)	0.010	18 (22.8)	22 (27.8)	0.918
	Ⅲ/Ⅳ	23 (36.5)	11 (17.5)		41 (45.6)	32 (35.6)		18 (22.8)	21 (26.6)	
dMMR	Yes	ND			3 (3.3)	16 (17.6)	0.003	ND		
	No				41 (45.1)	31 (34.1)				

MUC: Mucin; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; dMMR: Mismatch-repair deficiency; ND: Not determined; TNM: Tumor node metastasis.

sided location, absence of nodal metastasis, and lower TNM stage in PDA, and right-sided location in MUA. Furthermore, MUC5AC expression tended to be associated with older age in WMDA, PDA, and MUA, although the difference was not statistically significant. MUC5AC expression was not associated with any clinicopathologic variables in SRCC. These results are summarized in Table 3 (partly not shown).

Expression of the MMR proteins and the mucin core proteins in PDA

In PDA cases, MUC5AC positivity was significantly associated with right-sided location and lower TNM stage, and marginally associated with older age. Survival curve analysis also suggested a better prognosis for MUC5AC-positive PDA cases than for negative ones as described below. These are clinical features associated with high levels of microsatellite instability (MSI; MSI-H)[17-19]. A subset of sporadic CRC cases (approximately 10%-15%) is MSI-H; this is caused by inactivation of the DNA MMR system. Identifying MSI previously required molecular testing, although immunostaining for hMLH1 and hMSH2 has come to be accepted as a practical test to detect MSI[20,21]. Therefore, we investigated MMR status in PDA cases using immunohistochemistry. Tumors that did not express either of these two were considered MMR deficiency (dMMR).

dMMR was found in 19 PDA cases, 16 of 47 MUC5AC-positive cases and 3 of 44 MUC5AC-negative cases ($P = 0.003$). In contrast, there was no significant association between MUC2 expression and dMMR. Thus, dMMR showed statistically significant association with MUC5AC positivity, although it should be noted that dMMR was found in only one third of MUC5AC-positive tumors and dMMR was also found in one tenth of MUC5AC-negative tumors (Table 3).

Expression of the mucin core proteins and prognosis

The effects of clinicopathologic variables on recurrence/metastasis-free survival (RFS) and overall survival (OS) were investigated using Cox regression analysis for each CRC subtype. For WMDA, TNM stage was the only significant prognostic factor for RFS by univariate and multivariate analysis, but no significant predictor for OS was identified. Next, we included dMMR in the survival analysis for PDA. Univariate Cox regression analysis showed that TNM stage, MUC5AC expression, and dMMR were significant prognostic factors for RFS; however none of

Table 4 Prognostic significance of clinicopathologic variables in well-to-moderately differentiated adenocarcinoma and poorly differentiated adenocarcinoma

	Recurrence/metastasis				Death			
	Parameter		HR (95%CI)	*P* value	Parameter		HR (95%CI)	*P* value
WMDA	Univariate analysis				Univariate analysis			
		Age (over 65 yr)	2.215 (0.974-5.037)	0.058		Age (over 65 yr)	1.984 (0.794-4.955)	0.142
		Gender (male)	0.887 (0.398-1.975)	0.769		Gender (male)	2.194 (0.727-6.617)	0.163
		Location (right-sided)	1.268 (0.559-2.875)	0.570		Location (right-sided)	1.335 (0.524-3.402)	0.545
		TNM stage (Ⅲ/Ⅳ)	3.642 (1.446-9.170)	0.006		TNM stage (Ⅲ/Ⅳ)	2.632 (0.990-6.996)	0.052
		MUC2 positive	0.477 (0.210-1.082)	0.077		MUC2 positive	0.527 (0.207-1.341)	0.179
		MUC5AC positive	1.389 (0.613-3.145)	0.431		MUC5AC positive	1.803 (0.723-4.497)	0.206
	Multivariate analysis				Multivariate analysis			
		Age (over 65 yr)	2.220 (0.965-5.017)	0.061				
		TNM stage (Ⅲ/Ⅳ)	3.473 (1.370-8.805)	0.009				
		MUC2 positive	0.554 (0.243-1.265)	0.161				
PDA	Univariate analysis				Univariate analysis			
		Age (over 65 yr)	0.891 (0.495-1.602)	0.700		Age (over 65 yr)	0.985 (0.955-1.016)	0.331
		Gender (male)	0.624 (0.343-1.138)	0.124		Gender (male)	0.899 (0.484-1.667)	0.734
		Location (right-sided)	0.857 (0.474-1.549)	0.609		Location (right-sided)	0.686 (0.371-1.270)	0.231
		TNM stage (Ⅲ/Ⅳ)	2.647 (1.109-6.320)	0.028		TNM stage (Ⅲ/Ⅳ)	3.208 (1.236-8.330)	0.017
		MUC2 positive	0.936 (0.368-2.379)	0.890		MUC2 positive	1.009 (0.357-2.850)	0.987
		MUC5AC positive	0.433 (0.237-0.793)	0.007		MUC5AC positive	0.599 (0.323-1.112)	0.105
		dMMR	0.373 (0.164-0.847)	0.018		dMMR	0.352 (0.153-0.810)	0.014
	Multivariate analysis				Multivariate analysis			
		TNM stage (Ⅲ/Ⅳ)	1.698 (0.672-4.289)	0.263		TNM stage (Ⅲ/Ⅳ)	2.385 (0.886-6.421)	0.085
		MUC5AC positive	0.586 (0.307-1.117)	0.104		dMMR	0.466 (0.195-1.111)	0.085
		dMMR	0.228 (0.260-1.308)	0.175				

WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; dMMR: Mismatch-repair deficiency; TNM: Tumor node metastasis; MUC: Mucin.

these was significant by multivariate analysis. TNM stage and dMMR were also significant predictors for OS by univariate analysis but were not significant by multivariate analysis (Table 4).

For MUA, MUC2 expression was excluded from the analysis because there were very few MUC2-negative MUA cases (n = 4 out of 81). The TNM stage was the only significant predictor for RFS, and no variable was a significant predictor for OS (data not shown).

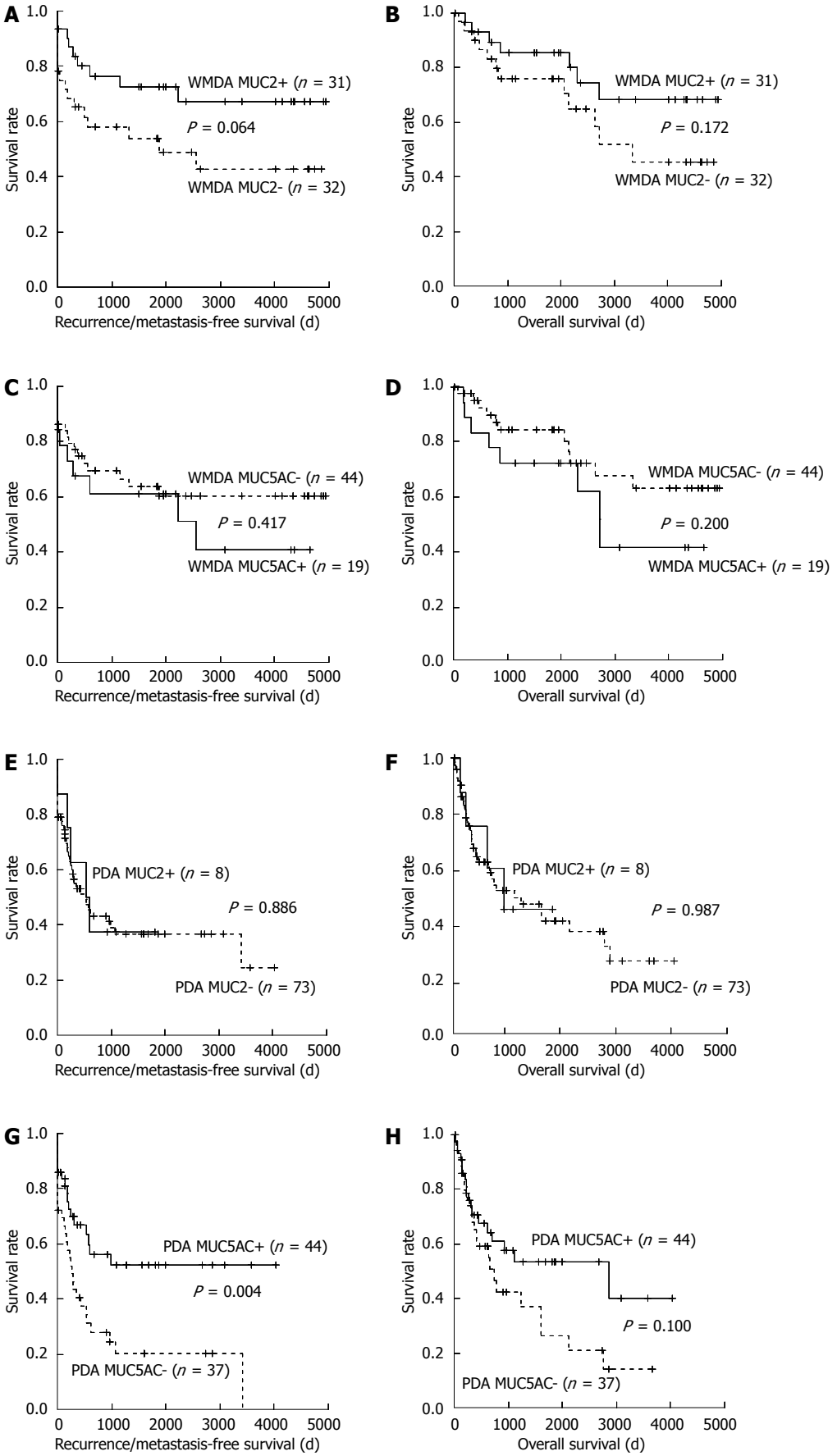
Thus, no significant associations were found between mucin expression and the prognosis of each CRC subtype by multivariate Cox analysis. However, as the data suggested marginal associations between mucin expression and prognosis, and from the need for subsequent discussion, Kaplan-Meier survival curves for associations with mucin expression were generated and assessed by log-rank test for each CRC subtype.

For this analysis of WMDA, marginally better RFS and OS with MUC2-positive tumors were found as compared with negative tumors. The prognosis of MUC5AC-positive WMDA cases tended to be worse as compared to those that were negative, although this difference was not significant (Figure 4A-D). There was also no difference with respect to MUC2 status in PDA. However, MUC5AC expression in PDA was significantly and marginally associated with a better prognosis in terms of RFS and OS, respectively (Figure 4E-H). Because MUC2 positivity was very high in MUA (77 of 81 tumors), its clinical significance in these settings was not investigated. MUC5AC expression in MUA did not affect its prognosis (Figure 4I and J).

DISCUSSION

MUC2 is normally expressed in the perinuclear cytoplasm of goblet cells in normal colonic mucosa. MUC2 is also expressed in adenomas and mucinous carcinomas[4]. MUC2 downregulation occurs in non-mucinous adenocar-cinomas that arise within adenomas, whereas cancers that are considered to develop de novo do not express MUC2[4]. Thus, MUC2 levels have been thought to be a predictor of malignant potential. However, despite a poor prognosis, higher levels of MUC2 expression were maintained in MUA and SRCC than in WMDA. This suggests the difficulty with using MUC2 levels as a differentiation marker in these subtypes.

Some investigators reported MUC2 expression in CRC in association with clinical significance, although the association between MUC2 expression and prognosis has been controversial. For example, Matsuda *et al*[9] analyzed 86 CRCs that included 82 WMDA tumors and 4 other variants and reported that the MUC2 expression level was not associated with advanced Dukes' stage and liver metastasis. Baldus *et al*[10] investigated 243 CRCs that included 213 grade Ⅰ-Ⅱ tumors and 30 grade Ⅲ tumors, which also included 22 MUA tumors, and reported that


A
Survival rate
WMDA MUC2+ (n = 31)
P = 0.064
WMDA MUC2- (n = 32)
Recurrence/metastasis-free survival (d)
B
WMDA MUC2+ (n = 31)
P = 0.172
WMDA MUC2- (n = 32)
Overall survival (d)
C
WMDA MUC5AC- (n = 44)
P = 0.417
WMDA MUC5AC+ (n = 19)
Recurrence/metastasis-free survival (d)
D
WMDA MUC5AC- (n = 44)
P = 0.200
WMDA MUC5AC+ (n = 19)
Overall survival (d)
E
PDA MUC2+ (n = 8)
P = 0.886
PDA MUC2- (n = 73)
Recurrence/metastasis-free survival (d)
F
PDA MUC2+ (n = 8)
P = 0.987
PDA MUC2- (n = 73)
Overall survival (d)
G
PDA MUC5AC+ (n = 44)
P = 0.004
PDA MUC5AC- (n = 37)
Recurrence/metastasis-free survival (d)
H
PDA MUC5AC+ (n = 44)
P = 0.100
PDA MUC5AC- (n = 37)
Overall survival (d)

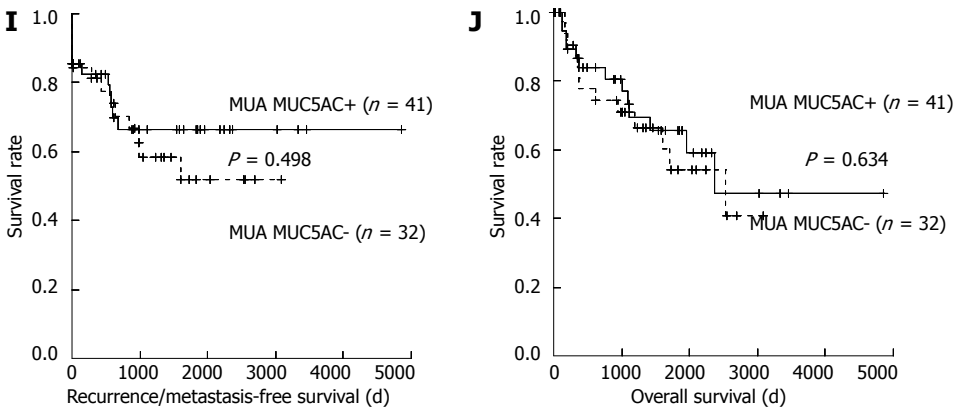


Figure 4 Survival curves. A-D: Well-to-moderately differentiated adenocarcinoma (WMDA); E-H: Poorly differentiated adenocarcinoma (PDA); I and J: Mucinous adenocarcinoma (MUA). A: Recurrence/metastasis-free; B: Overall survival curves with or without mucin (MUC) 2 expression of WMDA; C: Recurrence/metastasis-free; D: Overall survival curves with or without MUC5AC expression; E: Recurrence/metastasis-free; F: Overall survival curves with or without MUC2 expression; G: Recurrence/metastasis-free; H: Overall survival curves with or without MUC5AC expression; I: Recurrence/metastasis-free; J: Overall survival curves with or without MUC5AC expression. Curves were generated using the Kaplan-Meier method and compared by log-rank tests. *P* values were derived from comparing mucin-negative and -positive tumors.

MUC2 reactivity was not a marker for worse survival. In contrast, Kang *et al*[8] investigated 301 patients with stage Ⅱ-Ⅲ CRCs, including 200 well-to-moderately differentiated and 101 poorly differentiated cancers (also 266 nonmucinous and 35 mucinous) and reported that a loss of MUC2 expression was associated with a worse overall survival with CRC of stages Ⅱ and Ⅲ. Hanski *et al*[7] showed that a loss of MUC2 expression in CRC owing to promoter methylation was associated with liver and nodal metastasis. On the other hand, a loss of MUC2 expression in CRCs was associated with peritumoral lymphocyte infiltration[22]. Host responses, such as peritumoral lymphocyte infiltration, are known to be associated with a favorable CRC prognosis[23]. MUC2 mucin harbors the sialosyl-Tn antigen that mediates the inhibition of natural killer cell cytotoxicity[24].

However, MUC5AC expression is usually absent in normal colonic mucosa and is only occasionally found in pericancerous normally appearing colonic mucosa. Aberrant MUC5AC expression can be observed in a subset of adenomas and adenocarcinomas[11,25-27]. Microscopically, MUC5AC was detected primarily as focal staining in the cytoplasm and mucous droplets in goblet cells in the normally appearing colonic mucosa but was diffuse in the cytoplasm of cancer cells. MUC5AC expression levels are highest in adenoma but decrease with increasing degrees of dysplasia, and the positive rates for MUC5AC expression were lower in CRC than in adenoma[11,25-27]. These results suggest that MUC5AC may play a role in early carcinogenesis and its expression status can be used to classify CRC from the viewpoint of pathogenesis.

Biemer-Hüttmann *et al*[28] and Losi *et al*[29] reported associations between MUC5AC expression and MSI-H, which is linked with histological subtypes like PDA and MUA. Kocer *et al*[12] analyzed MUC5AC expression in 41 CRCs that included 33 adenocarcinomas, 5 mucinous carcinomas, and 3 neuroendocrine carcinomas. They reported that MUC5AC-negative CRCs had lower rates of disease-free status and of overall survival, but thy did not investigate associations between MSI and MUC5AC expression.

It has come to be well recognized that CRC comprises various carcinomas that originate from distinct pathogenetic pathways. Owing to this CRC heterogeneity, the significance of mucin core protein expression in CRC remains controversial. Thus, we performed these analyses for each histological CRC subtype.

In the present study, the expression profiles of MUC2 and MUC5AC in conventional adenocarcinoma (WMDA and PDA), MUA, and SRCC were similar to those in previous reports. High MUC2 expression levels in MUA and SRCC suggested distinct histogenetic pathways for these cancer cells, which maintained the feature of mucin-producing goblet cells, from conventional adenocarcinoma. In addition, the expression of the both mucin core proteins tended to decrease during the course of disease progression, from adenoma to carcinoma or from WMDA to PDA. Disease progression is usually accompanied by a decrease in cells of the goblet lineage.

These results are consistent with the hypothesis that the levels of the mucin core proteins may be a marker of malignancy potential in the adenoma-carcinoma sequence in both non-mucinous and mucinous carcinomas. We found that the prognostic significance of the mucin core proteins was different between MUC2 and MUC5AC. In our survival curve analysis, a tendency for better prognosis with MUC2-positive cases than for negative cases was observed in WMDA. A difference in prognosis was not evident from the MUC2 status in PDA.

However, MUC5AC expression in PDA was significantly associated with a better RFS and marginally associated with a better OS. MUC5AC was a significant factor for RFS by univariate Cox regression analysis. Although no significant effects of MUC5AC expression on RFS or OS were found by multivariate analysis, these results may have been because of the limited number of cases

or other unknown factors associated with this patient population. A larger study will be needed to clarify these points.

In comparison, MUC5AC expression was not a factor associated with better prognosis in WMDA and MUA. Aberrant MUC5AC expression is considered to be an early event in carcinogenesis. In addition, MUC5AC expression was significantly associated with right-sided location, absence of nodal metastasis, and a lower TNM stage and was marginally associated with older age in our PDA series. These clinical features as well as poor differentiation are characteristic of MSI-high tumors. MUC5AC expression in PDA was significantly associated with dMMR as shown by a loss of MMR protein expression (16 of 47 MUC5AC-positive cases *vs* 3 of 44 negative cases; $P = 0.003$). Unlike the report by Biemer-Hüttmann *et al*[28] stating that MUC2 expression was also associated with MSI-H, our PDA cases did not exhibit an association between the two. These results suggest that PDA also consists of heterogenous groups of cancers and that MUC5AC expression status may be one of the classification hallmarks.

To date, the mechanisms underlining aberrant MUC5AC expression in the colon have not been determined. During colon carcinogenesis, MUC5AC expression may be regarded as the re-expression of this fetal mucin. MUC5AC mucin is detected from the fourth month of gestation and is maximum during the sixth month[30]. On the other hand, the MUC5AC promoter was shown to be activated by various inflammation mediators[31]. It was also reported that tumor necrosis factor-α stimulated colon cancer HT-29 cells, which are a goblet cell line, to secrete MUC5AC mucin in a dose-dependent manner[32]. Forgue-Lafitte *et al*[33] reported that MUC5AC mucin was detectable in the mucus of ulcerative colitis patients who underwent surgery. In their series, 10 patients suffering from ulcerative colitis tested were positive for MUC5AC, which suggested that long-term chronic inflammation may induce the production of this mucin in the colonic epithelium. In addition, MUC5AC expression in the regenerating areas close to ulcerations in Crohn's disease suggests its involvement in tissue repair mechanisms[34]. CRC with MUC5AC expression may originate from precancerous lesions owing to long-standing inflammation caused by bacterial infection, inflammatory bowel disease, or other reasons.

In our study, we sometimes observed aberrant MUC5AC expression in normally-looking colonic mucosa at the interface between non-tumor and tumor tissue, where a strong anti-tumor inflammatory reaction had been observed. We speculate that this may be suggestive of the origin of a neoplasm with aberrant MUC5AC expression. Furthermore, it was previously reported that long-standing inflammation due to ulcerative colitis resulted in CRC with dMMR[35,36]. Taken together, a hypothesis of inflammation-related carcinogenesis may explain the pathogenesis of CRC from MUC5AC-positive precancerous lesions and an association between dMMR and MUC5AC expression.

In conclusion, we investigated MUC2 and MUC5AC expression status in each of the histological CRC subtypes. MUC2 levels were decreased and MUC5AC levels were increased from WMDA to PDA. MUA and SRCC maintained high MUC2 levels. The expressions of these mucins in PDA and MUA decreased during disease progression in the adenoma-carcinoma sequence. MUC5AC expression was closely associated with MMR deficiency in PDA. MUC2 and MUC5AC expression tended to be associated with a better prognosis in WMDA and PDA, respectively, although these were not statistically significant. Thus, the mucin proteins show distinct clinical significance according to the histological subtypes, and this may also suggest different pathogeneses for these tumors.

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COMMENTS

Background

Mucin (MUC)2 mucin is predominantly found in colorectal goblet cells and MUC5AC mucin is primarily expressed in gastric mucosa. Altered mucin expression may be correlated with the biological behavior of and, possibly, the prognosis for colorectal carcinoma (CRC).

Research frontiers

To date, MUC2 and MUC5AC expressions in CRC have been investigated for their association with clinicopathologic characteristics and prognosis. However, many contradictory results make it difficult to interpret the clinical significance of altered mucin expression, possibly owing to various CRC subtypes. The authors investigated mucin expression by immunohistochemistry in each of the histological CRC subtypes individually and assessed its significance.

Innovations and breakthroughs

MUC2 levels may be associated with malignant potential in conventional adenocarcinoma, whereas mucinous adenocarcinoma and signet-ring cell carcinoma retain high MUC2 levels, which suggests distinct pathogenesis. In comparison, MUC5AC expression may be an early event in tumorigenesis. MUC5AC expression in poorly differentiated adenocarcinoma (PDA) was closely associated with mismatch repair deficiency. MUC2 and MUC5AC expression tended to be associated with a better prognosis in well-to-moderately differentiated adenocarcinoma and PDA, respectively. Thus, the mucin proteins show different clinical significance according to the histological subtypes, and this may also suggest different pathogeneses of these tumors.

Applications

These results could be the basis for further studies to understand the pathogenesis of CRC. Immunohistochemical detection of MUC5AC may be useful for further subclassifications and for predicting a favorable prognosis for PDA.

Terminology

MUC2 and MUC5AC are the backbone proteins of secreted mucins. MUC2 is synthesized in goblet cells in the gastrointestinal tract and MUC5AC is normally expressed in gastric foveolar epithelium. The *MUC2* and *MUC5AC* genes encode gel-forming mucins and are located in a cluster on chromosome 11p15.5.

Peer review

This study is important and interesting. Although there are a large number of publications on the roles of MUC2 and MUC5AC expression in CRC, the results are conflicting. This study adds some refinements to these issues, such as MUC2 and MUC5AC expressions in terms of CRC patient survival, adenoma-

carcinoma sequence, and expression of MMR proteins, with regard to associations with clinicopathologic variables by offering its own evidence.

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ORIGINAL ARTICLE

Roles of BN52021 in platelet-activating factor pathway in inflammatory MS1 cells

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Abstract

AIM: To determine the effects of BN52021 on platelet-activating factor receptor (PAFR) signaling molecules under lipopolysaccharide (LPS)-induced inflammatory conditions in MS1 cells.

METHODS: MS1 cells (a mouse pancreatic islet endothelial cell line) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine and 100 μg/mL penicillin/streptomycin in 5% CO_2 at 37 ℃. After growth to confluency in media, the cells were processed for subsequent studies. The MS1 cells received 0, 0.1, 1 and 10 μg/mL LPS in this experiment. The viability/proliferation of the cells induced by LPS was observed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. Apoptosis and necrosis of the cells under the inflammatory condition described previously were observed using Hoechst 33342-propidium iodide staining. Adenylate cyclase (AC), phospholipase A_2 (PLA_2), phospholipase Cβ (PLCβ), protein tyrosine kinase (PTK), G protein-coupled receptor kinases (GRK) and p38-mitogen-activated protein kinase (p38 MAPK) mRNA in the PAFR signaling pathway were measured by real-time polymerase chain reaction. The protein expression level of phosphorylated AC (p-AC), phosphorylated PLA_2 (p-PLA_2), phosphorylated PTK (p-PTK), phosphorylated p38 MAPK (p-p38 MAPK), PLCβ and GRK was measured using Western blotting analysis.

RESULTS: The activity of MS1 cells incubated with different concentrations of LPS for 6 h decreased significantly in the 1 μg/mL LPS group (0.49 ± 0.10 *vs* 0.67 ± 0.13, $P < 0.05$) and 10 μg/mL LPS group (0.44 ± 0.10 *vs* 0.67 ± 0.13, $P < 0.001$), but not in 0.1 μg/mL group. When the incubation time was extended to 12 h (0.33 ± 0.05, 0.32 ± 0.03 and 0.25 ± 0.03 *vs* 0.69 ± 0.01) and 24 h (0.31 ± 0.01, 0.29 ± 0.03 and 0.25 ± 0.01 *vs* 0.63 ± 0.01), MS1 cell activity decreased in all LPS concentration groups compared with the blank control ($P < 0.001$). BN52021 significantly improved the cell activity when its concentration reached 50 μmol/L compared with the group that received LPS treatment alone, which was consistent with the results obtained from fluorescence staining. The mRNAs levels of AC (4.02 ± 0.14 *vs* 1.00 ± 0.13), GRK (2.63 ± 0.03 *vs* 1.00 ± 0.12), p38 MAPK (3.87 ± 0.07 *vs* 1.00 ± 0.17), PLA_2 (3.31 ± 0.12 *vs* 1.00 ± 0.12), PLCβ (2.09 ± 0.08 *vs* 1.00 ± 0.06) and PTK (1.85 ± 0.07 *vs* 1.00 ± 0.11) were up-regulated after LPS stimulation as compared with the blank control ($P < 0.05$). The up-regulated mRNAs including AC (2.35 ± 0.13 *vs* 3.87 ± 0.08), GRK (1.17 ± 0.14 *vs* 2.65 ± 0.12), p38 MAPK (1.48 ± 0.18 *vs* 4.30 ± 0.07), PLCβ (1.69 ± 0.10 *vs* 2.41 ± 0.13) and PLA_2 (1.87 ± 0.11 *vs* 2.96 ± 0.08)

were significantly suppressed by BN52021 except for that of PTK. The level of p-AC (1.11 ± 0.12 *vs* 0.65 ± 0.08), GRK (0.83 ± 0.07 *vs* 0.50 ± 0.03), PLCβ (0.83 ± 0.16 *vs* 0.50 ± 0.10) and p-p38 MAPK (0.74 ± 0.10 *vs* 0.38 ± 0.05) was up-regulated after LPS stimulation as compared with the blank control (P < 0.05). The up-regulated proteins, including p-AC (0.65 ± 0.15 *vs* 1.06 ± 0.14), GRK (0.47 ± 0.10 *vs* 0.80 ± 0.06), PLCβ (0.47 ± 0.04 *vs* 0.80 ± 0.19) and p-p38 MAPK (0.30 ± 0.10 *vs* 0.97 ± 0.05), was significantly suppressed by BN52021, but p-PLA_2 and p-PTK protein level were not suppressed.

CONCLUSION: BN52021 could effectively inhibit LPS-induced inflammation by down-regulating the mRNA and protein levels of AC, GRK, p38 MAPK, PLA_2 and PLCβ in the PAFR signaling pathway.

Key words: BN52021; Platelet-activating factor receptor; Signaling pathway; Inflammation; Pancreatitis

Core tip: Microcirculatory disorder is considered to be one of the possible mechanisms of pathogenesis of severe acute pancreatitis (SAP). Platelet-activating factor (PAF) is known to mediate microcirculatory disturbance and inflammation. Although BN52021, a PAF receptor antagonist, has demonstrated significant treatment effects on SAP, its mechanism has not been elucidated in detail. In this study, we examined the signaling molecules of the PAF receptor pathway to evaluate whether BN52021 has any influence on the inflammatory effects induced by lipopolysaccharide in MS1 cells, hoping to elucidate the mechanism underlying microcirculatory disturbances in the pathogenesis of SAP *in vitro*.

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease that can develop into severe AP (SAP)[1]. SAP refers to AP associated with organ failure and/or local complications such as necrosis, pseudocyst or abscess, which is a disease of high morbidity and mortality with an unpredictable clinical course[2,3]. There is no clinically effective therapeutic strategy for SAP, because the pathogenesis of the disease remains largely unclarified. The possible explanations for the pathogenesis of SAP include theories of self-digestion, leukocyte overactivation, microcirculatory disorder, bacterial shifting, and secondary infection, which is a second attack by immune functional change, cell apoptosis, oxygen-free radicals, and others from different aspects[4]. Accumulated evidence has proven that microcirculatory disorders are the key pathogenesis of AP. Many complications of SAP are due to the amplifying effects of microcirculatory disruption[5-10]. The inflammation of pancreatic microvascular endothelial cells induced by lipopolysaccharide is a suitable pancreatitis model to simulate the microcirculatory disturbances *in vitro*.

Platelet-activating factor (PAF), a bioactive phospholipid synthesized and secreted by a variety of cells including pancreatic acini and microvascular endothelium cells[11], is known to mediate many physiological responses such as microcirculatory disturbance and inflammation. AP causes the release of PAF, which induces systemic effects that contribute to circulatory disturbance and multiple organ failure[1]. PAF can significantly potentiate pancreatic tissue damage, increase serum amylase and lipase levels, cause scattered hemorrhages and may serve as a primary mediator of inflammation in the pathological progress of SAP[1,7,12]. A single injection of PAF into the superior pancreaticoduodenal artery of rabbits induces dose-dependent morphologic alterations of the pancreatic tissue and increased serum amylase levels[13]. Our previous research revealed that PAF was stably expressed in the rat pancreas tissue and played an important role in inflammatory response during the procession of SAP[4,14]. PAF could produce physiological and pathological effects by binding to its cell surface receptor, PAF receptor (PAFR). Flickinger *et al*[15] revealed specific localization of PAFR in the pancreatic vascular endothelium but not in other pancreatic cell types. Recent studies have demonstrated that bacterial lipopolysaccharide (LPS) can induce an increase in the surface expression of PAF receptors[16]. Our recent study demonstrated that BN52021 exerted biological effects through inhibiting the increased PAF level and binding potential with PAFR rather than through decreasing PAFR expression in the pancreatic tissue[17]. Through binding with PAFR, PAF may, through G-protein transduction, activate phospholipase C, phospholipase A2, adenylate cyclase and tyrosine protein kinase, leading to the occurrence and development of SAP[18].

PAFR antagonists can block a series of inflammatory injuries caused by PAF, thereby improving the AP prognosis as a preventive treatment[19]. Research on such a potential therapy has helped elucidate the role of PAF in AP[20]. It was observed that BN52021 extracted from Ginkgo biloba leaves could act as a potent antagonist of PAFR[21], and BN52021 can inhibit the PAF-induced cascade effect in inflammatory reactions, exhibiting an anti-shock effect by reducing the portal vein pressure of liver cirrhosis[22,23]. In experimental pancreatitis models and clinical trials, the administration of several PAF antagonists significantly reduced the level of serum amylase, leukocyte infiltration, and improved capillary blood flow in the pancreas and distant organs, as well as the renal and respiratory functions and the survival rate. BN52021 could significantly reduce vascular permeability, pancreatic edema, hyperamylasemia, diminute superoxide dismutase activity, and inhibit lipid peroxidation in the

pancreatic tissue. These changes were accompanied by a significant reduction of acinar cell vacuolization and a remarkable inhibition of inflammatory cell infiltration in the interacinar space[24,25]. Our recent studies have also shown a therapeutic effect of BN52021 on experimental SAP[26-28], but its mechanism is not yet fully understood.

In this study, we examined signaling molecules of the PAFR pathway to evaluate whether a PAF receptor antagonist (BN52021) had any influence on the inflammatory effects induced by lipopolysaccharide in MS1 cells, hoping to elucidate the mechanism underlying the microcirculatory disturbances in the pathogenesis of SAP *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Chemicals and reagents used in this study included BN52021, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS (Sigma-Aldrich, St. Louis, MO, United States); Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Carlsbad, CA, United States); the mouse primers for the *Adcy1* [adenylate cyclase (AC)], *Pla2g4a* [phospholipase A_2 (PLA_2)], *Plcb3* [phospholipase Cβ (PLCβ)], *Ptk7* [protein tyrosine kinase (PTK)], *Adrbk1* [G protein-coupled receptor kinases (GRK)], *Mapk14* [p38-mitogen-activated protein kinase (p38 MAPK)] and *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) genes (Beijing AuGCT DNA-SYN Biotechnology Co., Ltd., Beijing, China); rabbit polyclonal antibodies of phosphorylated PTK (p-PTK) and phosphorylated AC (p-AC) (Abcam, Cambridge, MA, United States); rabbit polyclonal antibodies for GRK_2 and phosphorylated p38 MAPK (p-p38 MAPK) (Epitomics, Burlingame, CA, United States); rabbit polyclonal antibody for phosphorylated PLA_2 (p-PLA_2) (Cell Signaling Technology, Beverly, MA, United States); rabbit polyclonal antibody for PLCβ (Santa Cruz, Dallas, TX, United States); rabbit polyclonal antibody for β-actin (Abmart, Arlington, MA, United States); protein molecular weight markers, reverse transcription-polymerase chain reaction (RT-PCR) kit and quantitative PCR kit (Beijing TransGen Biotech Co., Ltd, Beijing, China); and polyvinylidene fluoride (PVDF) membranes (BD Biosciences, BD Corporation, MA, United States).

Cell culture

MS1 cell line (a mouse pancreatic islet endothelial cell line firstly established in 1994) was purchased from Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine and 100 μg/mL penicillin/streptomycin in 5% CO_2 at 37 ℃. After grown to confluency in media, the cells were processed for subsequent studies.

MTT colorimetric assay

The viability/proliferation of the cells induced by LPS was observed using a MTT colorimetric assay as previously described[29]. MS1 cells received 0, 0.1, 1 and 10 μg/mL LPS in this experiment. Briefly, the cells were trypsinized with trypsin-ethylenediaminetetraacetic acid (EDTA), followed by incubation with DMEM in the presence of 10% FBS to inhibit trypsin activity. The cell pellets were then resuspended in DMEM with 10% FBS to a concentration of 1×10^4 cells/mL. Two hundred microliters of the cell suspension containing approximately 2000 cells was inoculated into selected wells of the 96-well plate. After the cells grew to 75% confluence, 20 μL of MTT solution was added to each well, and cultured for 4 h. Next, the medium was removed by inverting and tapping the plates, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. The spectrophotometric absorbance at 490 nm was measured by a Titertek Multiscan enzyme-linked immunosorbent assay reader. Each experiment was repeated at least three times. Every experimental condition was repeated at least in triplicate wells for each experiment.

Hoechst 33342/propidium iodide staining

The apoptosis and necrosis of the cells under the conditions described previously were observed by Hoechst 33342-propidium iodide (PI) staining[30]. MS1 cells were plated in a 6-well plate and co-incubated with media, LPS, LPS + DMSO and LPS + BN52021 when the cells achieved 90% confluence. The cells were washed twice with PBS. After the addition of 5 μL of Hoechst 33342 staining solution, the cells were stained with PI in the dark for 20-30 min at 4 ℃ and washed twice with PBS. Cells with blue and red fluorescence were examined under a fluorescence microscope.

Real-time quantitative RT-PCR

The mRNAs levels of AC, PLA_2, PLCβ, PTK, GRK and p38 MAPK were measured by real-time PCR. In detail, MS1 cells were plated in a 6-well plate and co-incubated with media, LPS, LPS + DMSO and LPS + BN52021 when the cells achieved 90% confluence. The cells were collected, and the total RNA was extracted with a Trizol RNA reagent kit according to the manufacturer's instructions. In addition, 2 μL (1 μg) of total RNA was added to the reverse transcription kit MIX system, and reverse-transcribed PCR was performed by random priming. The resulting complementary DNA amount was measured by quantitative PCR analysis using the GeneAmp 5700 Sequence Detection System and Step One Plus Real-Time PCR System (Applied Biosystems). The qPCR primer sequences are available online as indicated in Table 1. All expression data were normalized to the data for *Gapdh*. A no-template, double-distilled water control was included for each template. All samples were amplified simultaneously in triplicate in a single run. The relative quantitative gene expression was calculated as previously described and expressed as the percentage of the control level[31].

Western blotting

The protein expression level of p-AC, p-PLA_2, p-PTK,

Table 1 Specific primers for *Adcy1*, *Pla2g4a*, *Plcb3*, *Ptk7*, *Adrbk1*, *Mapk14* and *Gapdh* genes

Gene	Primer	Length (bp)	Annealing temperature (℃)
Adcy1			
Forward	5'-GACTTTGTTCTCCGAGTTG-3'	19	49
Reverse	5'-GTGCTATCCATCCGACTG-3'		
Pla2g4a			
Forward	5'-GAATAAAGGCTCTACAATGG-3'	20	49
Reverse	5'-GTTGTCGCTTTGGTACTC-3'		
Plcb3			
Forward	5'-CCTCAACTTCAACCGAGTT-3'	19	49
Reverse	5'-CAGAGTGAGGTACGGCTTG-3'		
Ptk7			
Forward	5'-CACTGCGATGTCACATTG-3'	18	49
Reverse	5'-CACTATGTTCGGGACTGG-3'		
Adrbk1			
Forward	5'-AAGCCAGCCAACATTCTC-3'	18	51
Reverse	5'-CCCTTCTGTAGGACTTCG-3'		
Mapk14			
Forward	5'-GGACCTGAACAACATCGTG-3'	19	50
Reverse	5'-CTAGGTTGCTGGGCTTTAG-3'		
Gapdh			
Forward	5'-CATCTTCCAGGAGCGAGAC-3'	19	50
Reverse	5'-GGCTAAGCAGTTGGTGGTG-3'		

Adcy1: Adenylate cyclase; *Pla2g4a*: Phospholipase A2; *Plcb3*: Phospholipase Cβ; *Ptk7*: Protein tyrosine kinase; *Adrbk1*: G protein-coupled receptor kinases; *Mapk14*: p38-mitogen-activated protein kinase; *Gapdh*: Glyceraldehyde 3-phosphate dehydrogenase.

p-p38 MAPK, PLCβ and GRK was measured using Western blotting analysis. In detail, MS1 cells were plated in a 6-well plate and co-incubated with media, LPS, LPS + DMSO and LPS + BN52021 for 24 h when the cells achieved 90% confluence. The cells were washed twice with 0.1 mol/L PBS and then lysed in RIPA lysis buffer (Tris-HCl 10 mmol/L, pH 7.4; NaCl 0.15 mmol/L; EDTA 0.5 mmol/L; phenylmethylsulfonyl fluoride 10 mmol/L; Tritonx-100 1%; dithiothreitol 40 mmol/L). The protein concentration of the lysate was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Beijing, China). Cell lysates containing 60 mg of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 12% polyacrylamide resolving gels. After electrophoresis, the proteins were transferred onto PVDF membranes, which were then blocked with 5% nonfat dry milk in TBS-0.05% Tween 20 (TBST) for 1 h at room temperature, washed in TBST for 10 min × 3, and incubated at 4 ℃ with gentle shaking overnight with rabbit primary antibodies against the protein of interest at corresponding dilutions, followed by incubation with horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G at 1:2000 dilution, incubation with 1 mL of enhanced chemiluminescence reagent for 3 min, and exposure to the film. The optical density of the protein of interest relative to that of β-actin was analyzed using Quantity One 4.6.2.

Statistical analysis

The data are expressed as the mean ± SE. The dose and time effects of LPS on the activity of MS1 cells were evaluated with a two-way analysis of variance (ANOVA). The differences between three or more groups were evaluated by one-way ANOVA. A P value less than 0.05 (2-tailed) was considered statistically significant. All tests were performed using the statistical software package GraphPad 5.0 (GraphPad Software Inc., San Diego, CA, United States).

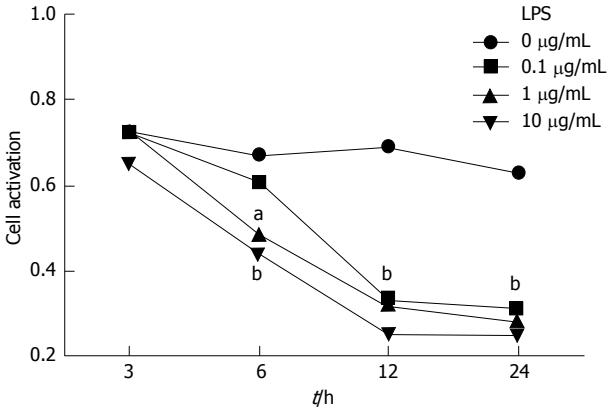

Figure 1 The optimal dose and duration of lipopolysaccharide stimulation were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. The cell survival rate was determined after incubation with 0 (saline) and 0.1, 1 and 10 μg/mL lipopolysaccharide (LPS) for 3, 6, 12 and 24 h. $^aP < 0.05$, $^bP < 0.01$ *vs* the saline group.

RESULTS

Dose and time effect of LPS on MS1 cell activity

MS1 cells received 0, 0.1, 1 and 10 μg/mL LPS to mimic the inflammation condition of AP *in vitro*. The optimal dose and duration of LPS stimulation were determined using the MTT method. As shown in Figure 1, there was no significant difference in MS1 cell activity between cells co-incubated with the different concentrations of LPS and control cells 3 h after culture ($P > 0.05$), but when the incubation time was extended to 6 h, MS1 cell activity decreased significantly in the 1 μg/mL LPS group (0.49 ± 0.10 *vs* 0.67 ± 0.13, $P < 0.05$) and 10 μg/mL LPS group (0.44 ± 0.10 *vs* 0.67 ± 0.13, $P < 0.001$), but not in the 0.1 μg/mL group ($P > 0.05$) compared with the control group. When the incubation time was extended to 12 h (0.33 ± 0.05, 0.32 ± 0.03 and 0.25 ± 0.03 *vs* 0.69 ± 0.01) and 24 h (0.31 ± 0.01, 0.29 ± 0.03 and 0.25 ± 0.01 *vs* 0.63 ± 0.01), MS1 cell activity decreased in all LPS concentration groups compared with the blank control ($P < 0.001$). Therefore, we chose the concentration 10 μg/mL LPS for the 24 h stimulation as the optimal protocol in the following experiments.

Dose effect of BN52021 on LPS-induced inflammation

The dose effect of BN52021 on LPS-induced inflammation was determined using the MTT method and Hoechst 33342/PI staining. The MS1 cell activity was significantly decreased 24 h after administration of 10 μg/mL LPS compared with the control group ($P < 0.01$). Pretreat-

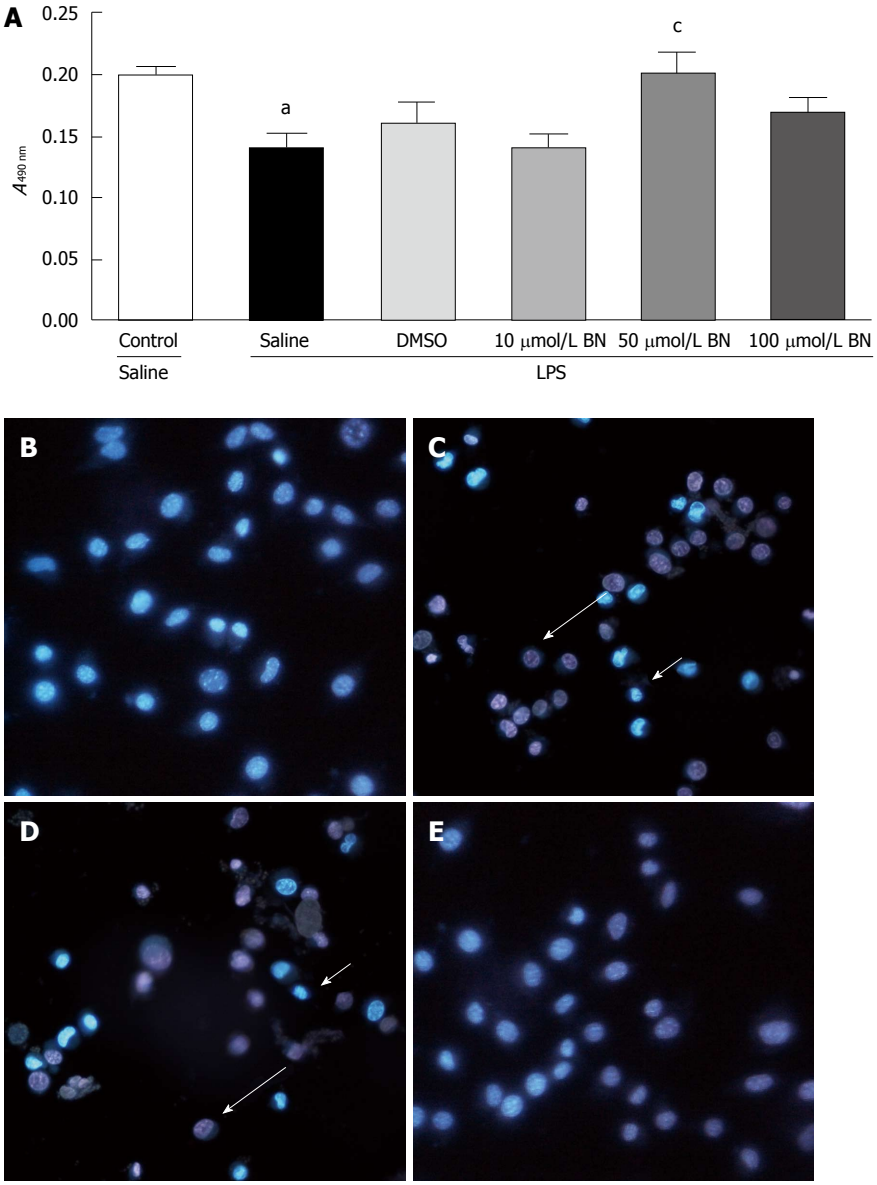

Figure 2 The dose effect of BN52021 on lipopolysaccharide-induced inflammation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method and Hoechst 33342/propidium iodide staining. MS1 cell activity at $A_{490\ nm}$ was significantly decreased 24 h after administration of 10 μg/mL lipopolysaccharide (LPS) *vs* the control group ($^aP < 0.05$). Pretreatment with BN52021 for 20 min before incubation with LPS significantly improved the MS1 cell activity at $A_{490\ nm}$ *vs* the group that received LPS treatment only when its concentration reached 50 μmol/L ($^cP < 0.05$) (A). Pretreatment with 50 μmol/L BN52021 for 20 min before incubation with LPS significantly improved MS1 cell activity *vs* the LPS + saline group, and the LPS + dimethyl sulfoxide (DMSO) group as determined Hoechst 33342/propidium iodide staining (B, C, D and E). The arrows indicate the apoptosis (short) and necrosis (long) of the cells.

ment with BN52021 20 min before incubation with LPS significantly improved the cell activity compared with the group receiving LPS only when its concentration reached 50 μmol/L, which was consistent with the results obtained by Hoechst 33342/PI staining ($P < 0.05$) (Figure 2). Therefore, the concentration of 50 μmol/L BN52021 was used for pretreatment in the following experiments.

Effect of BN52021 on PAFR signaling molecules at the mRNA level in LPS-induced inflammation

The mRNAs levels of AC (to 4.02 ± 0.14 folds), GRK (to 2.63 ± 0.03 folds), p38 MAPK (to 3.87 ± 0.07 folds), PLA_2 (to 3.31 ± 0.12 folds), PLCβ (to 2.09 ± 0.08 folds) and PTK (to 1.85 ± 0.07 folds) were up-regulated after LPS stimulation compared with the blank control ($P < 0.05$). The up-regulated mRNAs were significantly suppressed by BN52021, except for that of PTK (fold-change relative to control, 1.83 ± 0.13, $P > 0.05$), including that of AC (fold-change relative to control, down to 2.35 ± 0.13), GRK (down to 1.17 ± 0.14), p38 MAPK (down to 1.49 ± 0.18), PLCβ (down to 2.09 ± 0.08) and PLA_2 (down to 1.87 ± 0.11), as shown in Figure 3.

Effect of BN52021 on PAFR signaling molecules at the protein level in LPS-induced inflammation

The level of p-AC (fold-change relative to control, increase from 0.65 ± 0.08 to 1.11 ± 0.12), GRK (increase from 0.50 ± 0.03 to 0.83 ± 0.07), PLCβ (increase from 0.50 ± 0.10 to 0.83 ± 0.16) and p-P38 MAPK (increase from 0.38 ± 0.05 to 0.74 ± 0.10) was up-regulated after LPS stimulation compared with the blank control ($P < 0.05$). The up-regulated protein level was significantly suppressed by BN52021 for p-AC (decrease from 1.11 ± 0.12 to 0.65 ± 0.15), GRK (decrease from 0.83 ± 0.07 to 0.47 ± 0.10), PLCβ (decrease from 0.83 ± 0.16 to 0.47 ± 0.04) and p-p38 MAPK (decrease from 0.74 ± 0.10 to 0.30 ± 0.10). However, the level of p-PLA_2 and p-PTK was not significantly up-regulated after LPS stimulation and was not significantly altered by BN52021, as shown in Figure 4.

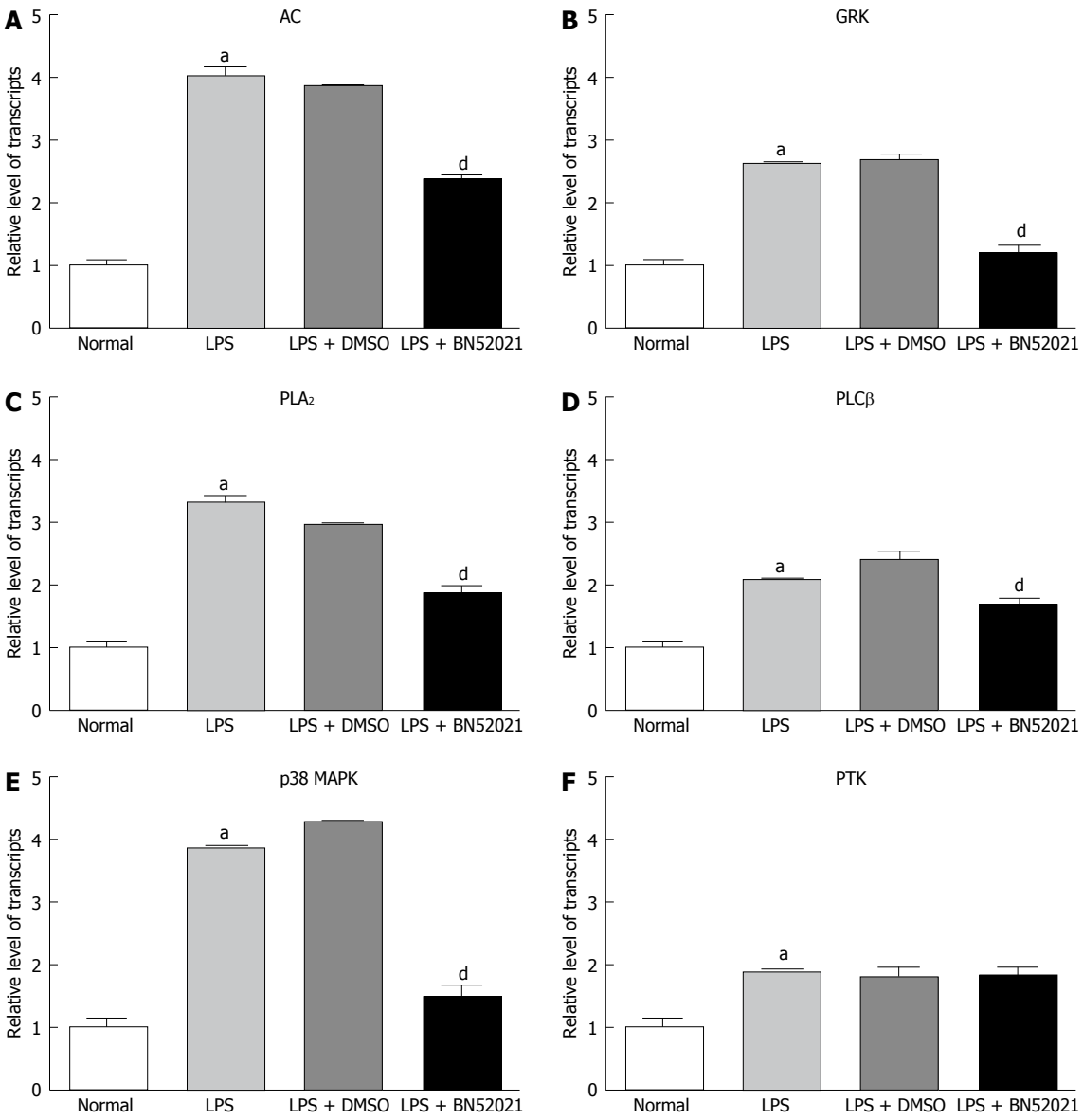

Figure 3 The effect of BN52021 on platelet-activating factor receptor signaling molecules at the mRNA level under lipopolysaccharide-induced inflammation. The mRNA level of adenylate cyclase (AC) (A), G protein-coupled receptor kinases (GRK) (B), phospholipase A_2 (PLA_2) (C), phospholipase Cβ (PLCβ) (D), p38-mitogen-activated protein kinase (p38 MAPK) (E) and protein tyrosine kinase (PTK) (F) was up-regulated after lipopolysaccharide (LPS) stimulation. The up-regulation of AC, GRK, p38 MAPK, PLCβ and PLA_2 mRNA was significantly suppressed by BN52021 except for that of PTK. $^aP < 0.05$ *vs* control; $^dP < 0.01$ *vs* the LPS + dimethyl sulfoxide (DMSO) groups.

DISCUSSION

In this study, we examined the signaling molecules of the PAFR pathway to evaluate whether the PAFR antagonist BN52021 had any influence on LPS-induced inflammation in MS1 cells. It was observed that BN52021 could sufficiently inhibit the inflammation, apoptosis and necrosis induced by LPS in pancreatic vascular endothelial cells. BN52021 could inhibit the up-regulation of signaling molecules in the PAFR pathway, which may help to explaining the mechanism underlying microcirculatory disturbance in the pathogenesis of AP.

PAF-induced microcirculatory disruption plays a key role in the pathogenesis of AP

Platelet-activating factor is a proinflammatory lipid mediator that plays a key role in many pathophysiological conditions, including asthma, ischemia, gastrointestinal ulceration, pancreatitis and multiple organ failure[32]. A number of experimental studies suggest that the pathogenesis of AP correlates with microcirculatory disorders. An experiment that constricted interlobular pancreatic arteries 2 min after intraductal infusion of sodium taurocholate indicated that microcirculatory changes are closely related to the process of AP[6]. Many complications of SAP are due to the amplifying effect of microcirculatory disruption[7]. PAF is one of the most important vasoactive mediators activated during the inflammatory response to pancreatic injury that can cause microcirculatory disorders in AP. Recent data suggest that PAF can directly modulate microvascular permeability and increase venular permeability[10]. Increased microvessel permeability

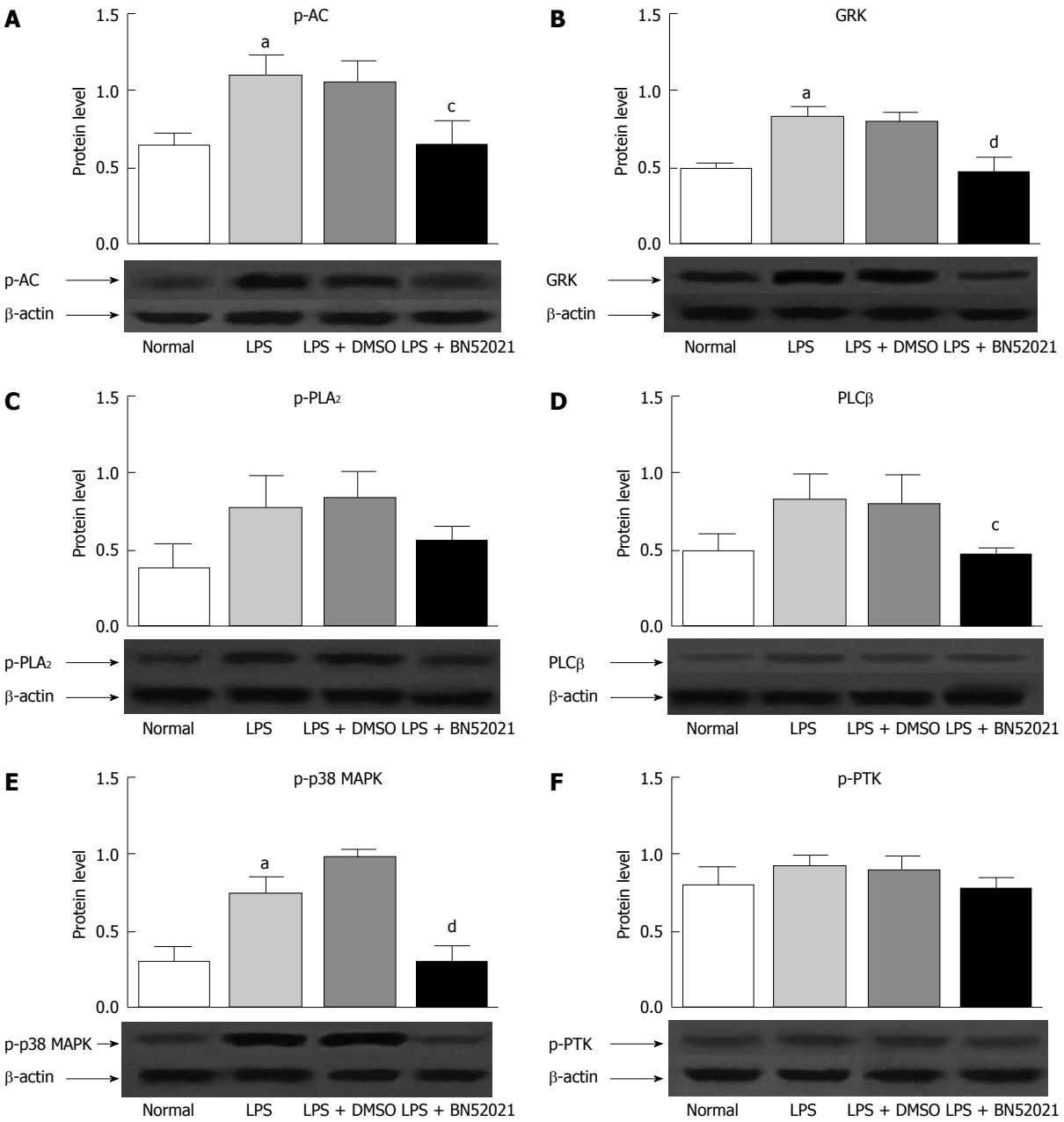

Figure 4 The effect of BN52021 on platelet-activating factor receptor signaling molecules at the protein level under lipopolysaccharide-induced inflammation. The protein level of p-adenylate cyclase (p-AC) (A), G protein-coupled receptor kinases (GRK) (B), p-phospholipase A_2 (p-PLA_2) (C), phospholipase Cβ (PLCβ) (D) and p-p38-mitogen-activated protein kinase (p-p38 MAPK) (E) was up-regulated after lipopolysaccharide (LPS) stimulation *vs* the blank control ($^aP < 0.05$). The up-regulation of p-AC, p-p38 MAPK, GRK and PLCβ protein levels was significantly suppressed by BN52021. However, p-PLA_2 and phosphorylated protein tyrosine kinase (p-PTK) protein levels were insignificantly up-regulated after LPS stimulation and were not significantly changed by BN52021 (F). $^cP < 0.05$, $^dP < 0.01$ *vs* LPS + dimethyl sulfoxide (DMSO) groups.

induced by PAF may be related directly to endothelial cell activation, adhesion molecule expression, and leukocyte activation[7,8]. Increased capillary permeability permits the sequestration of macromolecules and fluid, causing deficiency of circulating blood volume and microcirculatory disorders[7]. In addition, vasospasm and microthrombus formation due to hypercoagulability can also lead to the deterioration of pancreatic microcirculation and pancreatic necrosis[7]. The treatment of AP with PAF antagonists can significantly improve capillary blood flow in the pancreas and colon, renal and respiratory function, and the survival rate and can stabilize capillary permeability and decrease fluid loss into the third space[33,34]. As a preventive treatment, PAFR antagonists such as BN52021 can block a series of PAF-mediated inflammatory injuries, thus improving the prognosis of AP[1]. This protective effect of PAF antagonists further supports the role of PAF in microcirculatory disorders.

LPS-induced inflammation of pancreatic microvascular endothelial cells is a suitable pancreatitis model to simulate microcirculatory disturbance *in vitro*. The MS1 cell line is a mouse pancreatic islet endothelial cell line

first established in 1994. It can represent the pancreatic microvascular endothelium because previous studies[35] have verified that the pancreatic lobule is a structured and functional basic unit of pancreatic microcirculation, and insulo-acinar portal circulation represents the basic feature of the pancreatic microcirculation. Therefore, in this study, we examined the signaling molecules of the PAFR pathway in MS1 cells to evaluate whether the PAF receptor antagonist BN52021 had any influence on the LPS-induced inflammatory effect, hoping that it could help elucidate the mechanism underlying microcirculatory disturbance in the pathogenesis of SAP *in vitro*. Our results indicated that pretreatment with BN52021 for 20 min before incubation with LPS could significantly improve the MS1 cell activity compared with the group that received LPS treatment only.

PAFR signaling pathway plays a pivotal role in pancreatic proinflammatory response

In recent years, researchers have become concerned with the significance of the signal transduction pathway of PAF in the pathogenesis of AP[4,22,28], because it has been reported to induce morbidity and unacceptably high mortality[18]. However, the impact of a PAF receptor antagonist (BN52021) on the signaling molecules of the PAFR signaling pathway in pancreatic microvascular endothelial cells under the LPS-induced inflammatory condition remains unclear.

PAFR is almost ubiquitous in diverse type cells and acts not only on the local pancreas cells, including the pancreatic vascular endothelium, but also on distant organs, inducing systemic inflammatory response and multiple organ injury[15]. PAFR belongs to the G protein-coupled receptor subfamily[36]. By binding to its receptor, PAF activates the associated G protein, which, in turn, activates phosphoinositide hydrolysis by phosphoinositide specific phospholipase C, arachidonic acid release by phospholipase A_2, increases in intracellular Ca^{2+} concentration, activation of protein kinase C and PTK[37]. PAF has also been shown to activate MAPKs, including extracellular signal-regulated kinase[38-42], p38 MAPK[38,40,41], and c-Jun N-terminal kinase[43]. Deo *et al*[44] reported that PAF activated pertussis toxin-insensitive Gαq protein upon binding to its seven transmembrane receptors and adenylate cyclase, elevating cAMP levels, and thus activating protein kinase A in human umbilical vein endothelial cells. GRK plays a key role in the homologous desensitization of G protein-coupled receptor (GPCR) and GRK phosphorylate activated receptors, promoting high affinity binding of arrestins, thus precluding G protein coupling. Direct binding to active GPCRs activates GRKs so that they selectively phosphorylate only the activated form of the receptor regardless of the accessibility of the substrate peptides within it and their Ser/Thr-containing sequence[45]. Most GPCRs display a rapid loss of responsiveness in the continuing presence of chemoattractants in a process of desensitization that involves the phosphorylation of agonist-occupied GPCR by GRK[46]. The inflammation in pancreatic vascular endothelial cells induced by LPS was suppressed by BN52021. This finding might contribute to an understanding of the mechanism underlying the microcirculatory disturbances in the pathogenesis of SAP.

According to our results, the mRNA and protein levels of AC, GRK, p38 MAPK, PLA_2 and PTK were up-regulated after LPS stimulation compared with the blank control. The up-regulated AC, GRK, p38 MAPK and PLA_2 mRNA and protein levels were significantly suppressed by BN52021, suggesting that BN52021 could effectively inhibit the apoptosis and necrosis of MS1 cells under the LPS-induced inflammatory condition. The mechanism underlying the inhibition might relate to the suppression effect of BN52021 on the up-regulation of AC, GRK, p38 MAPK and PLA_2 mRNA and protein levels in the PAFR signaling pathway.

Other potential mechanisms of PAFR antagonism in AP treatment

It is known that PAFR is also able to interact with components of the bacterial wall, such as lipopolysaccharides[47] and phosphorylcholine[48]. The cell wall components exit the vasculature into the heart and brain, accumulating within endothelial cells, cardiomyocytes, and neurons in a PAFR-dependent way. The physiological consequences of the cell wall/PAFR interaction are cell specific, being noninflammatory in endothelial cells and neurons but causing a rapid loss of cardiomyocyte contractility that contributes to death. Thus, PAFR shepherds phosphorylcholine-containing bacterial components such as the cell wall into host cells from where the response ranges from quiescence to severe pathophysiology[48]. The explanation for the protective effect of BN-52021 cannot simply be attributed to the antagonism of LPS binding to PAFR or the prevention of PAF binding to its receptor. Therefore other potential mechanisms of PAFR antagonism in AP treatment must exist.

Bacterial translocation from the gastrointestinal tract to mesenteric lymph nodes and other extra intestinal organs is an important source of infection in AP. Preventing bacterial dissemination in early AP may have beneficial effects on the evolution of this disease[26,49]. PAF antagonist treatment decreases the bacterial spread to distant sites, suppresses elevation of interleukin (IL)-6 level, and has a significant effect on serum pancreatic enzymes and the histologic score of pancreatitis without reducing serum amylase and tumor necrosis factor alpha levels or ameliorating pancreatic damage in rats with AP[7,50]. In addition, BN52021 has been shown to have protective effect on slow mesenterioangial small arteriolar and venular blood flow velocity and dilated mesenterioangial small venular diameter in the early phase of AP[51]. Pretreatment with lexipafant could reduce the pancreatic endothelial barrier dysfunction and severity of pancreatitis-associated intestinal dysfunction as well as systemic concentrations of IL-1 and local leukocyte recruitment in experimental AP rats[52-54]. PAFR antagonism appears

to be involved in the maintenance of intestinal barrier integrity and the inhibition of cytokines release, such as IL-1 and IL-6[32]. Moreover, PAFR antagonists can also exert their effects by inhibiting the activity of neutrophils and depressing pulp peroxidase, competing for targets with PAF and inhibiting the activity of PAF, inhibiting increases in PAF in AP, and reducing plasma cytokines and inflammatory mediators, enzyme activity and the role of self-digestion of pancreatic tissue[1]. The involvement of the PAFR signaling pathway in these mechanisms needs to be further investigated.

The PAFR antagonist BN52021 could effectively inhibit LPS-induced inflammation, apoptosis and necrosis in pancreatic vascular endothelial cells. The mechanisms underlying the inhibition might be related to the suppression effect of BN52021 on the up-regulation of AC, GRK, p38 MAPK and PLCβ mRNA and protein levels in the PAFR signaling pathway, which may help to explain the mechanism underlying the microcirculatory disturbance in the pathogenesis of AP.

COMMENTS

Background

Microcirculatory disorder is considered to be one of the possible mechanisms of severe acute pancreatitis (SAP) pathogenesis. Platelet-activating factor (PAF), a bioactive phospholipid synthesized and secreted by a variety of cells including pancreatic acini and microvascular endothelium cells, is known to mediate many physiological responses, including microcirculatory disturbance and inflammation.

Research frontiers

Recent studies have demonstrated that PAF plays an important role in the pathological progress of SAP. Although BN52021, a PAF receptor antagonist, has demonstrated significant treatment effects against SAP, its effects on PAF receptor (PAFR) signaling molecules have not been elucidated in detail.

Innovations and breakthroughs

The authors found that BN52021 could effectively inhibit the apoptosis and necrosis of MS1 cells under lipopolysaccharide (LPS)-induced inflammatory conditions. The mechanism underlying the inhibitory effect may relate to the inhibitory effect of BN52021 on the up-regulation of adenylate cyclase, G protein-coupled receptor kinases, p38-mitogen-activated protein kinase, phospholipase A_2 and phospholipase Cβ mRNA and protein levels in the PAFR signaling pathway.

Applications

This study may contribute to a future strategy involving SAP treatment with BN52021 by investigating how PAF is induced and blocking its expression.

Terminology

PAF is a biologically active phospholipid mediator that plays its role by binding to PAFR, which is a unique G-protein-coupled seven transmembrane receptor, and the binding activates multiple intracellular signaling pathways. Ginkgolide B (code: BN52021) is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J) that are present in the whole extract of Ginkgo biloba leaves.

Peer review

This article attempts to elucidate the protective role of BN-52021 against LPS-induced apoptosis and necrosis in a pancreatic islet endothelial cell line. PAF is a crucial mediator of acute pancreatitis. Therefore the inhibition of its actions by BN-52021 is interesting from a pharmaceutical point of view. Because BN-52021 is a well-established antagonist of PAFR, the authors investigated its effect on certain members of the signal transduction pathways initiated by PAFR activation. The results are novel and interesting.

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ORIGINAL ARTICLE

Polydatin attenuated food allergy *via* store-operated calcium channels in mast cell

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Abstract

AIM: To investigate the effect of polydatin (PD), a resveratrol glucoside, on mast cell degranulation and anti-allergic activity.

METHODS: After the rats were orally sensitized with ovalbumin (OVA) for 48 d and underwent PD treatment for 4 d, all the rats were stimulated by 100 mg/mL OVA for 24 h and then sacrificed for the following experiments. The small intestines from all the groups were prepared for morphology examination by hematoxylin and eosin staining. We also used a smooth muscle organ bath to evaluate the motility of the small intestines. The OVA-specific immunoglobulin E (IgE) production and interleukin-4 (IL-4) levels in serum or supernatant of intestinal mucosa homogenates were analyzed by enzyme-linked immunosorbent assay (ELISA). Using toluidine blue stain, the activation and degranulation of isolated rat peritoneal mast cells (RPMCs) were analyzed. Release of histamine from RPMCs was measured by ELISA, and regulation of PD on intracellular Ca^{2+} mobilization was investigated by probing intracellular Ca^{2+} with fluo-4 fluorescent dye, with the signal recorded and analyzed.

RESULTS: We found that intragastric treatment with PD significantly reduced loss of mucosal barrier integrity in the small intestine. However, OVA-sensitization caused significant hyperactivity in the small intestine of allergic rats, which was attenuated by PD administration by 42% (1.26 ± 0.13 g *vs* OVA 2.18 ± 0.21 g, $P < 0.01$). PD therapy also inhibited IgE production (3.95 ± 0.53 ng/mL *vs* OVA 4.53 ± 0.52 ng/mL, $P < 0.05$) by suppressing the secretion of Th2-type cytokine, IL-4, by 34% (38.58 ± 4.41 pg/mL *vs* OVA 58.15 ± 6.24 pg/mL, $P < 0.01$). The ratio of degranulated mast cells, as indicated by vehicles (at least five) around the cells, dramatically increased in the OVA group by 5.5 fold (63.50% ± 15.51% *vs* phosphate-buffered saline 11.15% ± 8.26%, $P < 0.001$) and fell by 65% after PD treatment (21.95% ± 4.37% *vs* OVA 63.50% ± 15.51%, $P < 0.001$). PD mediated attenuation of mast cell degranulation was further confirmed by decreased histamine levels in both serum (5.98 ± 0.17 *vs* OVA 6.67 ± 0.12, $P < 0.05$) and intestinal mucosa homogenates (5.83 ± 0.91 *vs* OVA 7.35 ± 0.97, $P < 0.05$). Furthermore, we demonstrated that administration with PD significantly decreased mast cell degranulation due to reduced Ca^{2+} influx through store-operated calcium channels (SOCs) (2.35 ± 0.39 *vs* OVA 3.51 ± 0.38, $P < 0.01$).

CONCLUSION: Taken together, our data indicate that PD stabilizes mast cells by suppressing intracellular Ca^{2+} mobilization, mainly through inhibiting Ca^{2+} entry *via* SOCs, thus exerting a protective role against OVA-sensitized food allergy.

Key words: Polydatin; Food allergy; Mast cells; Store-operated calcium channels; Ca^{2+}

Core tip: In the present study, we have demonstrated for the first time that polydatin has the capacity for preventing pathogenesis of food allergy, which is dependent on regulation of Ca^{2+} mobilization *via* store-operated calcium channels in mast cells.

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INTRODUCTION

Food allergy (FA) is an adverse reaction mediated by immunoglobulin E (IgE) or non-IgE antibodies[1], which involves an abnormal response by the immune system to specific proteins in foods[2]. FA has been recognized as a worldwide health problem, especially in western countries, which is due to the severity of the reactions and its dramatic increase over the past three decades[3-5]. The majority of food allergies worldwide are caused by "eight main food allergens", including peanuts, tree nuts, eggs, milk, fish, crustacean shellfish, wheat, and soy[6]. It has been suggested that 25% of infants[7], 8% of children[3-5], and 2%-5% of adults[8] suffer from FA. However, the current understanding about the etiology of food allergies remains poor, and no effective treatment is available except the preventative measure of avoiding the offending food in the diet.

Mast cells play an essential role in the development of intestinal inflammatory disorders during food allergy. Cross-linking of the high-affinity IgE receptor (FcεRI) on mast cells by allergens results in degranulation, leukotriene generation, and cytokine synthesis. Degranulated mast cells release inflammatory mediators, including histamine and Th2 cytokines[9], which cause abnormal gut contractions and intestinal mucosa damage, which in term then result in abdominal pain, cramps, vomiting, and/or diarrhea[10]. It has been known that IgE-dependent mast cell degranulation relies on intracellular Ca^{2+} signaling[11,12]. Cytoplasmic Ca^{2+} mainly comes from the stored Ca^{2+} in the endoplasmic reticulum (ER) and extracellular Ca^{2+} through store-operated calcium channels (SOCs)[13]. Therefore, modulation of Ca^{2+} mobilization is a potential therapeutic strategy for stabilizing mast cells upon FcεRI activation and potentially offering a novel treatment method for allergic diseases.

Polydatin (PD), also known as polygoni cuspidati radix, is a natural component isolated from *Polygonum cuspidatum*. It has been determined as a resveratrol glucoside with a 3,4,5-trihydroxystilben-3-*D*-mono-*D*-glucoside molecular structure. Previous studies have demonstrated that PD has a therapeutic effect on the treatment of allergic diseases. Using the passive cutaneous anaphylaxis (PCA) model in mice, Lim *et al*[14] showed that PD reduced mast cell degranulation by suppressing phosphorylation of Syk and mitogen-activated protein kinases. On the other hand, Yuan *et al*[15] found that PD alleviated PCA in mice by stabilizing mast cells *via* the inhibition of Ca^{2+} release-activated Ca^{2+} channels. However, the therapeutic effect of PD on food allergies has not yet been determined.

In this study, we established ovalbumin (OVA)-induced food allergic models and evaluated the therapeutic effect of PD on food allergy. Furthermore, we explored the effect of PD on mast cell degranulation and found that the underlying mechanism was related to Ca^{2+} mobilization. Our research presented here is the first to reveal that PD can inhibit food allergy by suppressing mast cell degranulation *via* regulation of SOCs.

MATERIALS AND METHODS

Animals

Four-week old female Brown-Norway (BN) rats were purchased from Vital River Laboratories (Beijing, China) and housed in groups of four per cage in a controlled environment with a photoperiod of 12 h light to 12 h dark and a temperature of 20 ± 2 ℃. Sanitary controls were performed for all major rodent pathogens, with the results of these tests being uniformly negative. All the animal experimental procedures were approved by the Animal Care and Use Committee of Shenzhen University and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (publication No. 85-23, revised 1996).

Forty-eight Brown-Norway rats were randomly divided into four groups: control group (n = 12), OVA group (n = 12), OVA + PD group (n = 12), and PD group (n = 12). Each group received phosphate-buffered saline (PBS), OVA, or PD, as shown in Figure 1[16]. The control group received 1 mL PBS (0.1 mol/L) daily by gavage administration for 52 d, while the OVA group was orally treated with 1 mg OVA (1 mg/mL) for the first 48 d and 1 mL PBS (0.1 mol/L) from days 48 to 52. The OVA + PD group received PD (150 mg/mL × 1 mL daily per rat) oral treatment daily from days 49 to 52 after OVA sensitization. The PD group was not challenged by OVA. All the groups of rats were stimulated by 100 mg/mL OVA for 24 h at the end of day 52 and then sacrificed for the following experiments.

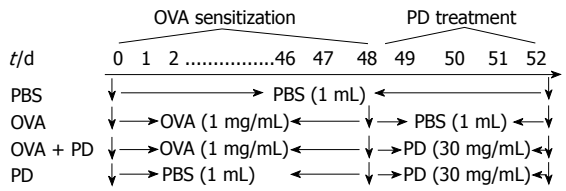

Figure 1 Protocol of ovalbumin sensitization/challenge and Formula-3 treatment. Rats were sensitized with ovalbumin (OVA) (1 mg/mL × 1 mL daily per rat) intragastrically for 48 d. For the polydatin (PD) treatment group, the rats were orally treated with PD (30 mg/mL × 1 mL daily per rat) from days 49 to 52 after OVA sensitization. All the rat groups were challenged by 100 mg/mL OVA for 24 h at the end of day 52 and then sacrificed. PBS: Phosphate-buffered saline.

Hematoxylin and eosin staining in small intestine tissues

The jejunal parts of the small intestine were isolated from the rats and embedded with paraffin. Sections (7 μm) were prepared and subjected to hematoxylin and eosin (HE) staining as previously reported[17].

Measurements of smooth muscle contractility

The tension of smooth muscle contractility was measured as previously reported[18]. Briefly, 2 cm long segments of the small intestine from the upper part of the jejunum to the lower part of the ileum were cut and mounted by hanging from triangle hooks. The hooks were connected to transducers from the upper end, and were inserted through the gut lumen from the lower end, allowing the circular muscle to contract. The tissue segments were incubated in chambers containing 20 mL Tyrode's solution (136 mmol/L NaCl, 5.4 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 0.33 mmol/L NaH_2PO_4, 1.8 mmol/L NaCl, 10.0 mmol/L glucose and 5.0 mmol/L HEPES), which was kept at 37 ℃ and constantly aerated with a mixture of 95% oxygen and 5% carbon dioxide. The initial tension load was set at 1.0 g, from which the segments spontaneously relaxed over time. The segments were allowed to stabilize for 30 min before they were stimulated with 1 mg/mL OVA for 3 min. *R0* was defined as the mean basal tension, when the segments were under rest conditions. ΔR denotes (*R1-R0*), where *R1* is the contract tension when the segments were stimulated with OVA.

Toluidine blue stain

Typical mast cells in rat small intestine tissue or peritoneal lavage solution (RPLS) were stained with toluidine blue stain as previously described[19]. Briefly, 200 L RPLS was air dried on cromolyn sodium pretreated slides and then covered with several drops of staining solution (toluidine blue stain dissolved in 70% ethanol). After 90 s, the staining solutions were washed away quickly with running tap water and the stained cells were examined and counted under a light microscope (Olympus, Japan).

Enzyme-linked immunosorbent assay

The contents of interleukin-4 (IL-4) (eBioscience Inc., CA, United States) and histamine (R & D Inc., MN, United States) in RPLS and serum were assayed by commercial enzyme-linked immunosorbent assay (ELISA) kits using paired antibodies according to the manufacturer's instructions. Serum IgE levels were also checked using a commercial ELISA kit (BD Pharmingen, CA, United States), following the manufacturer's instructions.

Rat peritoneal mast cell isolation

The BN rats were sacrificed after being anaesthetized by ether inhalation in air. Rat peritoneal mast cells (RPMCs) were obtained by peritoneal lavage and purified by density gradient fractionation as described previously[20,21]. Isolated RPMCs preparations contained > 98% mast cells and at least 98% of these cells were viable, as checked by metachromatic staining in 0.05% toluidine blue.

Ca^{2+} imaging by confocal microscope

Intracellular Ca^{2+} signal was measured as described previously with minor modification[22]. RPMCs or RBL-2H3 cells were incubated with 5 μmol/L Ca^{2+} fluorescent probe fluo-4 AM (Invitrogen, CA, United States) for 30 min at room temperature. After washing with Tyrode's solution three times, the dye inside the cells was allowed to de-esterify for 30 min at 37 ℃. It has been determined that nearly 95% of the fluorescent dye was retained in the cytoplasm. Fluorescent images of Ca^{2+} were obtained using an Olympus 1000 confocal microscope with a 40 × oil immersion lens (NA 1.3) (Olympus, Japan). The fluo-4 signal was excited at 488-nm and emitted at > 505 nm. Frame-scan images were acquired at a sampling rate of 15 ms per frame and 20 s per interval.

Image data were analyzed off-line using fv10-asw.2.1 software. A selected image from each image set was used as a template for designating the region of interest (ROI) within each cell. The integrated intracellular Ca^{2+} concentration was determined by calculating $\Delta F/F0$. *F0* was defined as the mean basal fluorescence intensity of the dye recorded during the first 5-10 scanning frames, when the cells were under rest conditions. ΔF denotes (*F-F0*), where *F* is the temporal fluorescence intensity. The $\Delta F/F0$ values within each ROI were plotted as a function of time (typical time-courses of Ca^{2+} response to thapsigargin or DNP-BSA stimulation in single RBL-2H3 cells). The amplitude of the Ca^{2+} response within each cell was quantified as the highest $\Delta F/F0$ level reached during the measurement period, which was averaged over all cells within each group.

Statistical analysis

Data are presented as mean ± SE. When two comparisons were obtained, Student's unpaired two tailed *t* test was used. When multiple comparisons were obtained, the analyses consisted of one-way analysis of variance for repeated measures and Student-Newman-Keuls multiple comparison test. A value of $P < 0.05$ was considered to be statistically significant.

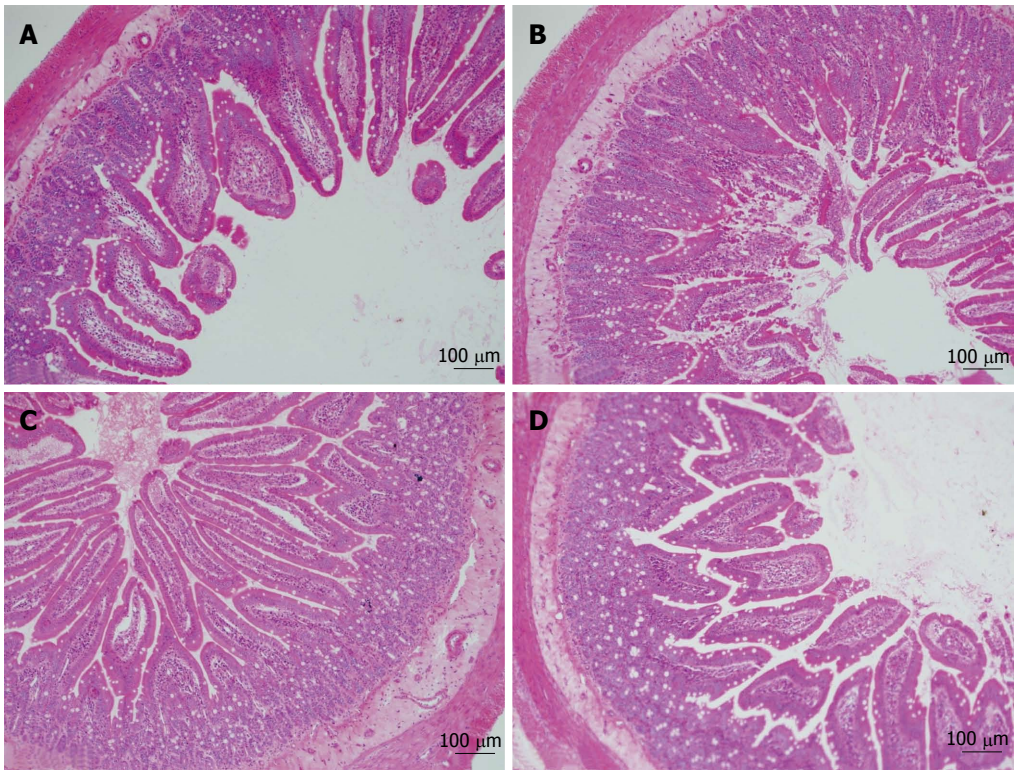

Figure 2 Polydatin attenuated tissue injury in small intestine caused by ovalbumin sensitization. A: Phosphate-buffered saline group; B: Ovalbumin (OVA) group; C: OVA + polydatin (PD) group; D: PD group. Morphology of intestinal jejunum was analyzed by hematoxylin and eosin staining. Representative images from three independent experiments are shown (magnification, × 63).

RESULTS

PD attenuated OVA-challenge caused small intestine abnormality in rats

In the present study, 1 mg OVA was used to sensitize BN rats orally and establish a food allergy model as previously described[16,23]. Loss of mucosal barrier integrity is a leading cause of food allergy[24]. Thus, we isolated jejunal fractions from the small intestine and checked tissue damage by HE staining. As shown in Figure 2, the results revealed that the intestinal mucosae were severely injured in the OVA group: the intestinal villi were eroded, and the swelling, shedding, and numbers of intestinal villi were significantly reduced. The morphological abnormality of the small intestine caused by OVA-sensitization was significantly attenuated by PD treatment.

The correlation between intestinal allergy and smooth muscle motility has been indicated by the fact that exposure to luminal allergen induces a state of proximal small intestinal hyperreactivity[25,26]. In order to evaluate the effects of PD on intestine motility, 2 cm intestine segments from each group were prepared, and the intestinal contraction tension was detected by smooth muscle organ bath. In response to 1 mg/mL OVA, intestinal segments isolated from OVA-allergic rats had a significant higher tension than the PBS group. The elevation of tissue tension caused by OVA sensitization was significantly attenuated by PD treatment by approximately 42% (Figure 3).

Treatment with PD decreased IL-4 levels and attenuated IgE production in OVA-sensitized group

The body weight of all rats in each group was monitored on days 0, 48 and 52. We found that basal body weight levels on day 0 were similar in all four groups. Compared to the PBS group, OVA sensitization significantly reduced body weight on day 48 (Figure 4A, left panel). After being treated with PD for 4 d, there was no significant difference between the PBS and OVA + PD groups (Figure 4A, left panel), which indicates that PD administration could maintain the body weight of an allergic rat at a normal level. The cytokine levels in the supernatant of the intestine mucosa were measured by ELISA. The results showed that the concentration of IL-4 in the OVA-challenged group was significantly higher than in the control group (58.15 ± 6.24 pg/mL *vs* 35.51 ± 5.48 pg/mL) (Figure 4B). Treatment with PD reduced the enhancement of IL-4 by 34%. Meanwhile, ELISA analysis showed that the concentration of OVA-specific IgE in serum was enhanced by 1.2 fold in the OVA group and PD therapy returned it to a normal level (Figure 4C).

PD reduced mast cell activation and degranulation in small intestine

Mast cell degranulation and histamine release are major factors in food allergy. The number and morphology of the mast cells in rat small intestine tissues (data not shown) or RPLS were examined by toluidine blue stain. In the OVA group, the number of mast cells was significantly increased and the cell size was much bigger, with more shrink on the cell membrane, bubbles in the cytoplasm, and degranulation vehicles around the cells (Figure 5A-D). *In vivo* administration with PD for 4 d reversed OVA-challenge-induced damage in mast cells. The ratio of degranulated mast cell, as indicated by vehicles (at least five) around the cells, dramatically increased in the OVA group by 5.5 fold and fell by 65% after PD treatment

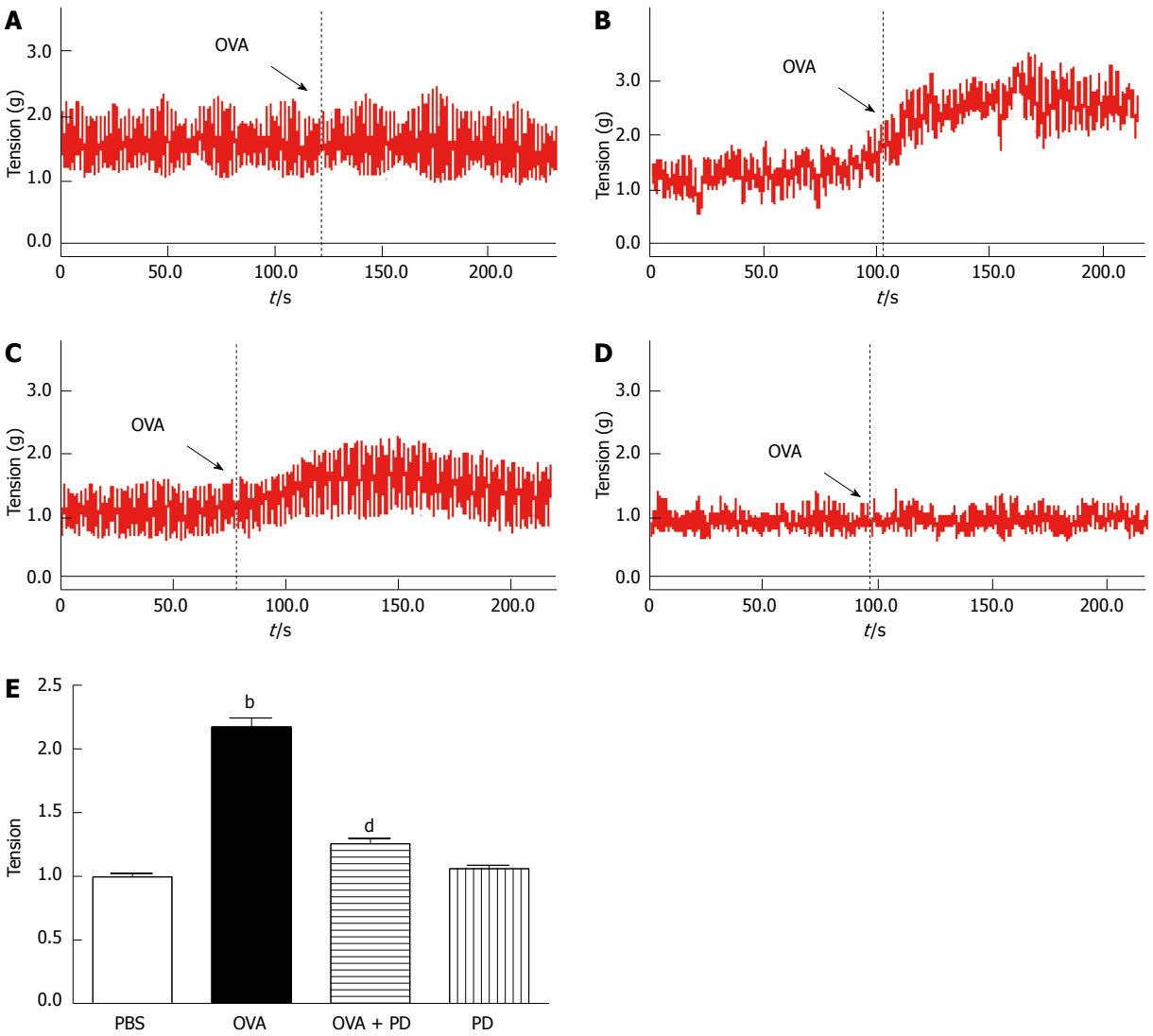

Figure 3 Polydatin attenuated small intestinal hyperreactivity in ovalbumin-allergic rats. A: Phosphate-buffered saline (PBS) group; B: Ovalbumin (OVA) group; C: OVA + polydatin (PD) group; D: PD group. n = 8, ^{b}P < 0.01 *vs* PBS group; ^{d}P < 0.01 *vs* OVA group. The tension of intestinal mobility was measured by smooth muscle organ bath, typical contraction curves (A-D), and the peak tension of each group (E).

(Figure 5E). PD mediated attenuation of mast cell degranulation was further confirmed by decreased histamine levels. It was found that histamine release in both serum and RPLS was significantly increased in the OVA-induced food allergic group, which was attenuated by PD therapy by approximately 11% and 20% respectively (Figure 5F).

PD inhibited mast cell degranulation by modulating Ca^{2+} mobilization through SOC channels

Rapid translocation of Ca^{2+} has been well-known to be essential for mast cell degranulation[27]. In a food allergic model, we also found that mast cell activation is related to stimulation of Ca^{2+} mobilization (data not published). To explore the underlying mechanism for the inhibitory effect of PD on mast cell degranulation, we isolated RPMCs and monitored intracellular Ca^{2+} with fluo-4 (5 mol/L). Using a standard Ca^{2+} add-back assay, in which intracellular Ca^{2+} stores were depleted by thapsigargin (TG), a sarcoplasmic/endoplasmic reticulum calcium ATPase (Ca^{2+} pump) blocker, in Ca^{2+}-free extracellular solution, after which the extracellular Ca^{2+} concentration was returned to 2 mmol/L[28]. Using this protocol, TG elicited two Ca^{2+} peaks, where the first one represented the ER Ca^{2+} release, and the second represented Ca^{2+} entry through activated SOCs. As shown in the first peaks in Figure 6A-D, when the cells were in Ca^{2+}-free solution, the TG-evoked Ca^{2+} amplitude was similar in all the groups, suggesting the amounts of Ca^{2+} released from ER are nearly the same. In the presence of 2 mmol/L extracellular Ca^{2+}, the TG-evoked Ca^{2+} influx was dramatically enhanced in OVA-sensitized RPMC by 1.5 fold, while PD treatment reduced the Ca^{2+} entry to normal level. The results indicate that PD attenuated OVA-induced Ca^{2+} influx elevation through SOCs.

DISCUSSION

The effect of resveratrol, a structural and functional

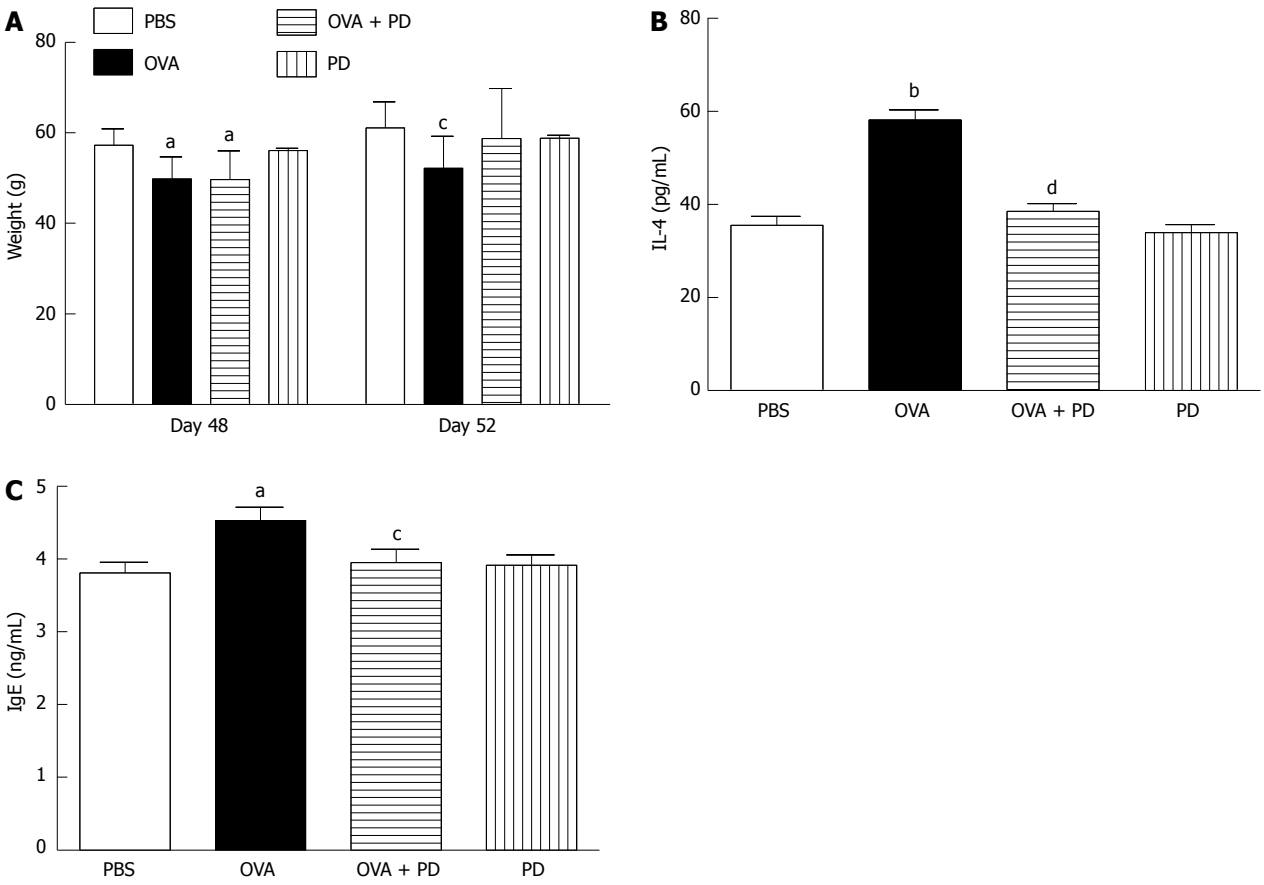

Figure 4 Polydatin suppressed interleukin-4 release and immunoglobulin E production in ovalbumin-allergic rats. A: Body weight of rats on day 48 [left panel, before polydatin (PD) treatment] or day 52 (right panel, after PD treatment) are shown; B: The cytokine levels in rat small intestine tissue or peritoneal lavage solution were analyzed by enzyme-linked immunosorbent assay; C: Statistical analysis of ovalbumin (OVA)-specific immunoglobulin E (IgE) in serum, which were collected from allergic rats administered with or without PD. $n = 8$. $^aP < 0.05$, $^bP < 0.01$ *vs* phosphate-buffered saline (PBS) group; $^cP < 0.05$, $^dP < 0.01$ *vs* OVA group.

analog of PD, on the regulation of intracellular Ca^{2+} signaling has been reported by several groups, although the results vary in different cell types[29,30]. Furthermore, a previous study in our lab identified PD as a novel mast cell stabilizer in passive cutaneous anaphylaxis mice[15]. There are two major findings in the present study. Firstly, using an *in vivo* food allergic model, we demonstrated that PD has therapeutic effects against food allergy by decreasing antigen-stimulated mast cell degranulation. Secondly, it was showed that PD suppressed Ca^{2+} mobilization by inhibiting Ca^{2+} entry through SOCs, which were the major contributors to PD-induced mast cell stabilization.

Food allergy is an immunological adverse reaction caused by food, which encompasses a range of disorders including IgE-mediated anaphylaxis, food protein-induced enterocolitis syndrome, and food-induced eosinophilic gastrointestinal disorders. Allergens from eggs seem to be one of the most frequent causes of food allergic reaction reported[31]. Thus, in this study, we used OVA to sensitize rats and establish a food allergic model. The allergic animal exhibited abnormal intestinal morphology and increased smooth muscle contractility, enhanced Th2 cytokine levels (IL-4), and OVA-specific IgE concentration. Our results are in line with other published data, as IL-4 has been reported to be the hallmark Th2-type cytokine with multiple immunological functions, including directing Th2 cell differentiation, triggering Ig class switching to IgE in B cells, driving mast cell expansion in intestines[32], and inducing an exaggerated contractile response in intestinal smooth muscle[33]. Furthermore, mast cell activation and degranulation, which was due to Ca^{2+} mobilization *via* SOCs, was also demonstrated.

Mast cells are the main effector cells in the pathogenesis of multiple allergic diseases, including asthma, allergic rhinitis, gastrointestinal allergy, and cutaneous anaphylaxis. The majority of mast cell studies have addressed their predominant role in acute allergic reactions (immediate hypersensitivity) and more recently, their roles in late-phase allergic reactions[34,35]. Therefore, developing new drugs capable of stabilizing mast cells would be valuable for treating diseases attributable to type I hypersensitivity reactions. Using the RBL-2H3 mast cell line, intensive research have been focused on looking for promising drugs to inhibit mast cell activation and degranulation, among which PD showed some latent effects[15]. In the present study, following administration with PD, the mast cell-dependent food allergic rats did not have apparent anaphylactic symptoms (data not shown) and had a marked decrease in Th2 responsiveness after oral challenge with OVA. We have found that PD significantly reduced IgE

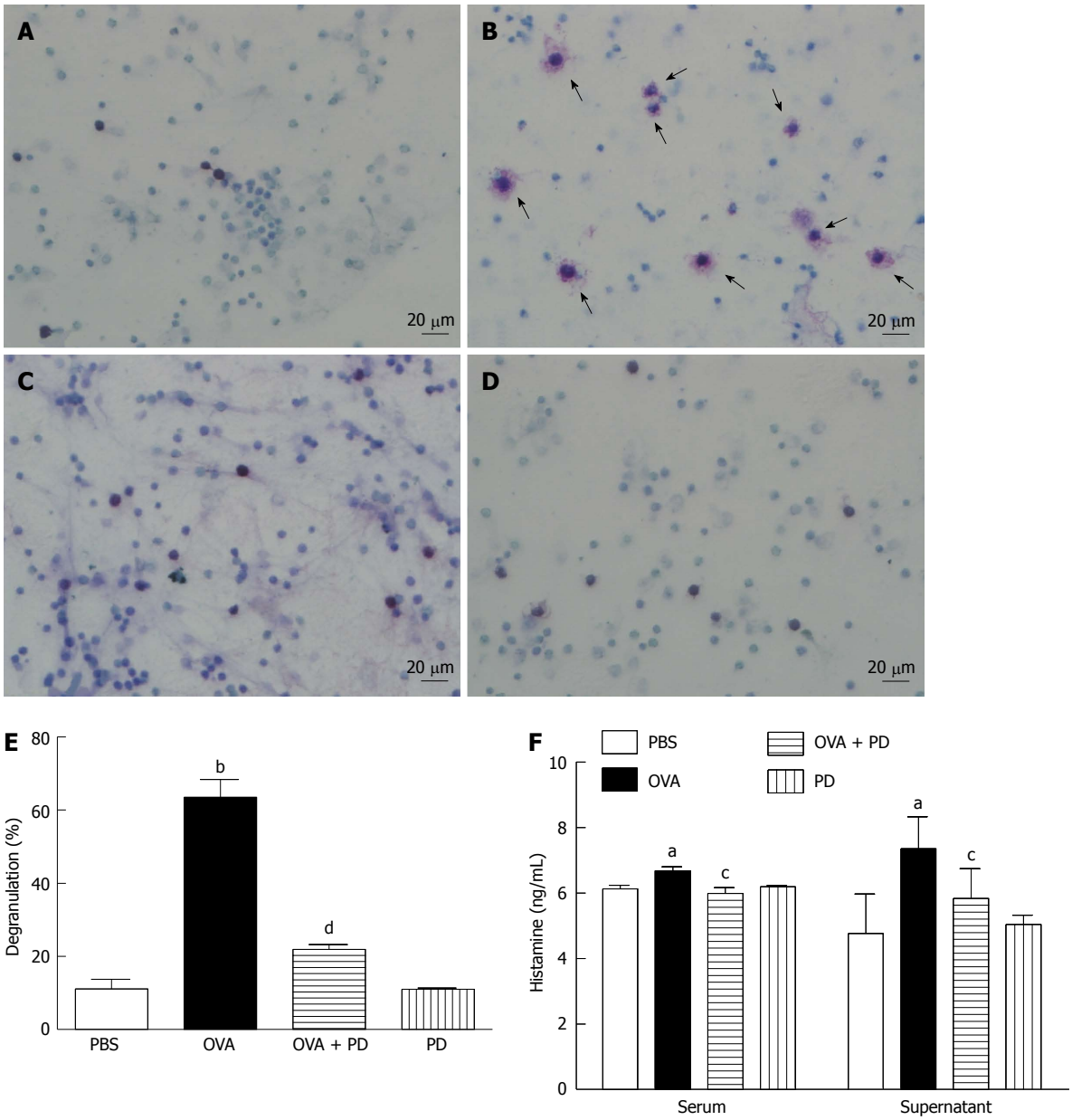

Figure 5 Polydatin therapy reduced mast cell degranulation and activation. A: Phosphate-buffered saline (PBS) group; B: Ovalbumin (OVA) group; C: OVA + polydatin (PD) group; D: PD group; E: Degranulated mast cells were counted from at least 500 total cells and the percentage of degranulation was calculated as degranulated cells against total cells; F: The release of histamine in serum (left panel), rat small intestine tissue, or peritoneal lavage solution (right panel) was measured by enzyme-linked immunosorbent assay. $n = 8$, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* PBS group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* OVA group. The mast cells in rat small intestine tissue or peritoneal lavage solution were identified by toluidine blue stain. Mast cells were considered degranulated if at least 5 granules appeared outside the cell body. Arrows indicate degranulated mast cells (magnification, × 250).

production by inhibiting Th2 cytokines release. On the other hand, PD showed the potential to block mast cell degranulation by decreasing Ca^{2+} influx *via* SOCs.

The importance of calcium influx in mast cell activation and degranulation has been well recognized[36]. The degranulation of mast cells is Ca^{2+} dependent, and an increase in intracellular Ca^{2+} characterized by Ca^{2+} entry through SOCs is essential for granules release[13,27,37]. In this study, we found that PD treatment significantly attenuated FcεRI-elicited intracellular Ca^{2+} increase, indicating that PD stabilized mast cells by suppressing Ca^{2+} mobilization. Furthermore, we found that PD inhibited Ca^{2+} entry through SOCs. Multiple mechanisms are involved in the regulation of SOC activity. It has recently been discovered that two subunits, STIM1 and Orai1, play a vital role in both the signaling and the permeation mechanisms for Ca^{2+} influx through SOCs. Overexpression of STIM1 together with Orai1 caused a dramatic increase in store-operated Ca^{2+} entry in RBL cells[38]. The mechanism underlying PD-mediated inhibition of SOCs activity remains unclear.

In summary, the present study established PD as a novel mast cell stabilizer, with the capacity for preventing pathogenesis of food allergy and perhaps other mast cell-

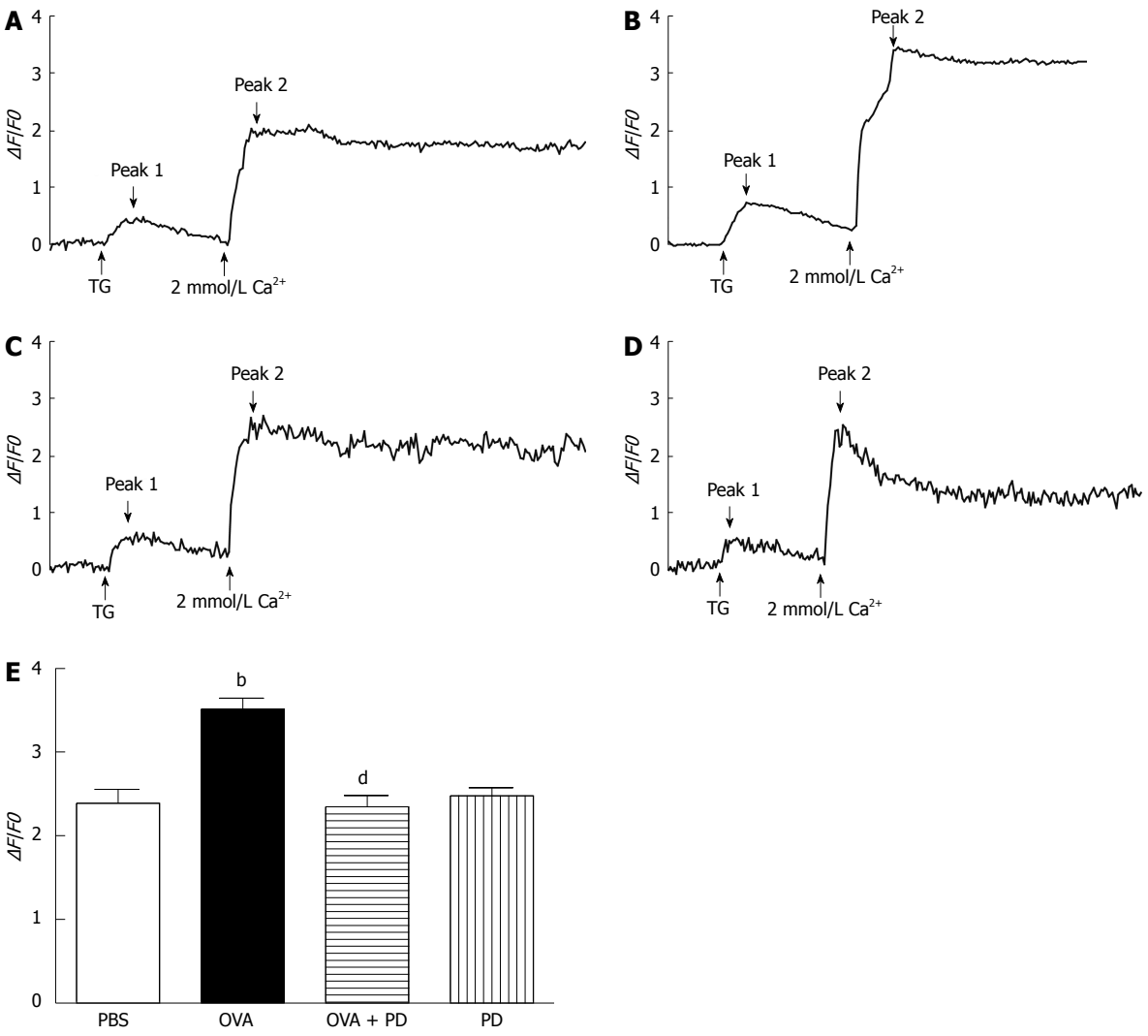

Figure 6 Polydatin reduced Ca^{2+} entry through store-operated calcium channels in food allergic mast cells. Typical responses of thapsigargin (TG)-evoked Ca^{2+} entry through store-operated calcium channels in rat peritoneal mast cells (RPMCs). A: Phosphate-buffered saline (PBS) group; B: Ovalbumin (OVA) group; C: OVA + polydatin (PD) group; D: PD group; E: Averaged peak amplitude of Ca^{2+} entry (second peak) as recorded in each group. $^{b}P < 0.01$ *vs* PBS group; $^{d}P < 0.01$ *vs* OVA group. RPMCs were isolated from Brown-Norway rat treated with or without PD. Intracellular Ca^{2+} was indicated by a fluo-4 fluorescent probe. Total cell numbers are 40-50 for each group, and the cells were from four independent experiments.

dependent allergic diseases through stabilizing mast cells. The underlying mechanism for PD-induced stabilization of mast cells is related to the inhibition of Ca^{2+} mobilization upon FcεRI activation.

COMMENTS

Background

The prevalence of food allergy has increased dramatically during the last three decades, but currently there is no satisfactory therapy except avoidance of the allergen in the diet. However, the offending foods causing an allergic effect are usually essential nutrients to human health. Therefore, to develop a new drug for food allergies is extremely important. Polydatin is a natural component isolated from *Polygonum cuspidatum*, and has been demonstrated to be effective in the treatment of allergic diseases.

Research frontiers

Polydatin is a sort of natural biological material and has been used as a medicine for many diseases. In the area of treatment of allergic diseases with polydatin, the research hotspot is how this product could stabilize mast cells and reduce allergic reactions in passive cutaneous anaphylaxis animals. The therapeutic effect of polydatin on food allergy has not yet been determined.

Innovations and breakthroughs

In previous studies in other laboratories and author's group, the extract of polydatin has been identified as a novel mast cell stabilizer in passive cutaneous anaphylaxis mice, in addition to its other new therapeutic effects, such as anticancer activity. Using an ovalbumin-sensitization mouse model, the authors are the first group to demonstrate that polydatin could attenuate food allergy by reducing mast cell degranulation. Furthermore, it was shown that polydatin suppressed Ca^{2+} mobilization by inhibiting Ca^{2+} entry through store-operated calcium channels, which were the major contributors to polydatin -induced mast cell stabilization.

Applications

The current results suggest that polydatin is a potential therapeutic drug that could be used in food allergy therapy.

Terminology

Food allergy mediated by immunoglobulin E (IgE) or non-IgE reaction, is an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food. It encompasses a range of disorders including IgE-mediated anaphylaxis, food protein-induced enterocolitis syndrome, and food-induced eosinophilic gastrointestinal disorders; Polydatin, also known as polygoni cuspidate radix, is a natural component isolated from *Po-*

lygonum cuspidatum. It has been determined as a resveratrol glucoside with a 3,4,5-trihydroxystilben-3-*D*-mono-*D*-glucoside molecular structure. It is traditionally used in South Korea, China, and Japan as a folk remedy for menoxenia, skin burns, gallstones, hepatitis, inflammation, and osteomyelitis.

Peer review

The authors investigated the effect of polydatin, a resveratrol glucoside, on mast cell degranulation and anti-allergic activity. This is a paper with some interesting value.

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BRIEF ARTICLE

A prospective study evaluating emotional disturbance in subjects undergoing defecating proctography

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Abstract

AIM: To investigate the prevalence of psychiatric illness in association with functional gastrointestinal disorders using defecating proctography (DP) and validated questionnaires.

METHODS: We prospectively evaluated 45 subjects referred for DP using hospital anxiety and depression scale (HADS), state trait anxiety inventory (STAI), patient health questionnaire 15-item somatic symptom severity scale (PHQ-15), validated questionnaires for sexual or physical abuse; post-traumatic stress disorder questionnaire (PTSD) and ROME-Ⅲ questionnaires for gastrointestinal complaints. DP results were considered negative if levator ani function was normal, rectoceles (if any) were < 4 cm and there was no evidence of intussusception, rectal prolapse, or other anatomic abnormality demonstrated. Subjects were subsequently divided into those with structural defects seen on DP (DP positive group) and those with a normal defecography study (DP negative group).

RESULTS: Forty five subjects were included in the study of which 20 subjects were classified as DP negative (44.4%). There was a striking prevalence of a history of sexual abuse in DP negative group compared to the DP positive group (n = 9, 5 respectively; P = 0.036). Further, subjects in the DP negative group scored significantly higher on the HADS anxiety (6.60 ± 1.00 *vs* 4.72 ± 0.40, P = 0.04) and depression scales (5.72 ± 1.00 *vs* 3.25 ± 0.46, P = 0.01). This correlated well with significantly higher scores on the STAI state anxiety scale (42.75 ± 3.16 *vs* 35.6 ± 2.00, P = 0.027), PHQ-15 questionnaire (13.15 ± 0.82 *vs* 10.76 ± 0.97, P = 0.038) and prevalence of PTSD (20% *vs* 4%, P = 0.045) among DP negative subjects. There was no difference between the groups in terms of STAI trait anxiety.

CONCLUSION: The findings of this prospective study demonstrate a significantly high degree of psychiatric ailments in patients with negative findings on DP who should be appropriately screened for a history of sexual abuse and symptoms of psychosocial distress.

Key words: Functional gastrointestinal disorders; Sexual abuse; Defecating proctography; Post-traumatic stress disorder questionnaire

Core tip: In this study, we used validated questionnaires in consort with defecating proctography and demonstrated that subjects undergoing defecating proctography who met ROME Ⅲ criteria for functional constipation have a high prevalence of psychiatric disorders and a significant history of sexual abuse. We also found an association between post-traumatic stress

disorder questionnaire, anxiety, history of sexual abuse and functional constipation. Taken together, these findings suggest that a very detailed history about psychiatric co-morbidities and traumatic experiences must be taken in selected patients complaining of constipation.

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INTRODUCTION

Patients with chronic gastrointestinal symptoms such as abdominal pain and constipation often display features suggestive of concomitant emotional disturbance[1]. Conversely, patients with a history of sexual or physical abuse have a high prevalence of gastrointestinal and genitourinary complaints[2].

There is evidence suggesting that patients with history of abuse and functional gastrointestinal disorders have a higher incidence of surgeries, lower quality of life and greater disability[3]. Given the lack of a surgically correctable etiology, surgical intervention may be inappropriate in these patients. In this context, we decided to evaluate patients with constipation, studying both anatomic and psychosocial variables using a variety of instruments.

We sought to assess the prevalence of abuse and psychiatric diagnoses in patients referred for defecating proctography (DP). Specifically, we sought to ascertain the prevalence of sexual abuse, physical abuse, anxiety, depression, somatization and post-traumatic stress disorder (PTSD) in subjects meeting criteria for functional constipation and without radiographic evidence of structural explanation for symptoms. We hypothesized that patients with no anatomic disorder of the pelvic floor evidenced by radiological parameters are likely to have a history of abuse and/or psychiatric diagnosis.

The purpose of the study was to use DP and a variety of validated questionnaires to assess the prevalence of psychiatric ailments in subjects with chronic constipation.

MATERIALS AND METHODS

Study design and setting

This prospective cohort study was undertaken at the Washington Hospital Center, a 926 bed tertiary care hospital in Washington DC. All subjects undergoing DP for the evaluation of lower gastrointestinal complaints were prospectively enrolled over 18 mo starting October 2010. Patients with obvious anatomical anomalies such as rectal prolapse, solitary rectal ulcers, fissures and fistulae were excluded.

At the time of enrollment, demographic information was recorded and each subject was assigned a unique identifying number. All other subject identifiers were removed. The study was approved by the institutional review board of Washington Hospital Center and all subjects gave written informed consent.

Immediately prior to undergoing the DP, all subjects were asked to complete a set of self-administered questionnaires.

Questionnaires

All subjects answered a total of 7 self-administered validated questionnaires that assessed a history of sexual or physical abuse, generalized anxiety disorder (state and trait anxiety), PTSD, depression and somatoform disorders. Gastrointestinal symptoms were assessed using the ROME Ⅲ criteria questionnaires that included the constipation module and irritable bowel syndrome module. All questionnaires were self-administered and met appropriate reliability and validity criteria. Further, each questionnaire was easily readable and understandable.

State-trait anxiety inventory: The state-trait anxiety inventory (STAI) is a self-administered test for evaluation of state and trait anxiety and has been used extensively in research and clinical practice. It has been translated into 30 languages and has since been extensively used and validated in the research literature[4]. For the purpose of our study the revised version of the test; "Form-Y" was used[5]. The STAI-Y is a self-administered test and takes 6-15 min to complete, depending on the subject's level of education. The S-anxiety scale (STAI Form Y-1) consists of 20 statements that evaluate how the respondent feels "right now, at this moment" using a 4 point Likert scale. The T-anxiety scale (STAI Form Y-2) consists of 20 statements that evaluate how individuals "generally feel". A score of over 40 on the STAI Form Y-1 and STAI Form Y-2 was considered diagnostic of state of anxiety and trait anxiety respectively.

Screening questionnaire for sexual and physical abuse history: This is a self-report questionnaire developed by Drossman *et al*[6] and has been validated against a detailed psychological interview[7]. The questionnaire has two sections to identify sexual abuse and physical abuse as a child or adult respectively. For the purpose of our study, no distinction was made between abuse as an adult or child.

Hospital anxiety and depression scale: The hospital anxiety and depression scale (HADS) is a self-administered questionnaire to assess generalized anxiety or depression[8] and takes approximately 2-5 min to complete. It has been extensively validated in patients with gastrointestinal disorders[9] in the in-patient[5] as well as the outpatient setting[10]. Each item is answered by the patient on a four point scale (0-3) with possible scores ranging from 0-21 for anxiety and 0-21 for depression. Any score above 11 is indicative of abnormal levels of anxiety or depres-

sion, thus a positive screen for the appropriate disorder.

Screening for somatoform disorders: We used the patient health questionnaire (PHQ-15) to screen for somatoform disorders in our subjects. The PHQ-15 is a 15 item scale addressing somatic symptoms during a 2 wk period on a scale of 0-2 with a maximum score of 30[11] and has been validated among patients with gastrointestinal complaints[12]. We compared the scores of the subjects based on the findings of the DP.

PTSD: We used the 4 item screen for PTSD in primary care developed by Prins *et al*[13]. This validated screening questionnaire[14] uses a binary yes/no response to a specific experience and a score of 3 or greater on the scale was defined as a positive case of PTSD.

ROME Ⅲ constipation module and ROME Ⅲ irritable bowel syndrome module: The ROME Ⅲ criteria were used to screen for irritable bowel syndrome (IBS) and constipation[15]. The ROME Ⅲ criteria are a system used to classify functional gastrointestinal disorders and we used validated self-administered questionnaires that are freely available for download[16]. The ROME Ⅲ criteria were used to rule out constipation predominant IBS as a cause of the patients symptoms and also to diagnose patients with true functional constipation.

Notably, all the questionnaires inquired about information which may have been potentially distressing to the subjects. Hence, one of the authors (Olden KW) who is a board-certified psychiatrist was available to the subjects in case emotional and mental distress was caused or detected by the protocol related questions. After completing the questionnaires, subjects underwent DP.

Single contrast DP

Defecating proctography was used to evaluate for anatomical defects that could explain the gastrointestinal symptoms in the subjects. Briefly, the study involved rectal administration of a radio-opaque semi-solid paste with the consistency of soft stool. The subject was then seated on a commode and made to excrete the material in a manner similar to defecation[17]. The radiological images taken during the evacuation process were interpreted by a radiologist who specialized in DP. The radiologist was blinded to the results of the psychosocial evaluation and did not interact with the subjects.

DP has been used extensively in patients with defecatory dysfunction, pelvic prolapse or puborectalis dysfunction[18-20] to visualize anatomic defects like internal or complete rectal prolapse, enterocele or rectocele. The quantification of the rectal evacuation is especially helpful in patients with pelvic floor dysfunction or dyssynergia and is recommended as a physiological means of assessing rectal dysfunction[21]. DP was considered "negative" for anatomical abnormalities if levator ani function was normal, rectoceles (if any) were < 4 cm[22] and there was no evidence of intussusception, rectal prolapse, or other anatomic abnormality demonstrated. Subjects were subsequently divided into those with structural defects seen on DP (DP positive group) and those with a normal defecography study (DP negative group). Responses to the questions were subsequently compared between the DP positive and the DP negative group.

Table 1 Demographic profile and psycho-social factors of subjects

Variables	DP positive group (n = 25)	DP negative group (n = 20)	P value
Demographics			
Males	3	5	> 0.050
Age (yr)	61.8 ± 2.8	58.1 ± 2.9	> 0.050
Assessment of psycho-social factors			
Sexual abuse	5	9	0.036
Physical abuse	1	2	> 0.050
Post traumatic stress disorder	1	4	0.045
STAI state anxiety	35.60 ± 2.00	42.75 ± 3.16	0.027
STAI trait anxiety	35.08 ± 1.76	38.06 ± 2.42	> 0.050
HADS anxiety	4.72 ± 0.40	6.60 ± 1.00	0.040
HADS depression	3.25 ± 0.46	5.72 ± 1.00	0.014
PHQ-15	10.76 ± 0.97	13.15 ± 0.82	0.038

All data are presented as mean ± SE. DP: Defecating proctography; HADS: Hospital anxiety and depression scale; STAI: State trait anxiety inventory; PHQ-15: Patient health questionnaire 15-item somatic symptom severity scale.

Statistical analysis

Unpaired Student's t test was used for analyzing demographic differences in demographic variables with continuous distribution while χ^2 test was used for analyzing categorical variables using GraphPad Prism software (v 5.0a, GraphPad Prism Inc., San Diego, CA, United States). A P value of < 0.05 was considered significant. All data are presented as mean ± SE.

RESULTS

A total of 45 patients were included in the study and completed the psychosocial evaluation prior to undergoing the DP. Thirty seven (82%) of the total subjects were females (Table 1). Forty four subjects (97.7%) were referred for DP for evaluation of constipation. One subject was referred for possible anismus (pelvic floor dyssynergia).

In 20 (44.4%) of the 45 subjects, the DP did not show any anatomical anomaly and these subjects were classified as DP negative (*i.e.*, negative for anatomical abnormalities on DP). The remaining 25 patients were classified as DP positive (*i.e.,* DP demonstrated anatomical abnormalities that could contribute to symptoms).

Anxiety and depression

Subjects in the DP negative group had strikingly high scores on the HADS anxiety and depression questionnaires (P = 0.04 and P = 0.01) compared to subjects in the DP positive group (Figure 1A and B). This correlated well with the significantly higher score on the STAI state

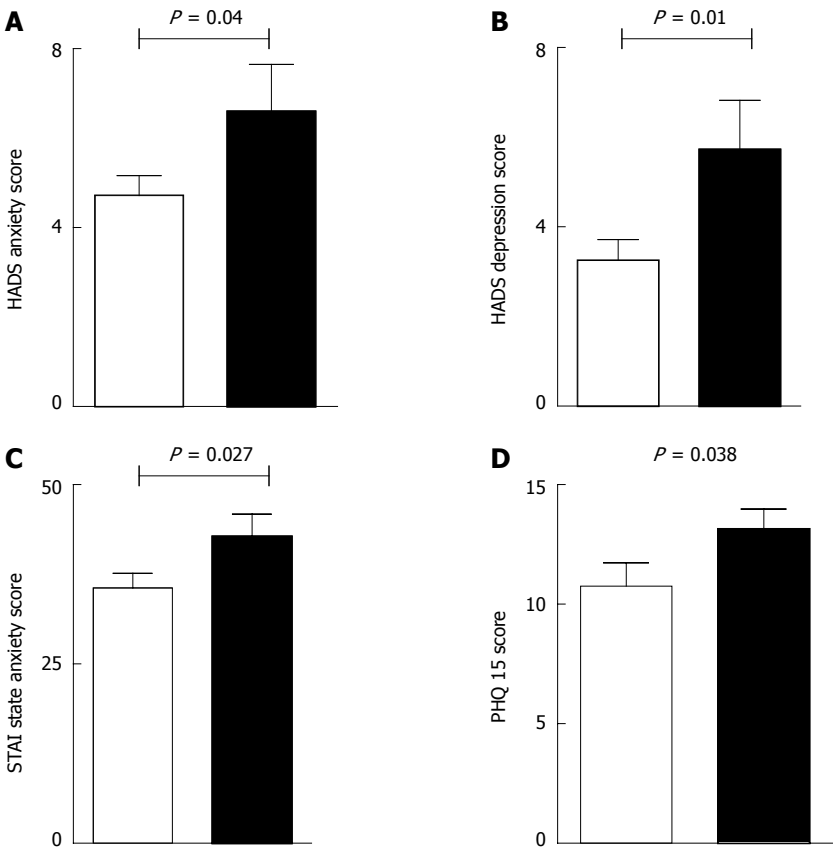

Figure 1 Subjects with no anatomical abnormalities seen on defecating proctography. Defecating proctography (DP negative; black column) have a significantly greater degree of psychiatric disorders as compared to the subjects with anatomical abnormalities on defecating proctography (DP positive; white column). A: Comparison of hospital anxiety and depression scale (HADS) anxiety; B: HADS depression; C: State trait anxiety inventory (STAI) state anxiety; D: Patient health questionnaire 15-item somatic symptom severity scale (PHQ-15) scores among DP positive and DP negative subjects using unpaired *t*-test is shown. All data are shown as mean ± SE.

anxiety questionnaire among subjects in the DP negative (P = 0.027) compared to the DP positive group (Figure 1C). Further, subjects in the DP negative group reported significantly worse PHQ-15 scores compared to the DP positive group (P = 0.038, Figure 1D).

Sexual abuse and PTSD

Notably, we found that a fair proportion of the subjects reported a history of sexual abuse. Nine of the 20 (45%) subjects in the DP negative group and 5 of the 25 (20%) subjects in the DP positive group reported a history of sexual abuse (P = 0.036, Figure 2A). This correlated well with a higher prevalence of PTSD among subjects in DP negative group (4 of 20 subjects, 20%) than the DP positive group (1 of 25 subjects, 4%; P = 0.04, Figure 2B).

Trait anxiety and physical abuse

There was no significant difference among the DP positive and DP negative groups in terms of STAI trait anxiety and prevalence of physical abuse. Further, there was no significant difference in the 2 groups in terms of prevalence of IBS or constipation assessed using the ROME Ⅲ questionnaires. Of note, all subjects included in the study had complaints of constipation and the results of the prevalence confirm the presenting complaints.

DISCUSSION

In this study, we demonstrated that subjects undergoing DP who met ROME Ⅲ criteria for functional constipation have a high prevalence of psychiatric disorders. Greater levels of state anxiety as well depression were found in the above mentioned population. We also found an association between PTSD, history of sexual abuse and functional constipation. Taken together, these findings suggest that a very detailed history about psychiatric co-morbidities and traumatic experiences must be taken in selected patients complaining of constipation.

We included subjects who were referred for DP as part of further work-up of constipation that was refractory to conservative management. It is pertinent to note that a majority of the referrals were from colo-rectal surgeons for pre-operative assessment. As the results of the study show, 44% of the patients (DP negative) did not have any surgically correctable cause and hence would not be appropriate candidates for surgical interventions.

Sexual abuse is very common among patients with functional disorders of the lower gastrointestinal tract[1] with one study showing prevalence of 40%. Further, abused patients were found to have constipation as the most common gastrointestinal complaint[23]. In our study,

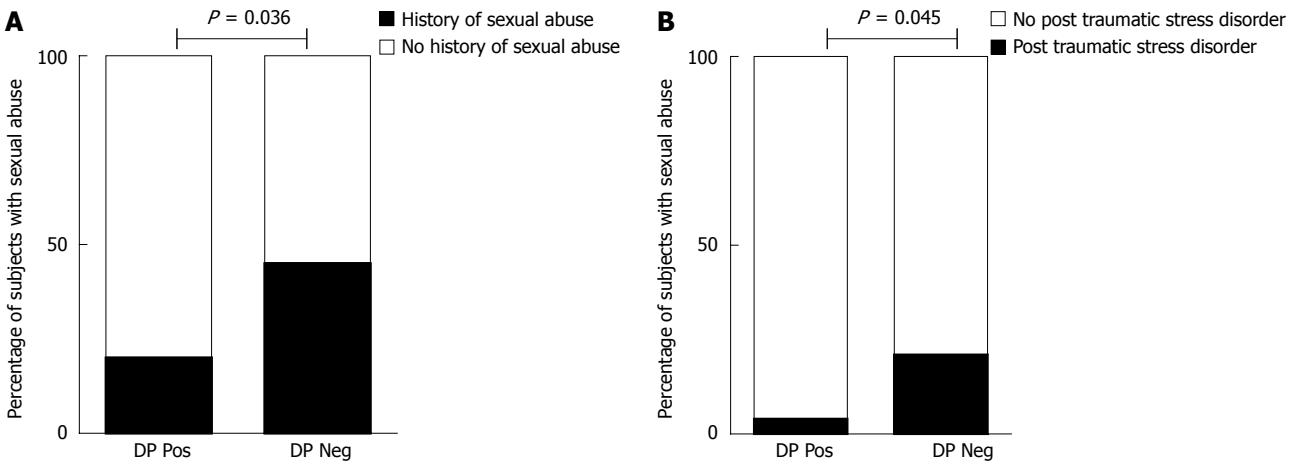

Figure 2 Subjects with no anatomical abnormalities seen on defecating proctography have a significantly greater prevalence of sexual abuse (A) and post-traumatic stress disorder (B) as compared to the subjects with anatomical abnormalities on defecating proctography. DP: Defecating proctography; Pos: Positive; Neg: Negative.

14 subjects had a history of sexual abuse of which a majority had constipation without any anatomical explanation. The difference in the prevalence of sexual abuse between the 2 groups was statistically significant. Notably, patients in the DP negative group had a significantly higher prevalence of PTSD, a condition closely associated with sexual abuse. Since a history of sexual abuse is a strong predictor of multiple surgeries and poor surgical outcomes for slow-transit constipation[24], a careful history is essential in this patient population.

There is a strong association between psychological dysfunction and gastro-intestinal disorders for which various therapeutic paradigms have been found to be effective. Cognitive behavior therapy, psychodynamic psychotherapy and pharmacological agents including anti-depressants been shown to be efficacious in managing the gastrointestinal symptoms in selected patients[1]. However, there needs to be a greater emphasis on the detection of psychological disturbance and eliciting a detailed history in these patients.

A novel aspect of our study was the use of DP for the evaluation of constipation and classifying subjects with organic (DP positive) *vs* functional (DP negative) gastrointestinal disorders. DP has been used extensively for evaluation of patients with evacuatory dysfunction[25] and is often considered the gold standard for imaging in patients with defecation disorders, most notably rectocele, enterocele, anismus and perineal descent[26]. Patients in the DP negative group would likely not benefit from surgical interventions given the functional nature of the symptoms detected by DP.

The findings of this prospective study demonstrate a significantly high degree of anxiety, depression, somatization, PTSD and sexual abuse in subjects with negative findings on DP. Our study confirms that psychological ailments can impact the lower gastrointestinal tract and is especially associated with constipation. We recommend that patients with refractory constipation and features suggestive of psychological ailment/abuse[3] should be appropriately screened for the aforementioned disorders.

COMMENTS

Background

Functional constipation is a common gastrointestinal ailment and is often associated with emotional disturbance. Defecating proctography (DP) is a radiological tool to visualize anatomical causes leading to gastrointestinal symptoms. This study assessed the prevalence of psychiatric ailments in patients with gastrointestinal symptoms who had been referred for defecating proctography.

Research frontiers

This study uses a novel approach of combining a radiological tool (DP) and multiple validated psychiatric instruments to assess the prevalence of psychiatric ailments causing gastrointestinal manifestations.

Innovations and breakthroughs

The study demonstrates that a significant proportion of subjects undergoing DP and met ROME Ⅲ criteria for functional constipation have psychiatric ailments. Further, there is an association between functional constipation and a history of sexual abuse.

Applications

It is suggested that a very detailed history about psychiatric co-morbidities and traumatic experiences must be taken in selected patients complaining of constipation. Further large scale studies are warranted to explore these associations.

Terminology

Defecating proctography is a radiological study that requires a subject to excrete a rectally administered contrast to visualize anatomical defects pertinent to rectal evacuation.

Peer review

The authors demonstrated a prospective study evaluating emotional disturbance in subjects undergoing defecating proctography. In my opinion, such a prospective study will give us more important information in this field.

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BRIEF ARTICLE

Gastroenterology training in a resource-limited setting: Zambia, Southern Africa

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Abstract

AIM: To evaluate need for and efficacy of a structured gastroenterology didactic session in expanding awareness and understanding of digestive disorders.

METHODS: A four-day symposium was developed with didactic sessions (days 1, 2) and practical endoscopy (days 3, 4). Didactic sessions included case presentations highlighting pathophysiology and management. One nurse and four practicing gastroenterologists from the United Kingdom led lectures and supervised workshops with audience participation. Practical endoscopy focused on diagnostic and therapeutic procedures and their application to diagnosis and treatment of ailments of the gastrointestinal tract. Pre- and post-workshop questionnaires were distributed to participants during didactic sessions. A pre-workshop questionnaire gauged expectations and identified objectives to be met at the symposium. Post-workshop questionnaires were administered to assess efficacy of each session. Participants graded sessions from 1 (poor) to 5 (excellent) on quality of case presentations, knowledge, clarity and mode of presentation. We assessed if time allotted to each topic was sufficient, value of sessions, impact on practice and interest in future symposiums.

RESULTS: There were 46 attendees on day 1: 41% undergraduates, 41% residents, 11% consultants and 4% unspecified. Day 2 (a Saturday) had 24 participants: 17% undergraduates, 71% residents, 9% consultants, 4% unspecified. Primary pre-workshop symposium expectation was to gain knowledge in: general gastroenterology (55.5%), practical endoscopy (13.8%), pediatric gastroenterology (5%), epidemiology of gastrointestinal disorders specific to Zambia (6%), and interaction with international speakers (6%). The post-symposium questionnaire was answered by 19 participants, of whom 95% felt specific aims were met; all would attend future conferences and recommend to others.

CONCLUSION: The beneficial effect of a structured symposium in developing countries warrants further attention as a mechanism to improve disease awareness in areas where resources are limited.

Key words: Gastroenterology training; Resource-limited country; Zambia; Specialist training; Postgraduate training; Hepatology

Core tip: The global burden of digestive diseases is increasing, yet formal training in gastroenterology is lacking in traditionally underserved areas such as the African continent. In this study we designed, implemented, and evaluated the effectiveness of a structured 4 d symposium focusing on general topics in the diagnosis and management of digestive disease. This

symposium was geared towards health care professionals and attendees reported improvement in their knowledgebase in gastrointestinal disorders. Structured symposiums are an effective and viable adjunct to medical education and their utility may be highest in regions where traditional academic medical resources are limited.

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INTRODUCTION

Zambia is a Southern African nation with a population of approximately 13 million people[1,2]. The University Teaching Hospital (UTH) in Lusaka has a capacity of approximately 1600 adult and 300 pediatric beds and is the main medical training institution in Zambia. UTH houses an endoscopy unit which serves as both an inpatient and ambulatory care facility providing both emergency and routine endoscopies. The unit is equipped with a Pentax video endoscopy suite which includes gastroscopes (including pediatric scope) and colonoscopes. The unit performs approximately 1000 gastrointestinal endoscopic procedures per year. Instrument cleaning and disinfection follows international guidelines (British Society of Gastroenterology)[3]. Continuing medical education is encouraged as staff regularly attend the South African Gastroenterology conference to maintain up to date proficiency. For a combination of epidemiologic (relatively lower prevalence of biliary disease compared with industrialized nations) and economic (lack of available funding) the unit does not currently carry out endoscopic retrograde cholangiopancreatography. However, there is a significant public health burden of luminal gastrointestinal and hepatology disease, and recent attention has turned to increasing the health care communities' awareness of these disorders[4-7]. Our objective was to develop and host a formal gastrointestinal/hepatology workshop to educate the healthcare sector and also evaluate its place as a mechanism to address the growing interest in this field. We review our experience in the development of the workshop and its impact on the health professionals working in a resource-limited setting.

MATERIALS AND METHODS

We hosted a course to improve understanding of gastrointestinal disorders. The specific aims of the conference were to promote a greater understanding of: (1) the pathophysiology and management of common gastrointestinal/hepatological disorders; (2) principles of endoscopy (upper and lower) including indication, risks and benefits; (3) clinical skills in endoscopy with emphasis on management and evaluation of varices, non-portal hypertensive related gastrointestinal bleeding and colonic polyp recognition and removal; and (4) maintenance of endoscopic efficiency and patient safety.

Lectures

Didactic sessions over the first two days introduced participants to essential pathophysiology and management of prevalent disorders emphasizing a multidisciplinary approach to patient care. The sessions were open to all participants and led by a panel of experts from the United Kingdom in conjunction with staff physicians at UTH. These lectures were focused on pertinent topics including: diarrheal disease, gastrointestinal emergencies, malnutrition, esophageal disease, abdominal pain and hepatology. Sessions were conducted in a case presentation format in which clinical findings and course were reviewed to illustrate key points in pathophysiology and management.

To illustrate, a case of cholera was used as an opportunity to review the physiology of secretory (toxin-mediated) diarrhea, which in turn served as a platform to discuss the rationale of oral rehydration therapy. As another example, a session was centered on gastrointestinal bleeding using cases of peptic ulcer bleeding and variceal bleeding due to schistosomiasis to illustrate principles of emergency management and differing endoscopic approaches to both non-portal hypertensive and portal hypertensive related bleeding. We ran focused hepatology sessions which included cases that fostered discussion on the applicability of gold-standard management with limited resources (for example, availability of vaccinations and access to ultrasound). Guidelines on the management of ascites, hepatitis B and encephalopathy were developed in break-out sessions. On day 2, patients with specific gastrointestinal and hepatology ailments were interviewed in front of the audience to elaborate points for discussion. Audience participation was actively encouraged throughout and sessions were designed to facilitate interaction and comparison of management strategies.

Prior to the workshop, we administered a questionnaire to gauge attendees' expectations and identify weak areas in their knowledge base. Post-workshop questionnaires on the quality of each session asked participants to grade sessions from 1 (poor) to 5 (excellent) on the following criteria: quality of the case presentations, knowledge, clarity and mode of presentation. We also assessed if the time allotted for each topic was considered sufficient, if participants felt presented information was applicable to their stage of training, if they felt there was a need for specific guidelines for the management of presented disorder in Zambia and finally if information learned at the presented session would change their personal management approach.

Practical endoscopy

Our practical sessions complemented lecture-based dis-

Table 1 Demographics of participants *n* (%)

Variables	Preconference	Day 1	Day 2
Gender			
Male		19 (41)	13 (52)
Female		18 (39)	7 (28)
Not stated		9 (20)	5 (20)
Training/specialty			
Undergraduate	17 (47)	19 (41)	4 (17)
Postgraduate	14 (39)	19 (41)	17 (63)
Consultants	4 (11)	5 (11)	4 (9)
Other	1 (3)	3 (7)	1 (4)
Total participants	36	46	24

Table 2 Preconference questionnaire *n* (%)

Expectations	
General GI	20 (56)
Practical endoscopy (adult/children)	5 (14)
Pediatric GI	2 (5)
Epidemiology of GI disorders specific to Zambia	4 (6)
Interaction with international visitors	4 (6)
None	1 (3)
Weak areas	
Hepatology	9 (19)
GI Bleeds (including peptic ulcer disease)	7 (15)
IBD	8 (17)
GI malignancy	4 (8)
Infectious Gastroenterology (including HIV)	6 (13)
Malnutrition	1 (2)
Malabsorption (including celiac)	2 (4)
Diarrheal disease	1 (2)
Fluid management	1 (2)
Autoimmune conditions	1 (2)
Colon pathology	2 (4)
Biliary disease (pancreatic and gallbladder)	1 (2)
Pediatric GI	2 (4)
Practical endoscopy skills	3 (6)

GI: Gastrointestinal; IBD: Inflammatory bowel disease; HIV: Human immunodeficiency virus.

cussion on management in the first two days. Participants were instructed on endoscope instruments and accessories, set-up of endoscopy equipment and preparation for endoscopy including patient safety and informed consent. The nurses were involved in the hands-on experience, and obtained additional training related to the patient preparation, aftercare and maintenance of equipment in the endoscopy unit.

There were 15 participants for the live cases. The first endoscopy day was a combination of adult and pediatric cases: esophageal variceal banding, duodenal polypectomy with hemoclip application for hemostasis and appropriate biopsies in a case of gastric ulcer. The colonoscopies included hematochezia and ulcerative colitis. All cases were followed by a case and management discussion.

RESULTS

Forty-six attendees answered our questionnaires on day 1: 41% undergraduates, 41% residents, 11% consultants and 4% unspecified. Day 2 had 24 participants: 17% undergraduates, 71% residents, 9% consultants, 4% unspecified. Attendees from neighboring countries included 3 physicians from Zimbabwe, 1 from Malawi and 1 from the Democratic Republic of Congo. The organizing committee included five visiting experts in gastroenterology from the United Kingdom, who led several of the didactic sessions. Table 1 describes the demographics.

Table 3 Evaluation of each session *n* (%)

Diarrheal disease	1 (poor)	2	3	4	5 (excellent)
Case presentation	0 (0)	0 (0)	1 (2)	25 (58)	17 (40)
Knowledge	0 (0)	0 (0)	0 (0)	21 (53)	19 (48)
Clarity	0 (0)	0 (0)	7 (18)	16 (41)	16 (41)
Mode of presentation	0 (0)	0 (0)	1 (3)	19 (49)	19 (49)
GI emergencies					
Case presentation	0 (0)	1 (3)	2 (6)	20 (59)	11 (32)
Knowledge	0 (0)	0 (0)	2 (6)	21 (62)	11 (32)
Clarity	0 (0)	0 (0)	4 (11)	17 (47)	15 (42)
Mode of presentation	0 (0)	0 (0)	1 (3)	19 (51)	16 (43)
Malnutrition					
Case presentation	0 (0)	0 (0)	1 (3)	29 (73)	10 (29)
Knowledge	0 (0)	0 (0)	1 (3)	22 (55)	17 (43)
Clarity	0 (0)	0 (0)	3 (8)	11 (64)	11 (28)
Mode of presentation	0 (0)	0 (0)	2 (5)	24 (60)	14 (35)
Oesophageal diseases					
Case presentation	0 (0)	0 (0)	0 (0)	6 (30)	14 (70)
Knowledge	0 (0)	0 (0)	0 (0)	9 (45)	11 (55)
Clarity	0 (0)	0 (0)	0 (0)	11 (55)	9 (45)
Mode of presentation	0 (0)	0 (0)	0 (0)	9 (45)	11 (55)
Abdominal pain					
Case presentation	0 (0)	0 (0)	0 (0)	6 (30)	14 (70)
Knowledge	0 (0)	0 (0)	1 (5)	5 (25)	14 (70)
Clarity	0 (0)	0 (0)	1 (5)	4 (20)	15 (75)
Mode of presentation	0 (0)	0 (0)	0 (0)	5 (25)	15 (75)
Hepatology					
Case presentation	0 (0)	0 (0)	1 (5)	7 (33)	13 (62)
Knowledge	0 (0)	0 (0)	2 (10)	7 (33)	12 (57)
Clarity	0 (0)	0 (0)	1 (3)	12 (57)	8 (38)
Mode of presentation	0 (0)	0 (0)	1 (5)	7 (33)	13 (62)

Participants graded sessions from 1 (poor) to 5 (excellent) on quality of case presentations, knowledge, clarity and mode of presentation.

Primary pre-workshop symposium expectations were to gain knowledge in: general gastroenterology (55.5%), practical endoscopy (13.8%), pediatric gastrointestinal (GI) disorders (5%), epidemiology of GI disorders specific to Zambia (6%), and interaction with international speakers (6%) (Table 2). The most common areas that participants thought their knowledge was weak were: hepatology (19%), inflammatory bowel disease (17%), gastrointestinal bleeds-including peptic ulcer disease (15%) and infectious gastroenterology-including human immunodeficiency virus (HIV) (13%) and gastrointestinal malignancy (8%) (Table 2). Sessions already planned by the time these responses were received covered the majority of these areas.

The sessions were on diarrheal disease, gastrointestinal emergencies, malnutrition, esophageal diseases, abdominal pain and hepatology (Table 3). The cumulative average percentage of respondents who scored sessions either good (4/5) or excellent (5/5) for the following

criteria: case presentation 97%, knowledge 96%, clarity 93%, mode of presentation 97%. The percentage of respondents who answered "Yes" to the following questions on each topic: was time allotted to this topic sufficient? 81%; did you find this session valuable for your stage of training? 98%; do we need to develop specific management guidelines? 95%; will this session change your management? 94%.

There were some variations between sessions although the overall quality was considered high. Significant numbers of respondents said that the time allocated was insufficient for diarrhoeal disease (33%), malnutrition (24%) and esophageal disease (25%). This demonstrates an interest and need for further training in these areas. The large majority (95%) who felt specific management guidelines should be developed on these topics is also indicative of the need for further work. The post-symposium questionnaire was answered by 19 participants, of whom 95% felt specific aims were met; 90% would pay for future conferences, all would attend future conferences and recommend to others.

DISCUSSION

There is a recognized shortage of general and specialized medical doctors in Zambia and Sub-Saharan African countries[8-10]. The detailed reasons for such shortages are beyond the scope for this paper, however one of the identified strategies to curtail this problem includes training opportunities and continued medical education[9,10]. Based on 2010 statistics, the health life expectancy at birth in Zambia is 49 years[11]. The dominant gastrointestinal and hepatological clinical problems are variceal bleeding due to schistosomiasis, esophageal strictures due to caustic substance ingestion, infectious diarrhea (often HIV related), peptic ulceration, hepatitis B, and gastrointestinal cancer (Kaposi sarcoma, esophageal, gastric and colon cancer)[12,13]. There is also a considerable burden of neurogastroenterological problems including achalasia, functional dyspepsia and irritable bowel syndrome. The most common cause of esophageal bleeding in patients presenting to our endoscopy unit is esophageal varices (25%), other etiologies are duodenal ulcer (17%) and gastric ulcers (21%); less frequent but significant causes are Kaposi's sarcoma (2%) and Mallory Weiss tear (1%)[12]. It has been estimated that more than 90% of schistosomiasis cases occur within Sub-Saharan Africa[14]. The prevalence in Zambia is 77% and the two predominant forms are *Schistosoma hematobium* and *Schistosoma mansoni*[15,16]. *Schistosoma mansoni* has been implicated in intestinal and liver disarray resulting in portal hypertension, esophageal varices, gastrointestinal bleed and rarely liver failure[17-19].

The estimated prevalence of HIV infection in Zambian adults aged 15-49 years is estimated between 10.3%-19.7%, leading to an increased burden of HIV enteropathy[20,21]. With this HIV burden there is also concern for the increasing trend of hepatitis B and hepatitis C[22]. There are no Zambian liver disease management guidelines, thus current practice follows guidelines of the World Health Organization[23], American Association for the Study of Liver disease[24] and European Society for the Study of Liver[25], which are not suitable in resource-limited settings and in an area where etiopathogenesis is different.

The incidence of gastric and esophageal cancer in adults younger than 45 years is higher than in United States or United Kingdom[12]. Gastric cancer in patients under 45 years accounts for 33% of cases, whilst esophageal cancer represented 16% of endoscopically diagnosed cases[12]. Survival from digestive disease is lower in developing countries, in those within the African continent[26], thus raising awareness and developing prevention programs are important and can be enhanced through educational symposia such as described in this paper. Caustic injuries, either suicidal or accidental, are another area of concern; with patients presenting late in the course with resultant gastric outlet obstruction (55%), esophageal strictures (30%), gastric ulcerations (21%)[27].

One of the aims of the workshop was to draw in other health care workers interested in gastroenterology and hepatology but working outside UTH so as to facilitate networking and optimize standards of care for GI diseases throughout the country, and positively impact the African continent. We successfully included physicians from neighbouring countries: 3 from Zimbabwe, 1 Malawi, 1 Congo. These surrounding nations benefited from this course because they share a similar disease burden as Zambia. Continued training in the field of gastroenterology in Zambia and other resource-limited areas, is necessary to enhance understanding of pathophysiology and management, thus improving overall patient care.

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COMMENTS

Background

Formal training in gastroenterology is lacking despite the huge burden of digestive disease across Africa. Given the disparity in the supply of formally-trained gastroenterologists and the ever increasing demand of citizens, the authors organized a structured four-day symposium focusing on gastrointestinal/hepatological case based presentations and introduction to endoscopy.

Research frontiers

Promotion of such educational activities should be encouraged not only to help physicians develop new perspectives on disease, but also to improve overall patient care.

Innovations and breakthroughs

In this work the authors organized the first gastroenterology symposium in Zambia, attracting students and physicians from neighbouring countries. The authors have set a foundation for similar activities in the future.

Applications

This could be replicated in other developing countries that face similar disease burdens and require improvements in undergraduate and postgraduate training.

Terminology

The symposium was an opportunity to teach, increase awareness of gastroin-

testinal and hepatological diseases whilst creating an environment for networking. This symposium addressed current knowledge and recent advances in gastrointestinal/hepatological disease.

Peer review

This article provides information and guidelines for setting up a structured symposium in a resource-limited setting. Equally important, this highlights the need for structured clinical gastroenterology/hepatology training programmes with adequate curricula that emphasize knowledge, skills, and scientific productivity.

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BRIEF ARTICLE

Cytokine profiles in patients receiving antioxidant therapy within the ANTICIPATE trial

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Abstract

AIM: To measure a broad profile of pro- and anti-inflammatory cytokines in patients with clinically proven chronic pancreatitis (CP) taking either antioxidant therapy or placebo as part of the larger ANTICIPATE study.

METHODS: Patients with chronic pancreatitis were recruited to the ANTICIPATE study following informed consent and were randomised to intervention with either antox version 1.2-based antioxidant therapy or placebo. After a separate ethics committee amendment a subgroup of 7 patients from either arm of the study were selected for additional analysis of cytokines. Cytokines were measured at baseline and after 6 mo of either antox therapy or placebo by biochip array and enzyme-linked immunosorbent assay.

RESULTS: Antioxidant therapy and placebo groups were well-matched in terms of age, gender, aetiology of CP, opiate use and disease duration. Baseline antioxidant levels were similar in patients allocated to the antioxidant group as compared to the group allocated to placebo. After 6 mo of antioxidant therapy there was significant elevation in vitamin C levels in the intervention group: 17.6 μg/mL (12.8-29.3 μg/mL) compared to 4.8 μg/mL (1.6-9.1 μg/mL) in placebo ($P < 0.001$; 95%CI: 9.0-20.2) with similar trends in selenium levels. There was no elevation in a broad array of pro- and anti-inflammatory cytokines in the antioxidant group compared to placebo [interleukin (IL)-1B, IL-4, IL-6, IL-10, tumor necrosis factor-α] either at baseline or after 6 mo of antioxidant therapy.

CONCLUSION: Cytokine levels were low at baseline and at 6 mo despite a significant elevation in plasma antioxidants. In patients with CP, with opiate-dependent abdominal pain, circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of inflammation.

Key words: Chronic pancreatitis; Antioxidant therapy; Cytokine

Core tip: This study examines cytokine levels in a subset of patients recruited from within the ANTICIPATE randomized controlled trial of antox for painful chronic pancreatitis. At baseline, pro- and anti-inflammatory cytokine levels were within the laboratory reference range in patients allocated to the antioxidant arm and those allocated to placebo. After 6 mo of intervention with antox, there was a significant elevation in antioxidant levels in patients in the active treatment arm. This was not associated with any change in either pro- or anti-inflammatory cytokine levels. In patients with chronic pancreatitis, with opiate-dependent abdominal pain, circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of inflammation.

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INTRODUCTION

The oxidative stress hypothesis proposed that cell injury in chronic pancreatitis (CP) was mediated at the acinar level by short-lived oxygen free radicals produced as a result of imbalance in the physiological processes producing these agents and those pathways involved in deactivating them[1]. A key component of this theory was that the methionine transsulfuration pathway which yields glutathione (important in the quenching of antioxidants) is overwhelmed in patients with CP as the detoxification of xenobiotics by cytochrome P450 led to overproduction of oxygen-derived free radicals[1]. There was evidence that the dietary intake of some patients with CP was deficient in selenium, methionine and vitamin C, key cofactors in these transsulfuration pathways[2]. This finding was supported by evidence showing that plasma/blood levels of circulating antioxidants were low in CP compared to control[3]. The logical completion of this paradigm was the development of antioxidant therapy - a pharmacological preparation containing methionine, vitamin C, vitamin E and selenium and designed to restore these critical co-factors to patients with CP[1]. Early clinical trials of antioxidant therapy failed to establish evidence of clinical efficacy and thus the treatment was not widely accepted. To address this issue, we conducted and reported the largest randomized controlled trial of antox for treatment of pain in chronic pancreatitis - the ANTICIPATE study[4]. In this, 356 patients with CP were screened for eligibility, 92 randomised and 70 completed intervention with 6 mo of antioxidant therapy or matched placebo. At the end of this period there was no difference between treatment and placebo in the primary endpoint of abdominal pain as assessed by a numerical rating scale or in secondary endpoints of pain assessed by pain diaries and quality of life assessed by validated questionnaire[4]. However, blood and plasma antioxidant levels were significantly elevated in patients in the treatment group[4]. In keeping with other clinical studies of antioxidant therapy in chronic pancreatitis with clinical endpoints there is little information on the effects of intervention on inflammatory markers.

The present study does provide unique data on cytokine profiles in patients with chronic advanced pancreatitis at their end disease stage receiving antioxidant therapy and in a matched cohort receiving placebo and provides negative results which should be regarded as important pilot data. Thus, although the principal findings were negative, the ANTICIPATE study provided a unique vehicle with which to assess the potential interaction between antioxidant therapy and cytokine markers of inflammation and fibrosis in chronic pancreatitis. To the best of our knowledge, this interaction has never previously been studied.

In chronic pancreatitis there is evidence that levels of platelet-derived growth factor-BB and transforming growth factor (TGF)-β1 are elevated and that these cytokines play an important role in pancreatic fibrosis[5]. Pancreatic stellate cells are activated by alcohol in CP and are key mediators of subsequent inflammatory changes and fibrosis with these changes being modulated by cytokines including epidermal growth factor[6,7]. Pancreatic ductal epithelium produces TGF-β which also mediates fibrosis[8]. Thus cytokines are known to be key mediators of inflammatory and fibrotic change in CP.

The aim of the present study was to examine circulating cytokine levels in a cohort of patients within the ANTICIPATE study. The principal endpoint was to assess whether there were differences between patients receiving antioxidant therapy and those receiving matched placebo.

MATERIALS AND METHODS

Study design

This is a case-control analysis of a sub-group of patients recruited from both arms of the ANTICIPATE double-blind, placebo-controlled, randomised trial of Antox version 1.2 (Pharma Nord, Morpeth, United Kingdom) in patients with painful chronic pancreatitis[4].

Setting

Tertiary care academic medical centre was eventually chosen as setting in which to implement the requirment.

Inclusion/exclusion criteria

The inclusion criteria were as for the main ANTICIPATE study and can be summarised as follows: patients with evidence of chronic pancreatitis on cross-sectional imaging together with evidence of impairment of pancreatic exocrine function as assessed by assay of faecal elastase. Patients who did not meet these criteria were excluded as were patients with evidence of malignancy. The inclusion/criteria for the main study are provided in detail elsewhere[4].

Identification and selection of study sub-group

Recruitment to ANTICIPATE commenced in February 2008 and a protocol amendment to permit additional enrolment to the present study was approved 6 mo later. Patients recruited to ANTICIPATE were allocated to receive either 6 mo intervention with antox or matched placebo in a randomised, double-blind, placebo-controlled fashion. Those patients selected to participate in this study signed an additional consent form. No additional inclusion or exclusion criteria were used. Allocation arm was unknown during the conduct of ANTICIPATE and patients were stratified at enrolment to this study by whether or not they had undergone prior pancreatic intervention (either surgical or endoscopic). Blood samples were drawn from 22 patients in the "prior intervention" stratification arm and from 15 in the "no prior intervention" stratification arm. Following the code break at the end of the clinical ANTICIPATE study, investigators were notified which patients had been allocated to active drug and which had

been allocated to placebo. At this point, a study population of 10 consecutive patients from each arm of the study was identified (total 20 patients). Allowing for loss to follow-up in 6 patients in whom blood samples for cytokine analysis were not taken after the original baseline assays a final study population of 7 patients treated with antox for 6 mo and 7 patients treated with placebo was obtained.

Assays

Full blood count (haemoglobin and white cell count) was measured at baseline and at 2, 4 and 6 mo. C-reactive protein (CRP) levels were also measured at these time points. Antioxidant levels comprising the following: selenium, vitamins C and E, β-carotene and glutathione were measured at baseline, study mid-point and at 6 mo. A range of cytokines were measured at baseline and at 6 mo as follows: pro-inflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor alpha; anti-inflammatory cytokines: IL-4, IL-10; chemokines: IL-8, IL-18, monocyte chemotactic protein 1; the T cell regulatory cytokine IL-2; the angiogenic signalling protein vascular endothelial growth factor (VEGF) and epidermal growth factor an important regulator of cellular proliferation, differentiation and survival[7].

Methods of measurement

Full blood count was measured by the haematology department of the Manchester Royal Infirmary with CRP being measured in the clinical biochemistry service and these results were available to clinicians to guide on-going management during the study. Antioxidant levels were measured by the pancreatic laboratory of the Manchester Royal Infirmary. The results of these assays were available during the study. Cytokine assays were undertaken by Bio-chip Arrays and enzyme-linked immunosorbent assay by Randox laboratories, Crumlin, Northern Ireland. These were analysed as a batch at the end of the study.

Sample collection

Non haemolysed and non-lipaemic serum and plasma were used for the Biochip array. Samples were collected into leak-proof, non-absorbent plastic containers. After collection, samples were aliquoted into containers and stored at -70 ℃. Repeated freeze/thaw cycles were avoided. Samples were labelled prior to transportation on dry ice to Randox laboratory, Crumlin, Northern Ireland *via* a secure, approved courier.

Interference

The effect of bilirubin, haemoglobin, triglycerides and lipids were assessed to establish the level at which the interference caused a significant increase or decrease in assay performance. The criterion set for this was that analyte recovery (all cytokines) should not vary from base recovery by more than 10%.

Ethics committee approvals

The original full study protocol was approved by the North West Regional Ethics Committee (MREC, 07/MRE08/13) and the United Kingdom Medicines and Health products Regulatory Agency (MHRA, 2006-006958-10). This cytokine subgroup study was approved by the North West Regional Ethics Committee as a separate amendment. The master study ANTICPATE from within which these patients were recruited was registered with the International Registry of Randomized Controlled trials and allocated the number ISRCTN-21047731.

Statistical analysis

Two by two tables were analysed by Fisher's exact test and non-parametric data by Mann-Whitney *U* test using the Statsdirect software package (version 2.6.5. www statsdirect.com).

RESULTS

Demographic and biochemical profiles

As in the parent study, the two groups were well-matched in terms of age and gender distribution. Alcohol was the dominant etiologic agent and a majority in both groups were cigarette smokers (Table 1).

Antioxidant profiles at baseline and at 6 mo

Baseline levels of vitamin C, vitamin A, whole blood glutathione transferase and red cell glutathione transferase were similar between groups and were also within the reference range for population normal as reported by the Pancreatic laboratory of the Manchester Royal Infirmary (Table 2). Levels were towards the lower range of normal. Although median vitamin E, β-carotene and selenium levels were below the range for population normal in the placebo group, this difference was not significant compared to the antioxidant group at baseline.

Haemoglobin, white cell count and CRP were within normal levels in both groups.

At 6 mo (Table 3) there was significant elevation of vitamin C and selenium levels in the antioxidant group compared to baseline and also compared to placebo at 6 mo. Vitamin A and E levels were also significantly elevated in patients receiving antioxidant therapy compared to those receiving placebo at 6 mo. A similar pattern was seen for β-carotene although these values did not attain significance.

There was no difference in haemoglobin, white cell count or CRP at 6 mo between antioxidant therapy and placebo or between antioxidant therapy and baseline.

There were also no differences in opiate usage.

Cytokine profiles

There was no difference between the antioxidant group and placebo at baseline in any of the cytokines measured in this study (Table 4). Similarly, there was no difference between antioxidant and placebo at 6 mo and no difference in the antioxidant group at 6 mo compared to the same group at baseline. IL-1B, IL-2, IL-4 and IL-10 me-

Table 1 Demographic profiles

Variables	Antioxidant (n = 7)	Placebo (n = 7)	P value
Age (yr), median (range)	46 (34-79)	46 (37-60)	0.92 (Mann-Whitney U)
Gender (male:female)	5:2	4:3	0.90 (Fisher's exact)
Aetiology	Alcohol 6; idiopathic 1	Alcohol 4; idiopathic 3	0.55 (Fisher's exact)
Disease duration (yr)	4 (1-5)	3 (2-13)	0.92 (Mann-Whitney U)
Body mass index (kg/m^2)	24.2 (18.8-36.7)	22.5 (22.9-32.8)	0.62 (Mann-Whitney U)
Alcohol (g/d), median (range)	175.5 (0-396)	138.6 (0-252)	0.43 (Mann-Whitney U)
Cigarette smoker (Y:N)	6:1	5:2	0.59 (Fisher's exact)
Diabetes mellitus (Y:N)	2:5	1:6	0.62 (Fisher's exact)
Faecal elastase (μg/g)	68 (15-500)	27 (15-500)	0.27 (Mann-Whitney U)
Opiate use (mg/d)	40 (30-300)	85 (0-120)	0.30 (Mann-Whitney U)

Laboratory reference range for faecal elastase report values < 200 μg/g as representing end-stage exocrine failure. All opiate intakes are reported as morphine equivalent.

Table 2 Baseline antioxidant profiles

Variables	Antioxidant (n = 7)	Placebo (n = 7)	Laboratory reference range	Median difference	P value (MWU)	95%CI
Vitamin C (μg/mL)	7.7 (0.7-13)	5.8 (2.4-9.9)	4-20	1.6	0.53	-3-6.1
Vitamin E (mg/L)	12.4 (5.4-20.9)	5.4 (3.6-15.2)	5.7-14.9	4.4	0.12	-2.6-11.1
β-carotene (mol/L)	35.9 (8-87)	11.6 (7-233)	19-254	15	0.55	-166-71
Vitamin A (mg/L)	0.60 (0.30-0.68)	0.40 (0.20-0.57)	0.4-1.2	0.16	0.07	-0.03-0.37
Selenium (μg/L)	82 (27-110)	49 (27-97)	83-152	27	0.22	-14-53
WGSH (μmol/L)	1361 (1229-1682)	1336 (1149-1585)	1078-1753	62.5	0.73	-118-290
WGSH/Hb (μmol/g)	9.2 (7.8-11.4)	9.7 (8.9-10.0)	7.5-12.2	-0.3	0.70	-1.4-1.5
WCC (10^9/L)	7.7 (6.4-10.3)	10 (5-15.9)	4-11	-1.4	0.38	-4.4-2.2
Hb (g/dL)	14.9 (13.4-15.8)	13.7 (12.4-16)	13-18	1	0.33	-0.6-2.1
CRP (mg/L)	3 (3-29)	7 (3-29)	0.3-5	-1	0.27	-7-3

WGSH: Whole blood glutathione; WGSH/Hb: Glutathione corrected for haemoglobin concentration; WCC: White cell count; Hb: Haemoglobin; CRP: C-reactive protein; MWU: Mann-Whitney U.

Table 3 Antioxidant profiles at 6 mo compared to baseline

Variables	Antioxidant (n = 7)	Placebo (n = 7)	P value (antioxidant *vs* baseline)	95%CI	P value (antioxidant *vs* placebo)	95%CI
Vitamin C (μg/mL)	17.6 (12.8-29.3)	4.8 (1.6-9.1)	0.001	-18.8-6.5	< 0.001	9.0-20.2
Vitamin E (mg/L)	17.8 (11.7-25.0)	5.0 (4.0-4.6)	0.160	-1.8-12.3	0.004	4.4-14.3
β-carotene (mol/L)	155.5 (23-478)	38.1 (8-204)	0.150	-189-35	0.244	-20-190
Vitamin A (mg/L)	0.5 (0.42-0.72)	0.3 (0.25-0.64)	0.910	-0.19-0.14	0.010	0.05-0.34
Selenium (μg/L)	109 (95-133)	48 (40-92)	0.007	-75-14	< 0.001	41-85
WCC (10^9/L)	6.7 (4.9-10.8)	7.4 (6-10.8)	0.330	-1.3-3.2	0.600	-3.3-1.9
Hb (g/dL)	14.2 (13.5-16.0)	12.7 (12.3-16.0)	0.510	-1-1.6	0.150	-1.3-2.6
CRP (mg/L)	3 (3-4)	6 (3-10)	0.190	0-3	0.070	-7-0
Opiate usage	20	55	0.210	-40-79	0.630	-56-60

WGSH: Whole blood glutathione; WGSH/Hb: Glutathione corrected for haemoglobin concentration; WCC: White cell count; Hb: Haemoglobin; CRP: C-reactive protein.

dian values were below the lower limit of the laboratory reference range at all sample points although individual patient sample values registered within the reference range. IL-6 and IL-8 values were within the reference range but towards the lower end at all sample points. VEGF showed higher values in the placebo group at both baseline and at 6 mo although this difference was not significant.

DISCUSSION

To the best of our knowledge, this study is the first to examine circulating cytokine levels in patients with chronic pancreatitis receiving antioxidant therapy and to compare these values to controls (also with CP) receiving matched placebo. When interpreting these findings, several important methodological sources of error should be emphasised. First, this is a small study with only 7 patients in each group. Thus it should be borne in mind that negative findings could represent a type Ⅱ error. A second source of error is the possibility of technical compromise in assay methodology as samples were transferred for analysis. As the majority of readings were low, could deterioration in sample quality have affected the assays?

Table 4 Cytokine levels in patients receiving antioxidant therapy compared to placebo

Cytokine	Laboratory range	Antioxidant therapy baseline (pg/mL)	Placebo baseline (pg/mL)	*P* value	95%CI	Antioxidant therapy at 6 mo (pg/mL)	Placebo at 6 mo (pg/mL)	*P* value	95%CI
IL-1β[1]	1.6-250	< 1.6	< 1.6			< 1.6	< 1.6		
IL-2	4.8-3000	2.6 (0-4.8)	3.1 (0-3.5)	0.83	-1.1-1.7	2.6 (0.0-4.8)	2.9 (0.0-3.2)	0.97	-2.6-2.3
IL-4	6.6-900	2.3 (2.2-6.6)	2.5 (2.1-6.6)	0.84	-3.7-3.9	2.5 (2.2-6.6)	2.8 (2.1-6.6)	0.81	-1.3-3.7
IL-6	1.2-900	1.9 (0.8-3.5)	1.8 (0.7-8.9)	0.99	-2.6-1.4	1.6 (1.0-2.3)	1.4 (0.9-7.6)	0.78	-3.9-0.7
IL-8	4.9-3000	10.1 (8.1-23.7)	8.5 (7.5-19.8)	0.46	-5.2-8.4	11.6 (8.1-19.3)	10.8 (6.6-18.6)	0.54	-2.7-18.7
IL-10[2]	1.8-1000	< 0.6	< 0.6			< 0.6	< 0.6		
TNFα	4.4-1500	3.5 (2.4-5.8)	3.5 (2.2-4.9)	0.71	-1.1-1.6	3.8 (2.4-4.7)	3.4 (2.5-4.5)	0.40	-0.7-1.1
IL-18	0-3000	388.6 (245.7-818.9)	457.9 (317.5-775.7)	0.71	-228-165	365.5 (211.5-879.8)	435.7 (349.1-570.1)	0.40	-238-299
VEGF	14.6-3000	116.2 (49.8-191.2)	243.1 (39.5-305.5)	0.32	-187-51	93.7 (35.1-173.9)	184.4 (34.1-299.9)	0.22	-206-34.7
EGF	2.9-900	28.4 (9.8-137.7)	23.4 (11.9-75.6)	0.99	-30.0-47.6	9.4 (3.1-65.2)	34.4 (2.3-139.8)	0.09	-66.8-5.5
MCP-1	13.2-1500	335.9 (267.1-423)	298.8 (229.5-685.1)	0.81	-134-106	296.5 (235.0-426.7)	326.4 (265.5-468.6)	0.38	-127-49

[1]All patients had interleukin (IL)-1β levels below the lower threshold of detection. IL-2: Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.64$ (95%CI: -1.5-2.5); IL-4: Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.89$ (95%CI: -3.7-3.7); IL-6: Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.25$ (95%CI: -0.4-1.7); IL-8: Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.69$ (95%CI: -17.8-4.9); [2]All patients had interleukin 10 below the lower threshold of detection. Transforming growth factor type β1 (TGFβ1): Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.46$ (95%CI: -39-74); tumor necrosis factor α (TNFα): Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.97$ (95%CI: -1.0-1.4); vascular endothelial growth factor (VEGF): Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.54$ (95%CI: -43.8- 81.0); epidermal growth factor (EGF): Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.05$ (95%CI: -0.22-59.8); monocyte chemotactic protein-1 (MCP-1): Antioxidant group at 6 mo *vs* antioxidant group at baseline, $P = 0.05$ (95%CI: -50.1-100.9).

Whilst this possibility cannot definitively be excluded, the commercial laboratory which undertook these assays works closely with the clinical biochemistry department of the Manchester Royal Infirmary and regularly undertakes analysis of externally drawn samples. Sample extraction, storage and transfer were in full compliance with established protocols. Further, laboratory markers of the inflammatory response measured in-hospital such as the white cell count and CRP were also normal providing indirect support. A third caveat is that cytokine levels measured in blood may not necessarily reflect their activity at the pancreatic parenchymal level. For example, Noh and colleagues demonstrated that IL-8 concentrations are elevated (compared to non-disease controls) in pancreatic juice collected by duodenoscopy[9].

Accepting these limitations, the present study does provide unique data on cytokine profiles in patients with chronic pancreatitis receiving antioxidant therapy and in a matched cohort receiving placebo and provides negative results which should be regarded as important pilot data. The first finding of interest is that at baseline, despite having radiological evidence of chronic pancreatitis, impairment of pancreatic exocrine function and a substantial requirement for opiate analgesia there was no elevation of circulatory pro- or anti-inflammatory cytokine levels. This is finding sits well with current paradigms of chronic pancreatitis which suggest that pain is not simply a product of inflammation and that it involves a complex interaction between inflammatory mediators and neural structures with alterations in nociception[10,11]. For example, fractalkine is a cell surface membrane-spanning adhesion molecule that can be cleaved to produce a soluble neuromodulatory chemokine which increases neuropathic pain through glial activation with expression correlating with the severity of pancreatic neuritis, fibrosis, intrapancreatic nerve fibre density and pain in chronic pancreatitis[12]. Fractalkine may be a better disease-specific chemokine in chronic pancreatitis although its relation to disease stage and response to therapy have yet to be elucidated[13].

In relation to cytokine profiles in chronic pancreatitis reported in other studies, the levels of IL-18 in our study are similar to those reported by Schneider and colleagues[14]. In terms of genotype, patients with alcohol-aetiology dominant, sporadic chronic pancreatitis do not have an increased frequency of functional polymorphisms in the *TGF-β1* gene, in the *IL-10* gene or in the intron 1 of the interferon-gamma gene[15].

In this study there was no relation between antioxidant therapy and cytokine levels. Thus, the significant elevations in plasma levels of antioxidants seen in the treatment group (and also in the main ANTICIPATE study and in other studies of antioxidant therapy) do not appear to interact with circulating cytokines. The low levels of cytokines probably reflect the results of sampling of an out-patient based population with clinically quiescent disease and in particular without evidence of a systemic inflammatory response.

In conclusion, this study has measured antioxidant profiles in patients with chronic pancreatitis receiving antioxidant therapy and compared these to patients receiving matched placebo. Cytokine levels were low at baseline and at 6 mo despite a significant elevation in plasma antioxidants. The study also demonstrates that circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of ongoing inflammation. It could be the result of the inflammation tissue damage caused long time ago.

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review of the final manuscript.

COMMENTS

Background

This study undertakes a subgroup analysis comparing pro- and anti-inflammatory cytokine levels in a sub-group of patients receiving either antioxidant therapy for chronic pancreatitis in the form of Antox (Pharmanord, Morpeth, United Kingdom) or matched placebo.

Research frontiers

The novel aspect of this study is that it is believed to be the first to examine pro- and anti-inflammatory cytokine levels in patients receiving antioxidant therapy for chronic pancreatitis and to compare these levels to those in patients receiving matched placebo.

Innovations and breakthroughs

The results show that pro-inflammatory cytokine levels were not elevated. This is potentially an important finding in that it shows that in patients with chronic pancreatitis, with established pain, inflammatory cytokine levels are not elevated.

Applications

The findings are preliminary and need to be reproduced in a larger validation dataset before more general acceptance.

Peer review

It is a very interesting paper. Considering that this paper employs patients from the ANTICIPATE study, it is desirable that the authors give the registration number of the main trial.

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BRIEF ARTICLE

Adipokines and C-reactive protein in relation to bone mineralization in pediatric nonalcoholic fatty liver disease

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Abstract

AIM: To investigate bone mineral density (BMD) in obese children with and without nonalcoholic fatty liver disease (NAFLD); and the association between BMD and serum adipokines, and high-sensitivity C-reactive protein (HSCRP).

METHODS: A case-control study was performed. Cases were 44 obese children with NAFLD. The diagnosis of NAFLD was based on magnetic resonance imaging (MRI) with high hepatic fat fraction (⩾ 5%). Other causes of chronic liver disease were ruled out. Controls were selected from obese children with normal levels of aminotransferases, and without MRI evidence of fatty liver as well as of other causes of chronic liver diseases. Controls were matched (1- to 1-basis) with the cases on age, gender, pubertal stage and as closely as possible on body mass index-SD score. All participants underwent clinical examination, laboratory tests, and whole body (WB) and lumbar spine (LS) BMD by dual energy X-ray absorptiometry. BMD *Z*-scores were calculated using race and gender specific LMS curves.

RESULTS: Obese children with NAFLD had a significantly lower LS BMD *Z*-score than those without NAFLD [mean, 0.55 (95%CI: 0.23-0.86) *vs* 1.29 (95%CI: 0.95-1.63); $P < 0.01$]. WB BMD *Z*-score was also decreased in obese children with NAFLD compared to obese children with no NAFLD, though borderline significance was observed [1.55 (95%CI: 1.23-1.87) *vs* 1.95 (95%CI: 1.67-2.10); $P = 0.06$]. Children with NAFLD had significantly higher HSCRP, lower adiponectin, but similar leptin levels. Thirty five of the 44 children with MRI-diagnosed NAFLD underwent liver biopsy. Among the children with biopsy-proven NAFLD, 20 (57%) had nonalcoholic steatohepatitis (NASH), while 15 (43%) no NASH. Compared to children without NASH, those with NASH had a significantly lower LS BMD *Z*-score [mean, 0.27 (95%CI: -0.17-0.71) *vs* 0.75 (95%CI: 0.13-1.39); $P < 0.05$] as well as a significantly lower WB BMD *Z*-score [1.38 (95%CI: 0.89-1.17) *vs* 1.93 (95%CI: 1.32-2.36); $P < 0.05$]. In multiple regression analysis, NASH (standardized β coefficient, -0.272; $P < 0.01$) and HSCRP (standardized β coefficient, -0.192; $P < 0.05$) were significantly and independently associated with LS BMD *Z*-score. Similar results were obtained when NAFLD (instead of NASH) was included in the model. WB BMD *Z*-scores were significantly and independently associated with NASH (standardized β coefficient, -0.248; $P < 0.05$) and fat mass (standardized β coefficient, -0.224; $P < 0.05$).

CONCLUSION: This study reveals that NAFLD is associated with low BMD in obese children, and that systemic, low-grade inflammation may accelerate loss of bone mass in patients with NAFLD.

Key words: Bone mineralization; Dual energy X-ray absorptiometry; Adipokines; C-reactive protein; Nonalcoholic fatty liver disease; Children

Core tip: Understanding the mechanisms underlying the relationship between nonalcoholic fatty liver disease (NAFLD) and low bone mineral density (BMD) is important to prevent poor bone mineralization in obese children. We showed that obese children with NAFLD have decreased BMD compared to obese children without liver involvement independently of adiposity, and that children with more severe histology have worse mineral status than children with more mild abnormalities. We also found a significant independent association of high sensitivity C-reactive protein with BMD scores, supporting the role of an inflammatory state which may accelerate loss of bone mass in patients with NAFLD.

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INTRODUCTION

Concurrent with the increasing rates of childhood obesity, nonalcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver disease in pediatric populations worldwide[1,2]. NAFLD comprises a disease spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), with varying degrees of inflammation and fibrosis, progressing to end-stage liver disease with cirrhosis and hepatocellular carcinoma[3]. NAFLD is strongly associated with obesity, insulin resistance, hypertension, and dyslipidemia, and is now regarded as the liver manifestation of the metabolic syndrome (MetS)[4]. Recently it has been suggested that NAFLD can be a cause of low bone mineral density (BMD) in obese children and adolescents[5-7]. However, the mechanisms explaining this relationship are not completely understood[8]. Obesity-induced low-grade systemic inflammation, a key component in the pathogenesis of insulin resistance and NAFLD, may negatively influence bone health[9,10]. Expanded and inflamed visceral adipose tissue releases a wide array of molecules potentially involved in the development of insulin resistance, including free fatty acids, tumor necrosis factor (TNF)-α, and other proinflammatory cytokines[11-14]. In the presence of increased free fatty acid flux and chronic, low-grade inflammation, the liver is both the target of and a contributor to systemic inflammatory changes[15]. Indeed, in a number of case-control studies, circulating levels of several inflammatory markers [*i.e.*, C-reactive protein (CRP), interleukin (IL)-6, monocyte chemotactic protein 1 and TNF-α], procoagulant factors, and oxidative stress markers were found to be highest in patients with NASH, intermediate in those with simple steatosis, and lowest in control subjects without steatosis, and the differences were independent of obesity and other potentially confounding factors[16].

Adipose tissue also produces adipokines, which are pleiotropic molecules that not only regulate food intake and energy metabolism but also are implicated in the complex interactions between fat and bone[17,18]. Leptin, produced in bone marrow adipocytes and osteoblastic cells, regulates appetite and weight, osteoblast proliferation and differentiation *in vitro*[19-21], and osteoclasts[19,22,23]. Its receptor is expressed in osteoblasts[19,24]. Adiponectin, exclusively expressed by adipocytes, is inversely related to visceral fat mass and body mass index (BMI)[25] and regulates metabolism and inflammatory pathways[26]. Adiponectin affects osteoblast directly and osteoclast indirectly. It stimulates the proliferation and differentiation of human osteoblasts *via* the p38 mitogen-activated protein kinase signaling pathway[27]. In contrast, adiponectin indirectly influences osteoclasts by stimulating the receptor activator of nuclear factor-κB ligand (RANKL) and inhibiting osteoprotegerin production in osteoblasts[28]. Some studies have shown a negative association between adiponectin and BMD, independent of fat mass or BMI[29].

The aims of this study were to evaluate: (1) BMD in obese children with and without NAFLD; and (2) the association between BMD and the serum adipokines, leptin and adiponectin, and a circulating marker of systemic inflammation, high-sensitivity C-reactive protein (HSCRP), using multiple regression.

MATERIALS AND METHODS

Study design and patients

A case-control study was performed. Cases were Caucasian obese children (BMI above the 95th percentile for age and gender) seen at the Hepatology outpatient Clinic of the Department of Pediatrics, Sapienza University of Rome, Italy. The diagnosis of NAFLD was based on magnetic resonance imaging (MRI) with high hepatic fat fraction (HFF ⩾ 5%). Other causes of chronic liver disease, including hepatic virus infections (hepatitis A-E and G, cytomegalovirus, and Epstein-Barr virus), autoimmune hepatitis, metabolic liver disease, α-1-antitrypsin deficiency, cystic fibrosis, Wilson's disease, hemochromatosis, and celiac disease were ruled out with appropriate tests. Exclusion criteria were also smoking habits, and history of type 1 or 2 diabetes, renal disease, total parenteral nutrition, use of hepatotoxic medications, and chronic alcohol intake. Finally, children were excluded for conditions that could have adversely influenced BMD including glucocorticoid therapy, hypothyroidism, Cushing's disease; history of long bone fractures; indwelling hardware; and abnormality of the skeleton or spine[30,31].

Controls were selected from Caucasian obese children with normal levels of aminotransferases, and without

MRI evidence of fatty liver (HFF < 5%) as well as of other causes of chronic liver diseases (see above). Controls were also excluded if they had smoking habits, history of type 1 or 2 diabetes, renal disease, chronic alcohol intake, and any condition known to influence BMD[30,31]. Controls were then matched (1- to 1-basis) with the cases on age, gender, pubertal stage and as closely as possible on BMI-SD score (SDS).

The research protocol was approved by the Hospital Ethics Committee, and informed consent was obtained from subjects' parents before assessment.

Clinical and laboratory data

All participants underwent physical examination including measurements of weight, standing height, BMI and determination of the stage of puberty, and laboratory tests. The pubertal stage was categorized into two groups (prepubertal: boys with pubic hair and gonadal stage Ⅰ, and girls with pubic hair stage and breast stage Ⅰ; pubertal: boys with pubic hair and gonadal stage ⩾ Ⅱ and girls with pubic hair stage and breast stage ⩾ Ⅱ). The degree of obesity was quantified using Cole's least mean-square method, which normalizes the skewed distribution of BMI and expresses BMI as SDS[32]. Blood samples were taken, after an overnight fast, for estimation of glucose, insulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), HSCRP, leptin, and adiponectin.

Analyses of glucose, insulin, ALT, AST, and HSCRP were conducted by COBAS 6000 (Roche Diagnostics). Insulin concentrations were measured on cobas e 601 module (Electrochemiluminescence Technology, Roche Diagnostics), while the remaining analytes on cobas e 501 clinical chemistry module (Photometric Technology), according to the instructions of the manufacturer. The degree of insulin resistance was determined by a homeostasis model assessment of insulin resistance (HOMA-IR)[33]. Scores were calculated as the product of the fasting serum insulin level (μU/mL) and the fasting serum glucose level (mmol/L), divided by 22.5. A RIA was used to measure human (total) leptin (DRG Diagnostica, Marburg, Germany; detection limit, 0.5 ng/mL; inter- and intra-assay CVs, 3.0%-6.2% and 3.4%-8.3%, respectively), and adiponectin (DRG Diagnostica, Marburg, Germany; detection limit, 1 ng/mL; inter- and intra-assay CVs, 6.9%-9.2% and 1.8%-6.2%, respectively).

MRI for liver fat quantification

The amount of hepatic fat content (% HFF) was measured by MRI using the two-point Dixon method as modified by Fishbein[34], as previously described and validated[35]. MRI results were interpreted by an experienced radiologist who was blinded to clinical and laboratory findings.

Lumbar spine and whole body dual energy X-ray absorptiometry scans

Anteroposterior lumbar spine (L_1-L_4), and whole body scans were obtained from all cases and controls using a Hologic QDR-4500W (Waltham, MA, United States) in the fan beam mode with a multidetector system. All subjects were measured on the same machine. The measurements were performed by using standard positioning techniques. Quality control was performed daily using the Hologic anthropomorphic spine, and weekly with the whole body phantom. In our department, the precision error for BMD measurements is less than 1% for the spine phantom, and less than 2.5% for the whole body phantom. The data were analyzed using the software version 11.2. Spine scans were analyzed with low-density software[36]. BMD *Z*-scores for whole body (WB) and for lumbar spine (LS) were calculated using race and gender specific LMS curves[37]. Whole body DXA results (BMD, fat mass and lean mass) shown in this study represent values excluding the skull[38].

Liver biopsy

The clinical indication for biopsy was either to assess the presence of NASH and degree of fibrosis or other likely independent or competing liver diseases. Percutaneous needle liver biopsy was performed as previously described[35]. The main histologic features of NAFLD were scored according to the scoring system developed by the NASH Clinical Research Network (CRN)[39]. Features of steatosis, lobular inflammation, and hepatocyte ballooning were combined to obtain the NAFLD activity score. As recommended by a recent NASH CRN article[40], a microscopic diagnosis, based on overall injury pattern (*i.e.*, steatosis, hepatocyte ballooning, and inflammation), as well as the presence of additional lesions (*e.g.*, zonality of lesions, portal inflammation, and fibrosis), has been assigned to each case[41]. Accordingly, biopsies were subdivided into not-NASH and definite NASH subcategories[41].

Statistical analysis

Statistical analyses were performed using the SPSS package. The data are expressed either as frequencies or as means with 95%CI. Insulin, leptin and adiponectin levels were distributed with a long tail to the right (positive skew), but their logarithms were approximately normally distributed. Mean differences in anthropometric, laboratory and body composition variables between subjects were assessed by using the *t* test. Linear regression analysis was used to identify variables associated with BMD. Then, a stepwise multiple linear regression analysis (including all variables significantly associated with BMD) was used to determine the independent variables associated with BMD. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Study subjects

Forty four obese children with MRI-diagnosed NAFLD were matched to 44 obese children without evidence of liver disease. By study design cases and controls were matched for age, gender, pubertal stage and BMI-SDS. The mean age of cases and controls was 12.5 (SD 1.8) years. Both cases and controls included 20 girls and 24

Table 1 Characteristics of obese children by liver status

Variables	NAFLD (n = 44)	Non-NAFLD (n = 44)	P value
Lean mass, kg	25.8 (24.0-30.0)	26.5 (24.0-29.0)	NS
Fat mass, kg	18.7 (17.0-21.0)	16.8 (15.1-19.0)	NS
Percentage body fat	40.2% (39.0%-41.0%)	38.0% (36.0%-40.0%)	NS
Aspartate amino-transferase, U/L	34 (30-38)	24 (22-26)	< 0.0010
Alanine amino-transferase, U/L	45 (35-55)	20 (18-22)	< 0.0001
Glucose, mmol/L	4.89 (4.69-5.10)	4.88 (4.77-5.02)	NS
Insulin, μU/mL	31.2 (21.9-40.6)	20.1 (16.2-24.1)	< 0.0100
HOMA-IR values	4.27 (3.40-5.10)	3.45 (2.97-4.01)	< 0.0100
Leptin, μg/L	19.5 (15.8-23.1)	20.8 (18.2-23.4)	NS
Adiponectin, μg/L	9.0 (7.3–11.0)	12.9 (10.6–15.4)	< 0.0500
HSCRP, μg/L	3310 (2785-3836)	2165 (1710-2620)	< 0.0100
Hepatic fat fraction (%)	17.0 (11.8-22.3)	1.6 (1.0-3.1)	< 0.0001

Results are expressed as n (%), mean (95%CI), or geometric mean (95%CI) for log-transformed variables. NS: Not significant; HOMA-IR: Homeostasis model assessment of insulin resistance; HSCRP: High-sensitivity C-reactive protein; NAFLD: Nonalcoholic fatty liver disease.

boys, and five prepubertal children. The mean BMI-SDS of cases and controls was 2.19 (SD 0.16) and 2.17 (SD 0.16), respectively. The clinical and laboratory characteristics for cases and controls are shown in Table 1. There were no differences between children with and without NAFLD with respect to lean and fat mass. Compared to the non-NAFLD group, children with NAFLD had significantly higher ALT, AST, insulin concentrations, HOMA-IR values, and HSCRP levels, but lower adiponectin concentrations. There were no significant differences between the two groups with respect to glucose as well as leptin.

Histological findings in children with NAFLD

Liver biopsy was obtained in 35 of the 44 children with MRI-diagnosed NAFLD, with parental refusal in 9 cases. The 35 children did not differ from those having only liver MRI with respect to age, gender, body composition, metabolic parameters, and bone measures.

Among patients with biopsy-proven NAFLD, 20 (57%) had definite NASH, while 15 (43%) no NASH. No statistically significant differences in body composition as well as in laboratory parameters such as glucose, insulin, leptin, adiponectin levels, and HOMA-IR values were found between children with NASH and those with simple steatosis. AST [mean, 41 U/L (95%CI: 34-48) *vs* 26 U/L (95%CI: 22-29); $P < 0.001$)], ALT [mean, 58 U/L (95%CI: 41-75) *vs* 30 U/L (95%CI: 20-45); $P < 0.001$)] as well as HFF [mean, 24.8% (95%CI: 19.5-30.2) *vs* 15.7% (95%CI: 5.6-28.8); $P < 0.001$)] were significantly higher in patients with NASH compared to children without NASH. HSCRP was also higher [mean, 4055 μg/L (95%CI: 2690-5419) *vs* 2870 μg/L (95%CI: 1794-3936); $P = 0.07$], although did not reach statistically significance.

Bone measures

Obese children with NAFLD had a significantly lower LS BMD Z-score than those without NAFLD [mean, 0.55 (95%CI: 0.23-0.86) *vs* 1.29 (95%CI: 0.95-1.63); $P < 0.01$] (Figure 1A). WB BMD Z-score was also decreased in obese children with NAFLD compared to obese children with no NAFLD, though borderline significance was observed [1.55 (95%CI: 1.23-1.87) *vs* 1.95 (95%CI: 1.67-2.10); $P = 0.06$] (Figure 1B). Among children with biopsy-proven NAFLD, those with NASH had a significantly lower LS BMD Z-score than children without NASH [mean, 0.27 (95%CI: -0.17-0.71) *vs* 0.75 (95%CI: 0.13-1.39); $P < 0.05$] (Figure 1C). Moreover, children with NASH had a significantly lower WB BMD Z-score than children without NASH [1.38 (95%CI: 0.89-1.17) *vs* 1.93 (95%CI: 1.32-2.36); $P < 0.05$] (Figure 1D).

Table 2 Multivariate analysis of the variables associated with lumbar spine and whole body bone mineral density Z-score in obese children

Variables	Standardized coefficient[1]	P value
LS BMD Z-score		
NAFLD	-0.230	< 0.01
HSCRP, μg/L	-0.195	< 0.05
WB BMD Z-score		
NAFLD	-0.218	< 0.05
Fat mass, kg	-0.225	< 0.05

[1]Included in the model were age, gender, pubertal stage, nonalcoholic fatty liver disease (NAFLD), and all variables significantly associated with lumbar spine or whole body bone mineral density (BMD) Z-score in univariate analysis [*i.e.*, high-sensitivity C-reactive protein (HSCRP) and leptin levels or fat mass].

In univariate analysis, LS BMD Z-score correlated negatively with NAFLD (standardized β coefficient, -0.202; $P < 0.01$) and HSCRP (standardized β coefficient, -0.212; $P < 0.05$). In contrast, leptin was positively associated with lumbar BMD (standardized β coefficient, -0.204; $P < 0.05$). No correlation was found between LS BMD Z-score and insulin as well as HOMA-IR. Likewise, neither BMI-SDS nor lean mass nor fat mass were correlated with LS BMD Z-score. After including in the model all the significant variables as well as age, gender, pubertal status, NAFLD (standardized β coefficient, -0.230; $P < 0.01$) and HSCRP (standardized β coefficient, -0.195; $P < 0.05$) remained significantly and independently associated with LS BMD Z-score (Table 2).

WB BMD Z-score was negatively associated with NAFLD (standardized β coefficient, -0.207; $P < 0.05$), fat mass (standardized β coefficient, -0.222; $P < 0.05$), and HSCRP (standardized β coefficient, -0.216; $P < 0.05$). No correlation was found between WB BMD Z-score and insulin as well as HOMA-IR. Likewise, neither BMI-SDS nor lean mass were correlated with WB BMD Z-score. After including in the model all the significant variables as well as age, gender, pubertal status, NAFLD (standardized β coefficient, -0.218; $P < 0.05$) and fat mass (standardized β coefficient, -0.225; $P < 0.05$) remained significantly and independently associated with WB BMD Z-score (Table 2).

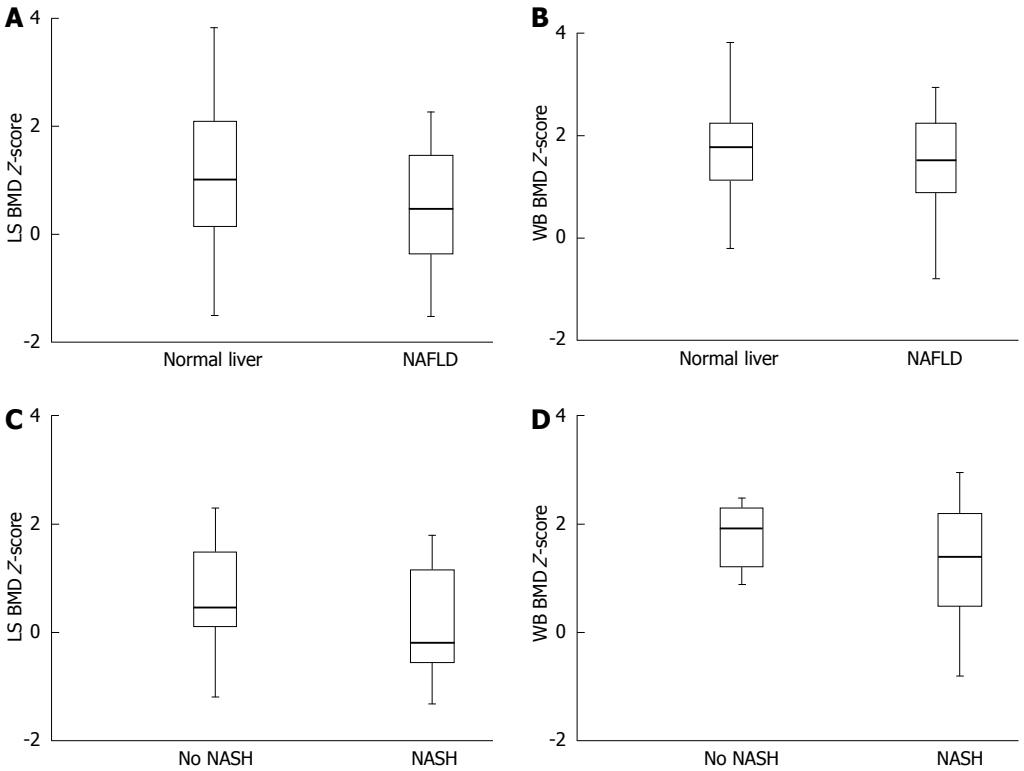

Figure 1 Bone measures. A: Lumbar spine bone mineral density Z-score (LS BMD Z-score) for obese children with and without nonalcoholic fatty liver disease (NAFLD). Box-plots give the median value (bold), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers); B: Whole body bone mineral density Z-score (WB BMD Z-score) for obese children with and without NAFLD. Box-plots give the median value (bold), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers); C: LS BMD Z-score for obese children with biopsy-proven NAFLD subdivided into those with and without nonalcoholic steatohepatitis (NASH). Box-plots give the median value (bold), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers); D: WB BMD Z-score for obese children with biopsy-proven NAFLD subdivided into those with and without NASH. Box-plots give the median value (bold), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers).

DISCUSSION

In this study, we showed that obese children with NAFLD had decreased LS BMD and WB BMD compared to obese children without liver involvement independently of adiposity, and that children with more severe histology had worse bone mineral status than children with more mild abnormalities. Furthermore, we found a significant independent association of HSCRP with BMD scores, supporting the role of an inflammatory state which may accelerate loss of bone mass in patients with NAFLD.

Growing evidence suggests the presence of a complex interplay between the skeleton and numerous homeostatic processes, including energy balance, insulin resistance, obesity and MetS[8]. Recent years have also witnessed an increased awareness of the clinical and epidemiological association between NAFLD and bone health, both in terms of reduced BMD and an increased risk of osteoporosis[8]. To our knowledge, such an association has been so far independently reported by five studies in both children and adults[5-7,42,43].

With respect to studies in adults, Moon *et al*[42] showed that in postmenopausal women ultrasound-diagnosed NAFLD was significantly associated with low lumbar BMD and this significance was maintained after adjusting for the concerned variables including age, BMI, ALT, smoking status, and alcohol consumption, and even after taking the presence of MetS into account. However, in premenopausal women, there was no such relationship. Yet, in the study by Purnak *et al*[43] involving 102 adult patients with ultrasound-diagnosed NAFLD and 54 healthy controls, there were no statistically significant differences in BMD measurements between the two groups. However, in a subgroup of patients with NAFLD, the presence of elevated serum ALT and HSCRP levels, which were suggestive of NASH, was associated with lower BMD.

With respect to studies in children, Pirgon *et al*[5] reported a negative association between BMD and insulin resistance in obese adolescents both with (n = 42) and without (n = 40) ultrasound-diagnosed NAFLD, although the obese adolescents with NAFLD had lower spine BMD Z-scores than their non-NAFLD counterparts. The Authors suggested that NAFLD could exert a negative impact on BMD in obese adolescents, probably *via* an increased insulin resistance. In the study by Pardee *et al*[6], poor bone mineralization was common among the 38 obese children with biopsy-proven NAFLD, but not among the 38 obese children without evidence of liver disease. Cases and controls were matched for age, gender, race, ethnicity, height and weight. Among children with NAFLD, 17 (45%) had BMD Z-scores ≤ -2.0, compared to none of the

controls ($P < 0.0001$). Importantly, among those children with NAFLD, children with NASH had a significantly ($P < 0.05$) lower BMD *Z*-score (-2.37) than children with NAFLD who did not have NASH (-1.58)[6]. These differences persisted after controlling for total per cent body fat. In the study by Campos *et al*[7], a 1-year interdisciplinary weight loss therapy was able to promote changes in the metabolic profile of 40 obese adolescents with (n = 18) or without (n = 22) ultrasound-diagnosed NAFLD, including a decrease in the BMI, body fat, visceral and subcutaneous fat, insulin concentration, HOMA-IR, and an increase in lean mass. At baseline, NAFLD group presented statistically lower values of bone mineral content (BMC); however, after one year of interdisciplinary therapy, there was an increase of BMC, reaching similar values of non-NAFLD group. Campos *et al*[7] suggest the importance of this kind of intervention to regulate bone mineral metabolism as result of an increased BMC and improved inflammatory state. Together, these studies indicate that NAFLD, in particular NASH, is associated with poor bone health.

Obesity and bone mineralization in children remains a topic of great interest, as data are conflicting regarding whether obesity in this age group is detrimental or protective to bone. Previous studies have suggested that body weight might improve bone mineralization in overweight adolescents by increasing the mechanical load on weight-bearing bones[44,45]. In terms of which component(s) of body weight underlie this association, the association between bone and lean mass has been found to be strongest[46]. Some studies have also suggested that fat mass may stimulate bone accrual in growing children, but these results have remained inconsistent showing both positive[47,48] and negative associations[49-51]. In multiple regression analysis, we found that fat mass had a negative association with WB BMD *Z*-score, while none of the anthropometric variables had an effect on LS BMD *Z*-scores. The basis for the negative effect of fat on WB BMD *Z*-score observed in the present study is unknown. We found that serum adipokines such as leptin and adiponectin were not significantly correlated with BMD *Z*-scores. In that vein, a recent systematic review of the literature concerning the influence of adipokines on BMD, rarely identified leptin as an independent predictor of BMD when BMI or fat mass parameters were included in the multivariate regression models[29]. Yet, in that systematic review, results were discordant for adiponectin[29]. Some studies showed a negative association between adiponectin and BMD, independent of fat mass or BMI[29]. Nevertheless, other studies did not find such associations[29]. There are possible explanations for this apparent discrepancy. Many variables, such as estrogen levels, proinflammatory cytokines, and preanalytical variability of adipokine dosage may interfere with adiponectin and bone.

Systemic inflammation is well known to contribute to low BMD in several diseases states[52-54]. CRP is a sensitive systemic marker of inflammation and tissue damage[55]. It is only produced by hepatocytes, predominantly under transcriptional control by IL-6, although other sites of local CRP synthesis and possible secretion have been suggested. Raised CRP levels are associated with many features of insulin resistance or MetS[56]. This may reflect, in part, the fact that adipocytes are the source of a substantial portion of IL-6 production[57]. On the other hand, inflammatory cytokines up-regulate the RANKL, leading to increased bone resorption and reduced BMD[58]. Some studies have suggested that an elevated CRP is associated with osteoporosis and non-traumatic fractures[9,10]. Our study suggests that HSCRP level is independently associated with LS BMD *Z*-scores in obese children with NAFLD. This finding is consistent with the hypothesis of a tight interplay between low-grade inflammation and bone turnover, even in patients with NAFLD.

COMMENTS

Background

In parallel with epidemic obesity, nonalcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver disease in both pediatric and adult patients worldwide. Liver disease can be cause of low bone mineral density (BMD). However, the mechanisms explaining this relationship are still not completely understood.

Research frontiers

A better understanding of the factors that may influence bone mineral status in NAFLD may open a new frontier to fight two highly prevalent conditions like NAFLD and osteoporosis.

Innovations and breakthroughs

Recent years have witnessed an increased awareness of the clinical and epidemiological association between NAFLD and bone health, both in terms of reduced BMD and an increased risk of osteoporosis. Given the high prevalence of NAFLD and the adverse consequences of low BMD in childhood, understanding the mechanisms underlying the relationship between NAFLD and low BMD is important to prevent poor bone mineralization in this potentially vulnerable population. In this study, authors showed that obese children with NAFLD have decreased BMD compared to obese children without liver involvement independently of adiposity, and that children with more severe histology have worse mineral status than children with more mild abnormalities. They also found a significant independent association of high sensitivity C-reactive protein with BMD scores, supporting the role of an inflammatory state which may accelerate loss of bone mass in patients with NAFLD.

Applications

The presence of systemic inflammation may have important implications for the long-term skeletal health of children with NAFLD, and particularly those with nonalcoholic steatohepatitis (NASH).

Terminology

NAFLD comprises a disease spectrum ranging from simple fatty liver to NASH, with varying degrees of inflammation and fibrosis, progressing to end-stage liver disease with cirrhosis and hepatocellular carcinoma. Bone density (or BMD) is a medical term normally referring to the amount of mineral matter per square centimeter of bones. Bone density (or BMD) is used in clinical medicine as an indirect indicator of osteoporosis and fracture risk.

Peer review

In this paper, authors compared lumbar spine (LS) and whole body (WB) BMD measured by dual energy X-ray absorptiometry scans between 44 pediatric patients with magnetic resonance imaging diagnosed NAFLD and controls matched 1:1 for age, gender, and pubertal stage and body mass. They found that LS-BMD *Z* score was lower in NAFLD than in controls; Thirty three NAFLD patients were biopsied; LS and WB BMD *Z* score were lower in NASH than in non-NASH children. At multivariate analysis LS-BMD was independently associated with NASH and C-reactive protein levels. They conclude that NAFLD is associated with low BMD in obese children, and systemic low grade inflammation may play a role in such a relationship.

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BRIEF ARTICLE

Unusual histopathological findings in appendectomy specimens from patients with suspected acute appendicitis

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Abstract

AIM: To investigate the prevalence and implications of unusual histopathological findings in appendectomy specimens from patients with suspected acute appendicitis.

METHODS: The demographic and histopathological data of 1621 patients (⩾ 16 years-old) who underwent appendectomy to treat an initial diagnosis of acute appendicitis between January 1999 and November 2011 were retrospectively assessed. Microscopic findings were used to classify the patients under six categories: appendix vermiformis, phlegmonous appendicitis, gangrenous appendicitis, perforated appendicitis, supurative appendicitis, and unusual histopathologic findings. The demographic and clinicopathologic characteristics of patients with unusual histopathologic findings were evaluated in detail, and re-analysis of archived resected appendix specimens was carried out.

RESULTS: A total of 912 males and 709 females, from 16 to 94 years old, were included in the study and comprised 789 cases of suppurative appendicitis, 370 cases of appendix vermiformis, 243 cases of perforated gangrenous appendicitis, 53 cases of flegmaneous appendicitis, 32 cases of gangrenous appendicitis, and 134 (8.3%) cases of unusual histopathological findings. The unusual histopathological findings included fibrous obliteration (n = 62), enterobius vermicularis (n = 31), eosinophilic infiltration (n = 10), mucinous cystadenoma (n = 8), carcinoid tumor (n = 6), granulomatous inflammation (n = 5), adenocarcinoma (n = 4; one of them mucinous), and mucocele (n = 3), adenomatous polyp (n = 1), taenia sup (n = 1), ascaris lumbricoides (n = 1), appendiceal diverticula (n = 1), and B cell non-hodgkin lymphoma (n = 1). None of the 11 patients with subsequent diagnosis of tumor were suspected of cancer prior to the appendectomy.

CONCLUSION: Even when the macroscopic appearance of appendectomy specimens is normal, histopathological assessment will allow early diagnosis of many unusual diseases.

Key words: Appendicitis; Appendectomy specimens; Histopathology; Unusual findings; Appendiceal malignancy

Core tip: Appendectomy is one of the most frequently performed surgical procedures worldwide. Although most of the resected appendectomy specimens show typical histopathologic findings, some (< 2%) show unusual histopathologic findings. The most common of these unusual features are primary or secondary appendiceal malignancies, mucocele, enterobisis, schistosomiasis, ascariasis, tuberculosis, amobiasis, and entometriosis. While some of the patients with unusual histopatholic findings require close follow-up and/or additional surgical treatment, others also necessitate antimicrobial therapy. Infectious appendicitis is respon-

sible for a significant majority of the most commonly observed unusual features, especially in cases from developing nations in geographic regions with tropical and sub-tropical climates. Therefore, regardless of the underlying etiology, the results from histopathological examination of the resected appendectomy specimen may help guide the subsequent management of cases to prevent serious appendicular diseases.

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INTRODUCTION

Appendicitis remains one of the most common acute conditions of the abdomen, and suspected cases are frequently treated with emergency appendectomy[1]. The complete organ excision not only allows for definitive diagnosis but also significantly reduces the risk of life-threatening complications, such as perforation, plastron and sepsis. However, the surgical procedure itself is very invasive, representing additional risks to the patient's morbidity and mortality as well as remarkable costs to the healthcare providers. Epidemiologic studies have revealed that the incidence of acute appendicitis roughly parallels that of lymphoid development, with the peak incidence occurring between the ages of 10 and 30 years old. The most important causative factor of acute appendicitis appears to be development of luminal obstruction. In addition, several factors have been implicated as causative etiologies of this underlying feature, and show an age-related trend[1-5]. For example, lymphoid hyperplasia is the most common factor identified in patients under 20 years old, while fecalith plugs are the most common factor identified in the elderly. Apart from these usual factors, numerous other less frequent (and thus "unusual") factors have been identified as having caused the clinical symptoms that indicated the suspicion of acute appendicitis with or without histopathologic evidence for acute appendicitis[1,3,4]. The primary objective of this study was to assess the incidence and implications of unusual histopathological findings detected in appendectomy specimens from patients who received surgery to address an initial diagnosis of acute appendicitis.

MATERIALS AND METHODS

In this retrospective study, the electronic records of the Inonu University Medical Faculty Department of Surgery were searched to identify all patients who underwent appendectomy to treat an initial diagnosis of acute appendicitis between January 1999 and November 2011. The recorded demographic and histopathological data extracted for each patient included age, sex, appendectomy surgery date, and macroscopic and microscopic properties of appendix vermicularis. Patients who had received the appendectomy incidental to other surgeries, such as colorectal or gynecological cancer surgery or trauma surgery, were excluded from study enrollment. In addition, pediatric patients younger than 16 years old were also excluded from study enrollment. Four researchers working independently collected the demographic and pathologic data of all patients fitting the inclusion criteria in excel spreadsheets, which were then adjudicated and analyzed by the group.

Using the microscopic findings of each patient's appendectomy specimen that were recorded in the pathology report, the patients were classified into one of six categories: (1) appendix vermiformis; (2) phlegmonous appendicitis; (3) gangrenous appendicitis; (4) perforated appendicitis; (5) suppurative appendicitis; and (6) unusual histopathologic findings. The archived appendectomy specimens (pathology blocks and microscopic slides) were retrieved for the 134 patients in group 6 and were re-evaluated by two experienced pathologists. The patient data for each demographic or histopathologic characteristic were summarized as mean ± SD, and incidence of a characteristic within a particular group was calculated as percentage of the entire study population.

RESULTS

General characteristics of patients undergoing appendectomy for suspected acute appendicitis

A total of 1621 patients underwent appendectomy to treat an initial diagnosis of acute appendicitis. The mean age of these patients was 36.7 ± 17.4 years (range: 16-94 years) and the male-to-female ratio was nearly equal (912: 709) but with a slight male bias (56.3% males). According to the histopathological findings, 789 patients had suppurative appendicitis, 370 had appendix vermiformis, 243 had perforated gangrenous appendicitis, 134 had unusual histopathological findings, 53 had flegmaneous appendicitis, and 32 had gangrenous appendicitis. Overall, the majorities (67.3%) of the patients were ≤ 40 years old, and 13.5% were ≥ 61 years old. There was also an age bias towards patients ≤ 40 years old for those in the negative appendicitis group (64.6% of the 370 patients), with only 17.5% in that group being ≥ 61 years old. Clinicopathologic characteristics of the 1621 patients who underwent appendectomy for clinical signs of acute appendicitis are summarized in Table 1.

Characteristics of patients who showed unusual histopathologic findings in appendectomy specimens

One-hundred-and-thirty-four (8.3%) of the patients who received appendectomy to treat the initial diagnosis of acute appendicitis had unusual histopathological findings in their appendectomy specimens. This group of patients had a mean age of 48.4 ± 19.5 years old, and the male-to-

Table 1 Clinicopathologic characteristics of the 1621 patients who underwent appendectomy *n* (%)

Patient characteristics	Results
Patients	1621
Sex	
Male	912 (56.3)
Female	709 (43.7)
Age in years, mean (range)	
Overall	36.7 ± 17.4 (16-94)
Male	36.2 ± 17.5 (16-89)
Female	37.3 ± 17.3 (16-94)
Distribution of patients according to age range (yr)	
16-20	275
21-30	526
31-40	290
41-50	165
51-60	146
61-70	134
⩾ 71	85
Histopathologic findings	
Suppurative appendicitis	789 (48.7)
Appendix vermiformis	370 (22.8)
Gangrenous appendicitis-perforated	243 (15.0)
Unusual histopathologic findings	134 (8.3)
Phlegmonous appendicitis	53
Gangrenous appendicitis	32
Age distribution of the 370 patients with negative appendectomy (yr)	
16-20	67
21-30	111
31-40	61
41-50	35
51-60	32
61-70	43
⩾ 71	21

female ratio was relatively equal (60:74) but with a slight female bias (55.2% females). The mean age of the males (50.9 ± 19.3 years old; range: 16-87 years) was slightly higher than that of the females (46.4 ± 19.5 years old; range: 16-94 years). Unlike any of the five other pathology groups, the group with unusual histopathological findings had a majority (60.4%) of patients > 40 years old.

The histopathologic findings of these 134 patients with unusual histopathological findings included fibrous obliteration (n = 62; Figure 1A), enterobius vermicularis (n = 31; Figure 1B), eosinophilic infiltration (n = 10), mucinous cystadenoma (n = 8; Figure 1C), carcinoid tumor (n = 6; Figure 1D), granulomatous infiltration (n = 5; Figure 1E), adenocarcinoma (n = 4; Figure 1F-G), mucocele (n = 3; Figure 1H), adenomatous polyp (n = 1), taenia sup (n = 1; Figure 1I), ascaris lumbricoides (n = 1), appendiceal diverticula (n = 1), and B cell non-Hodgkins lymphoma (NHL) (n = 1; Figure 1J).

Ninety-six of the 134 total patients with unusual histopathologic findings showed no evidence of inflammatory cell infiltration. However, 21 of these 96 cases had additional inflammation-related findings, including lymphoid hyperplasia (n = 18) and ovarian cyst rupture (n = 3). The remaining 38 of the 134 total patients did show evidence of inflammatory cell infiltration to varying degrees. Among those patients, five were histologically confirmed as having perforated appendicitis and three as having gangrenous appendicitis. Five of 134 patients showed evidence of classical granulomas and multinucleated giant cells formed by epitheloid histiocytes. Staining with erlich ziehl-nielsen and periodic acid-schiff revealed a complete absence of microorganisms; thus all cases were reported as granulomatous appendicitis. Taenia sup eggs were detected in one specimen, although the adult form of the parasite was not detected and the case was not specified as *Taenia saginata* or *Taenia solium*. Clinicopathologic features of the 134 appendectomized patients with unusual histopathological findings are summarized in Table 2.

Malignancy was detected in the appendectomy specimens of 11 of the 134 patients of this group. The mean age of these patients was 49.1 ± 16.7 years old (range: 21-74 years), and the majority was female (4:7). There was no suspicion of cancer prior to the appendectomy surgery for any of these patients. Histopathological findings, however, indicated carcinoid tumor (n = 6), adenocarcinoma (n = 4) and B cell NHL (n = 1). Standard appendectomy was carried out in five of the six patients with carcinoid tumor, and the tumor diameters of these cases ranged from 5-25 mm; only the sixth case underwent subsequent right hemicolectomy procedure following the cancer diagnosis. Detailed tumor data could be retrieved for only one of the four patients with adenocarcinoma (age range: 51-74 years) but all of these patients underwent subsequent right hemicolectomy following the cancer diagnosis. The one patient diagnosed with B cell NHL was referred to the Medical Oncology Department following the appendectomy surgery, and medical records indicate that the patient was in remission at the 1-year follow-up. Detailed characteristics of the 11 appendectomized patients with histologically-diagnosed appendicular malignancy are summarized in Table 3.

DISCUSSION

Acute appendicitis manifests upon inflammation of the inner lining of the appendix vermiformis, which can spread to other parts of the organ. This condition may be brought on by several different physiopathological processes, but luminal obstruction is considered the most important triggering factor of the underlying inflammation[1-5]. Although lymphoid hyperplasia and fecaliths are the most frequently observed etiologies of luminal obstruction, other, less frequent factors have been observed in patients with symptoms of acute appendicitis. According to the literature, the most common of these unusual factors are mucinous cystadenoma or mucocele[6-9], carcinoid tumor[9-12], granulomatous diseases[13-15], enterobiasis[1,5,16,17], taeniasis[3,18-20], ascariasis[4,21], diverticulitis[22-25], primary or secondary adenocarcinoma[26-30], lymphoma[26,31,32], and neurogenic appendicopathy[33,34]. In addition, the study by Akbulut *et al*[1] reported cases associated with eosinophilic granuloma, amebiasis, actinomycosis, schistosomiasis, balantidiasis, tuberculosis,

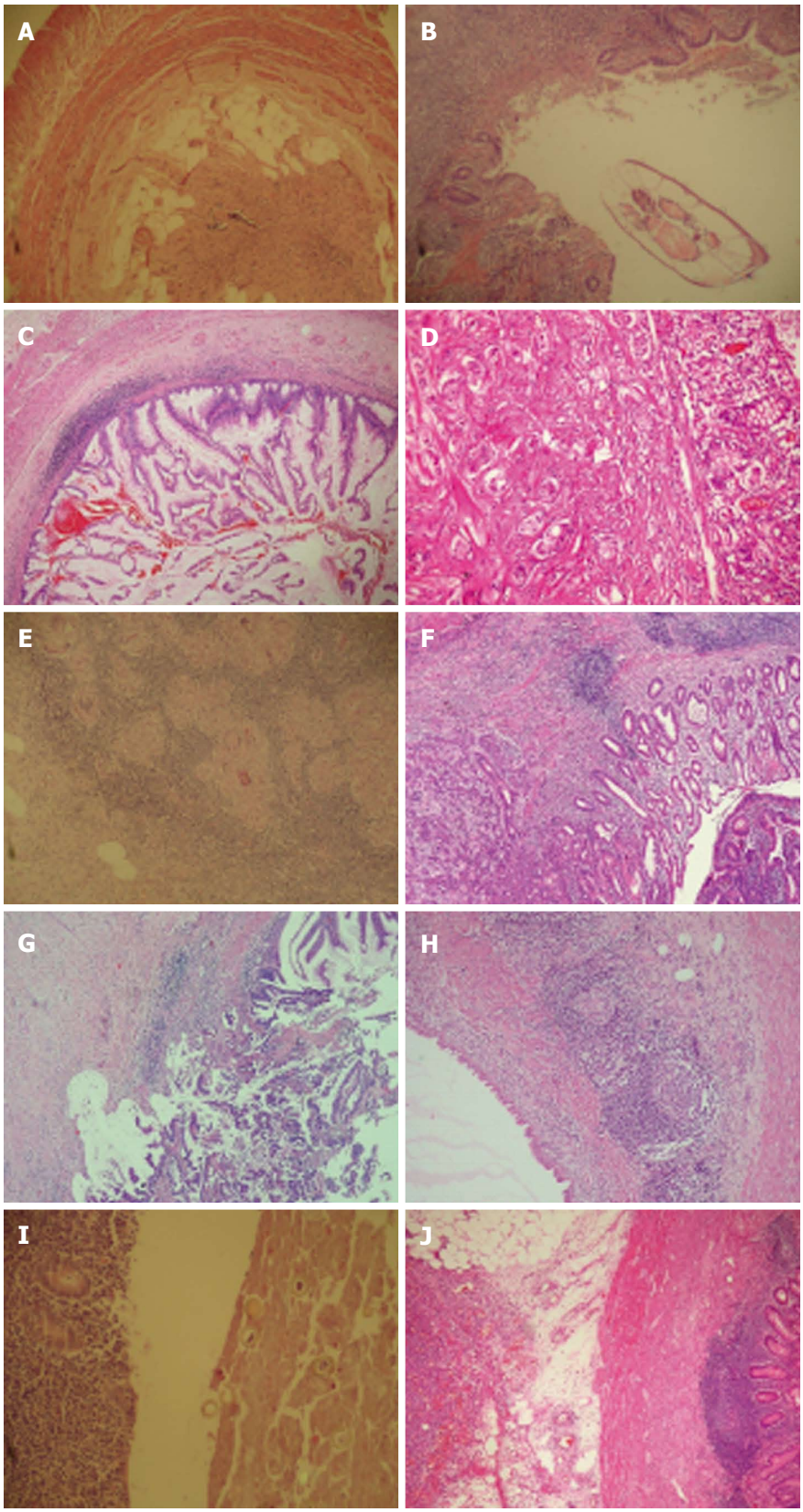

Figure 1 Unusual histopathologic findings. A: Appendix vermiformis showing fibrous obliteration [hematoxylin and eosin (HE) × 40]; B: View of the enterobius vermiformis in the lumen of appendix vermiformis (HE × 100); C: Mucinous cystadenoma showing proliferation of neoplastic adenomatous epithelium, which exhibits low-grade dysplasia (HE × 100); D: Carcinoid tumor of the appendix showing rounded nests and tubules of tumor cells with uniform nuclei (HE × 200); E: Granulomatous inflammation. Submucosal granuloma with central necrosis (HE × 40); F: Moderately differentiated adenocarcinoma showing infiltration of the mucosa and submucosa of the appendiceal wall (HE × 100); G: Adenocarcinoma of the appendix showing associated mucocele on the top right side (HE × 100); H: Mucocele showing a unilocular dilated appendiceal wall lined with flattened epithelial cells (HE × 100); I: Eggs of Taenia sup are present in the lumen of appendix vermiformis (HE × 100); J: Serosa of the appendiceal wall showing diffuse large B cell lymphoma infiltration (HE × 40).

Table 2 Detailed characteristics of 134 patients with unusual histopathological findings

Patient characteristic	Results
Patients	134
Sex, *n* (%)	
Male	60 (44.8)
Female	74 (55.2)
Age in years, mean ± SD (range)	
Overall	48.4 ± 19.5 (16-94)
Male	50.9 ± 19.3 (16-87)
Female	46.4 ± 19.5 (16-94)
Histopathologic findings, *n*	
Fibrous obliteration	62
Enterobius vermicularis	31
Eosinophilic infiltration	10
Mucinous cystadenoma	8
Carcinoid tumor	6
Granulomatous inflammation	5
Adenocarcinoma	3
Mucinous adenocarcinoma	1
Mucocele	3
Adenomatous polyp	1
Taenia saginata	1
Ascaris lumbricoides	1
Non-Hodgkin's lymphoma (B cell)	1
Appendicular diverticulitis	1
Age distribution of 134 patients with unusual findings (yr)	
16-20	8
21-30	30
31-40	15
41-50	16
51-60	22
61-70	22
⩾ 71	21

adenovirus, melanosis, neurofibroma, endometriosis, adenomatous or hyperplastic polyps, villous or tubulovillous adenoma, gastrointestinal stromal tumor, leukemia, and foreign body reactions.

Appendiceal tumors, which have been reported in < 3% of all appendectomy specimens, are rarely associated with manifestation of clinical symptomology. Thus, this condition is most often recognized incidentally, either during an abdominal operation or general pathological examination of a resected appendix specimen. The most frequently diagnosed type of appendiceal primary malignant lesion is the carcinoid tumor. Although it accounts for about 60% of all appendiceal tumors, its incidence in patients undergoing appendectomy is only 0.30%-2.27%. Most of the carcinoid tumors are located at the tip of the appendix and are < 1 cm in diameter. Fortunately, malignancy and metastasis of these tumors are very rare, and usually only involve tumors that exceed 1 cm. Therefore, simple appendectomy is considered sufficient management for these tumors. The risk of metastasis jumps up to 85%, however, once the tumor size reaches 2 cm or larger, in which case a formal right hemicolectomy is recommended[10-12]. In our patient series, the incidence of appendiceal carcinoid (0.37%) was similar to that in the overall literature.

Primary adenocarcinoma of the appendix is an extraordinarily rare tumor, with overall incidence in the literature between 0.01% and 0.20%. However, this tumor is most likely to occur in persons between 50 and 55 years old. Adenocarcinomas generally show aggressive behavior, the pattern of which has been likened to colonic adenocarcinomas. Therefore, appendicular adenocarcinomas are often treated by oncologic resection with right hemicolectomy[10,26,29]. In our patient series, only four patients presented with this tumor type, giving an incidence of 0.25% that is similar to that in the overall literature. In addition, these patients were within the age range of 51 and 74 years old (mean ± SD, 64.0 ± 8.3 years).

Appendiceal mucinous adenocarcinoma, also known as mucinous cystadenocarcinoma, is another rare condition of the appendix. This tumor type, however, is most often associated with a second malignancy of the gastrointestinal tract and the most common manifestation is symptoms of acute appendicitis. Like the other appendix-related cancers, diagnosis of mucinous adenocarcinoma is usually only made upon the subsequent pathological evaluation of a resected appendiceal specimen[27,28].

Mucocele is a condition in which mucoid material accumulates in the intraluminal region of the appendix, eventually causing obstructive dilatation of the organ. However, the occlusion of the appendiceal lumen may also be caused by endometriosis or carcinoid tumors[8,9]. The overall incidence of this condition in the literature ranges from 0.2% to 0.7%. Currently, four histologic types of appendiceal mucocele are recognized, and these include (in order of incidence): Mucinous cystadenoma, mucosal hyperplasia, mucinous cystadenocarcinoma, and retention cyst[1,6,7]. Up to one-half of mucocele cases are asymptomatic and the condition is incidentally diagnosed by histological examination of tissues from appendectomy, or sometimes during a laparotomy surgery. Appendectomy is the standard of care for mucinous cystadenoma, whereas a cystadenocarcinoma requires a right hemicolectomy[1,6,7].

The gastrointestinal tract is the most common site for extranodal lymphomas, accounting for 30%-45% of all extranodal cases. The incidence of primary appendiceal lymphoma is extremely low, and has been estimated at between 0.015% and 0.05%[26,31,32]. Cases of appendiceal lymphoma most often occur in young adults, between the ages of 20 and 40 years old. The usual manifestation of symptoms of acute appendicitis explain its diagnosis most frequently occurring following appendectomy and upon histopathologic analysis of the resected organ. Unfortunately, the rarity of the disease has impeded establishment of evidence-based guidelines for treatment.

The incidence of neurogenic appendicopathy is estimated to be about 30%. Although this process is often described as fibrous obliteration, recent studies have demonstrated that the occlusive proliferation is predominantly neurogenic in some cases. As of yet, the pathogenesis of this condition remains to be fully elucidated, but some studies have indicated that it may actually be secondary to hyperplasia of neuroendocrine cells. Differential diagnosis between appendiceal neuroma and acute

Table 3 Detailed characteristics of the 11 patients with appendicular malignancy

No.	Age (yr)	Sex	Primary tumor type	Tumor size (mm)	Pleomorphism	Mitosis (HPF)	Necrosis	Parietal spread	Surgical approach
1	55	M	B-NHL	CD20(+), CD79a(+)					Appendectomy
2	64	F	Adenoca	40				Muscularis propria	Appendectomy-right hemicolectomy
3	67	M	Adenoca	15					Appendectomy-right hemicolectomy
4	74	F	Adenoca	50				Serosa	Appendectomy-right hemicolectomy
5	51	M	Adenoca	50				Serosa	Appendectomy-right hemicolectomy
6	41	F	Carcinoid	25	Minimal	1/10	No	Mesoappendix	Appendectomy-right hemicolectomy
7	28	F	Carcinoid	5	Minimal	1/10	No	Submucosa	Appendectomy
8	28	F	Carcinoid	8	Minimal	0/10	No	Mesoappendix	Appendectomy
9	60	F	Carcinoid	10	Moderate	2/10	No	Mesoappendix	Appendectomy
10	21	M	Carcinoid	12	Moderate	2/10	No	Mesoappendix	Appendectomy
11	52	F	Carcinoid	13	Moderate	1/10	No	Muscularis propria	Appendectomy

HPF: High power field; M: Male; F: Female; B-NHL: B cell non-Hodgkins lymphoma.

appendicitis is relatively subjective and depends upon a patient's clinical history, symptomology, and findings from laboratory and physical examination. Accordingly, most appendiceal neuromas are incidentally indicated by histological evidence of fibrous obliteration in appendix specimens of otherwise asymptomatic patients[1,33,34]. In the current patient series, the incidence of fibrous obliteration was only 3.7%, which is lower than in the overall literature.

Enterobius vermicularis, commonly known as the pinworm, is a widespread parasitic infection that is estimated to affect up to 200 million people worldwide. The association of pinworm infection and appendicitis was first made in the late 19th century. While the reported incidence of pinworm infections in appendectomy specimens from patients with presumed appendicitis has ranged from 0.2% to 41.8%, inflammation is often associated with pinworm infection in the appendix[1,5,16,17]. In the current patient series, the incidence of pinworms in the appendectomy specimens was 1.9%, which is similar to the overall literature.

Taeniasis manifests upon intestinal infection with helminths. The first sign of infection is usually a segment of the parasite that appears in the stool. Taenia sup infection of the appendix, in particular, is so rare that the situation invites a case report. In general, cases of taeniasis do not necessitate identification of the specific species in order to initiate appropriate treatment, and a single dose of praziquantel can efficiently clear the infection[3,18-20].

Ascaris lumbricoides is one of the most common human helminthic pathogens infecting humans worldwide; however, epidemiologic studies have revealed that the highest prevalence of ascariasis occurs in tropical and semitropical countries. In the human host, the worm can establish residence in the gastrointestinal region from the stomach to the ileocecal valve, but up to 99% of the cases reported have worms localized to the jejunum and proximal ileum. Infections involving the appendix are only rarely seen. The ability of a roundworm to migrate to the appendix, thereby causing appendicitis, is controversial. The physical and physiological effects of such a migration may indeed simulate other physiopathogenic processes that promote appendicitis, but are believed less likely to be the direct cause of it[1,4,21].

Granulomatous appendicitis is another rare condition that may be discovered incidentally in a patient with a clinical presentation of acute appendicitis. The reported incidence in Western countries has ranged from 0.14% to 0.30%, and is higher (1.3%-2.3%) in underdeveloped countries[13,14]. The criteria for diagnosis are similar to those of other diseases of the gastrointestinal tract, and include granulomatous inflamation, transmural lymphoid aggregates, and fissuring-type ulcers. Various infectious agents (such as *Yersinia* spp., *Mycobacterium tuberculosis*, and *Schistosoma* spp.) and non-infectious factors (such as Crohn's disease and sarcoidosis) have been implicated as causative factors of this condition[1,14-16]. In the current series of patients, granulomatous inflammation was observed in only 0.3%. As tuberculosis is endemic in the region where our hospital is located, all of the patients had been tested accordingly; yet, no findings related to tuberculosis were encountered.

Appendiceal diverticulum is another very rare clinical entity, and the incidence is reported between 0.004% and 2.1%. The diverticula may occur as singlets or in multiples, but generally involve the distal third of the appendix, on its mesenteric side, and their size is usually < 0.5 cm. Cases of appendiceal diverticula are routinely classified as either congenital or acquired. While the congenital form (considered a true diverticulum) is extremely rare, the acquired form (a pseudodiverticulum consisting of mucosa and submucosa herniated through vascular clefts in the muscular layer) are encountered much more often. Four clinical variations of either form of this condition have been described, and include the appendiceal diverticula without inflammation, acute appendicitis with diverticula, acute appendiceal diverticulitis with acute appendicitis, and isolated acute diverticulitis. However, all

four forms are generally asymptomatic, with the related complications of perforation and inflammation causing the abdominal pain that mimics acute appendicitis[22-25].

Considering the overall case reports in the literature and the case series presented herein, it is clear that even when the macroscopic appearance of a resected appendix is normal, histopathological assessment of specimens will allow early diagnosis of malign and infectious appendiceal diseases.

COMMENTS

Background

Appendicitis is one of the most common acute surgical conditions of the abdominal cavity. While this clinicopathologic condition may manifest from several underlying etiologies, luminal obstruction is the essential triggering factor for development of the inflammatory process. Although lymphoid hyperplasia and fecaliths are the most common cause of luminal obstruction, other less commonly observed factors, such as infectious and malignant appendiceal diseases, may also underlie this pathogenic condition.

Research frontiers

According to the literature, the most common of unusual histopathologic findings are mucinous cystadenoma or mucocele, carcinoid tumor, granulomatous diseases, enterobiasis, taeniasis, ascariasis, diverticulitis, primary or secondary adenocarcinoma, lymphoma, and neurogenic appendicopathy, eosinophilic granuloma, amebiasis, actinomycosis, schistosomiasis, balantidiasis, tuberculosis, adenovirus, melanosis, neurofibroma, endometriosis, adenomatous or hyperplastic polyps, villous or tubulovillous adenoma, gastrointestinal stromal tumor, leukemia, and foreign body reactions. In this study, the authors conducted and investigation of the incidence and implications of unusual histopathological findings detected in resected appendectomy specimens obtained from patients who underwent surgery for suspected acute appendicitis.

Innovations and breakthroughs

The authors emphasize and strongly recommend that all appendectomy specimens be examined by histopathological analysis, even if specimens have a normal gross appearance.

Peer review

This is a quite well-done manuscript of appropriate interest and with images of reasonable quality.

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BRIEF ARTICLE

CYP1A1, *GSTM1*, *GSTT1* and *NQO1* polymorphisms and colorectal adenomas in Japanese men

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Abstract

AIM: To investigate the role of functional genetic polymorphisms of metabolic enzymes of tobacco carcinogens in the development of colorectal adenomas.

METHODS: The study subjects were 455 patients with colorectal adenomas and 1052 controls with no polyps who underwent total colonoscopy in a preretirement health examination at two Self Defense Forces hospitals. The genetic polymorphisms studied were *CYP1A1*2A* (rs 4646903), *CYP1A1*2C* (rs 1048943), *GSTM1* (null or non-null genotype), *GSTT1* (null or non-null genotype) and *NQO1* C609T (rs 1800566). Genotypes were determined by the polymerase chain reaction (PCR)-restriction fragment length polymorphism or PCR method using genomic DNA extracted from the buffy coat. Cigarette smoking and other lifestyle factors were ascertained by a self-administered questionnaire. The associations of the polymorphisms with colorectal adenomas were examined by means of OR and 95%CI, which were derived from logistic regression analysis. Statistical adjustment was made for smoking, alcohol use, body mass index and other factors. The gene-gene interaction and effect modification of smoking were evaluated by the likelihood ratio test.

RESULTS: None of the five polymorphisms showed a significant association with colorectal adenomas, nor was the combination of *GSTM1* and *GSTT1*. A borderline significant interaction was observed for the combination of *CYP1A1*2C* and *NQO1* (P = 0.051). The OR associated with *CYP1A1*2C* was significantly lower than unity among individuals with the *NQO1* 609CC genotype. The adjusted OR for the combination of the *CYP1A1*2C* allele and *NQO1* 609CC genotype was 0.61 (95%CI: 0.42-0.91). Although the interaction was not statistically significant (P = 0.24), the OR for individuals carrying the *CYP1A1*2C* allele and *GSTT1* null genotype decreased significantly compared with those who had neither *CYP1A1*2C* allele nor *GSTT1* null genotype (adjusted OR: 0.69, 95%CI: 0.49-0.97). Smoking did not modify the associations of the individual polymorphisms with colorectal adenomas. There was no measurable effect modification of smoking even regarding the combination of the genetic polymorphisms of the phase Ⅰ and phase Ⅱ enzymes.

CONCLUSION: Combination of the *CYP1A1*2C* and *NQO1* 609CC genotypes was associated with a decreased risk of colorectal adenomas regardless of smoking status.

Key words: Colorectal adenoma; Smoking; Polymorphism; *CYP1A1*; *GSTM1*; *GSTT1*; *NQO1*

Core tip: The study investigated the associations of *CYP1A1*2A*, *CYP1A1*2C*, *GSTM1*, *GSTT1* and *NQO1* C609T polymorphisms with colorectal adenomas among 455 cases of colorectal adenomas and 1052 controls with no polyps. None of the five polymorphisms showed a significant association with colorectal adenomas, nor was the combination of *GSTM1* and *GSTT1*. A borderline significant interaction was observed for the combination of *CYP1A1*2C* and *NQO1*. Combination of the *CYP1A1*2C* and *NQO1* 609CC genotypes was associated with a decreased risk of colorectal adenomas regardless of smoking status.

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INTRODUCTION

Colorectal cancer is one of the most common cancers, accounting for approximately 10% of incident cancer cases worldwide[1]. Colorectal adenoma is a well-established precursor lesion of colorectal cancer[2,3]. Cigarette smoking has been related to increased risk of colorectal adenomas, whereas the association between smoking and colorectal cancer risk is rather inconsistent and much weaker[4-6]. Despite the consistent association between smoking and colorectal adenomas, biological mechanisms for the association remain unknown. Tobacco smoke contains various types of carcinogens such as polycyclic aromatic hydrocarbons, heterocyclic amines, aromatic amines and *N*-nitrosamines, which are activated by phase I enzymes and/or detoxified by phase Ⅱ enzymes, and thus functional genetic polymorphisms of the metabolic enzymes are of interest in colorectal carcinogenesis[7].

CYP1A1 is a phase I enzyme responsible for bioactivation of tobacco carcinogens. Two functional polymorphisms are known in the *CYP1A1* gene[8,9]. One is the 3698T>C substitution (*CYP1A1*2A*, rs 4646903) that creates an *Msp*I restriction site in the 3'-flanking region, and the other is the 2454A>G substitution (*CYP1A1*2C*, rs 1048943) that results in an amino acid change (Ile462Val) in exon 7. The *CYP1A1*2A* and *CYP1A1*2C* alleles are linked to higher inducibility of the enzyme, and have been associated with an increased risk of lung cancer and less consistently of other tobacco-related cancers[8,9]. Several studies reported an increased risk of colorectal cancer associated with *CYP1A1*2A*[10] and *CYP1A1*2C*[11], while others showed no association of either *CYP1A1*2A* or *CYP1A1*2C* with colorectal cancer[12-15] or adenomas[16]. Isoforms of the glutathione S-transferase (GST) are involved in detoxification of chemical carcinogens and environmental toxic compounds[17,18]. *GSTM1* and *GSTT1* polymorphisms have been studied most intensively in relation to tobacco-related cancers. The *GSTM1* and *GSTT1* null genotypes result in a complete loss of enzyme function[17,18]. A meta-analysis suggested an increased risk of colorectal cancer associated with the null genotype of *GSTT1*, but not of *GSTM1*[19], while another meta-analysis showed no association of either the *GSTM1* or *GSTT1* null genotype with colorectal cancer or adenomas[20]. Some recent studies have shown an increased risk of colorectal cancer associated with the *GSTM1* and *GSTT1* null genotypes in combination[21,22], but others failed to show such an increase in the risk of colorectal cancer[14,15] or adenomas[23].

NAD(P)H-quinone oxidoreductase 1 (NQO1) is involved in detoxification through two electron reductions of quinones to hydroquinones, while NQO1 can also activate procarcinogens in tobacco smoke[24]. The 609C>T polymorphism (rs 1800566) that causes an amino acid substitution (Pro187Ser) results in loss of NQO1 activity[24]. A meta-analysis reported that *NQO1* 609C>T was associated with a small increase in the risk of colorectal cancer in Caucasians[25], but a recent large Japanese study failed to corroborate such an association[15]. Homozygosity of the *NQO1* 609T allele was shown to be positively associated with colorectal adenomas[26]. Heavy smokers carrying both the *CYP1A1*2C* and *NQO1* 609T variant alleles showed a substantial increase in the risk of adenomas[26].

To clarify the role of *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* polymorphisms in colorectal carcinogenesis with reference to smoking, we examined the associations of these polymorphisms with colorectal adenomas and the effect of smoking on the associations between the polymorphisms and colorectal adenomas. A particular emphasis was placed on the combination of genetic polymorphisms of phase I and phase Ⅱ enzymes, because the literature is sparse on the influence of gene-gene interactions on the risk of colorectal cancer and adenomas.

MATERIALS AND METHODS

Subjects

Study subjects were male officials in the Self Defense Forces who received a preretirement health examination at the Self Defense Forces Fukuoka Hospital or Kumamoto Hospital during the period from January 1997 to March 2001. The preretirement health examination is a nationwide program that offers a comprehensive medical examination including colonoscopy to persons retiring from the Self Defense Forces. Details of the preretirement health examination have been described elsewhere[27,28]. The subjects were Japanese in ethnicity. A 7-mL fasting venous blood sample was donated for the purpose of medical research with written informed consent. The study was approved by the Ethics Committee of Kyushu University Faculty of Medical Sciences.

The present study included 455 cases of histologically confirmed colorectal adenoma and 1052 controls with no

polyps who underwent total colonoscopy. In a consecutive series of 2454 men, five refused to participate in the study and we excluded 77 who did not undergo colonoscopy. Further exclusions were 242 men with a history of colectomy (n = 17), colorectal polypectomy (n = 212), malignant neoplasm (n = 27) or inflammatory bowel disease (n = 1). For the remaining 2135 men, colonoscopic findings were classified as polyp (n = 938, 43.9%), colorectal cancer (n = 1, 0.0%), non-polyp benign lesion (n = 123, 5.8%) and normal (n = 1073, 50.3%). Of the 938 men with colorectal polyps, 461 were found to have adenoma without *in situ* or invasive carcinoma. The controls comprised 1067 men who underwent a complete colonoscopy among the 1196 men with normal colonoscopy or non-polyp benign lesions. DNA was not available for 21 men (6 cases and 15 controls), and 455 cases and 1052 controls remained in the analysis.

Lifestyle questionnaire

Smoking habits, alcohol consumption, physical activity and other lifestyle factors were ascertained by a self-administered questionnaire, with a supplemental interview for unanswered questions given prior to colonoscopy. Details of the lifestyle questions have been described elsewhere[27,28]. Lifetime exposure to cigarette smoking was expressed by cigarette-years, which were calculated as the product of total years of smoking and the average number of cigarettes per day. Cigarette smoking was classified into 0, 1-399, 400-799 and ⩾ 800 cigarette-years. Daily intake of ethanol was estimated for current alcohol drinkers based on consumption frequencies and amounts of five types of alcoholic beverages on average in the past year. Alcohol use was categorized into never, past and current use with a consumption of < 30, 30-59 or ⩾ 60 mL of ethanol per day. Body mass index was categorized into four levels (< 22.5, 22.5-24.9, 25.0-27.4 and ⩾ 27.5 kg/m^2). The categories for alcohol use and body mass index were arbitrary, but in accordance with those used in the previous studies[27,28]. Leisure-time physical activity was expressed as the sum of products of intensity score (metabolic equivalent) and amount of time for at most three types of regular exercise, and was categorized by quartiles in the control group.

Genotyping

DNA was extracted from the buffy coat by use of a commercial kit (Qiagen GmbH, Hilden, Germany). Genotyping was carried out by the polymerase chain reaction (PCR)-restriction fragment length polymorphism or PCR method, with agarose-gel electrophoresis and visualization by ethidium bromide. The PCR was performed in a mixture of 10 μL containing 1 μL template DNA with a concentration of 50-150 ng/μL. The PCR for *CYP1A1*2A* polymorphism was done using the primers described by Sivaraman *et al*[10], and the 340-bp PCR product was digested with *Msp*I, which resulted in fragments of 200 and 140 bp for the *CYP1A1*2A* allele. The *CYP1A1*2C* polymorphism was genotyped using the primers previously specified[29], with digestion by restriction enzyme *Hin*cII. The 187-bp product was cleaved into three fragments (120, 48 and 19 bp) in the presence of the *CYP1A1*2C* allele, and otherwise into two fragments (139 and 48 bp). *GSTM1* and *GSTT1* polymorphisms were determined by the multiplex PCR using the primers for *GSTM1*, *GSTT1* and albumin as described previously[30]. Genotyping for *NQO1* 609C>T was performed as described previously[31]. The 230-bp PCR product was digested with *Hin*fI, resulting in fragments of 195 and 35 bp for the 609C allele and fragments of 151, 44 and 35 bp for the 609T allele. The assay was repeated at most three times when the PCR was unsuccessful or when the migration pattern on the gel was aberrant.

Statistical analysis

Deviation of genotype frequency from the Hardy-Weinberg equilibrium was tested by χ^2 test with one degree of freedom using the Stata version 10 (Stata Corporation, College Station, TX, United States). The associations between the polymorphisms and colorectal adenomas were assessed by means of OR and 95%CI, which were derived from logistic regression analysis. Statistical adjustment was made for age (continuous variable), hospital (dichotomous variable), rank in the Self Defense Forces (low, middle and high), cigarette smoking, alcohol use, body mass index, physical activity and parental colorectal cancer. The gene-gene interaction and effect modification of smoking were evaluated by the likelihood ratio test. In the analysis of the effect modification of smoking, smoking status was categorized into < 400 and ⩾ 400 cigarette-years, *i.e.*, < 20 and ⩾ 20 pack-years, because an increased risk of adenomas associated with smoking was discernible only in the latter categories (see below). Statistical significance was declared if two-sided P was < 0.05. Statistical analysis was performed with SAS version 9.2 (SAS Institute, Cary, NC, United States).

RESULTS

Selected characteristics of the cases and controls are summarized in Table 1. The age range was 50-57 years for the cases and 47-59 years for the controls. The cases had a greater body mass index and a lower physical activity in leisure time than the controls. Heavy smoking and high alcohol consumption were more frequent in the cases than in the controls.

Among the controls, the frequencies of the *CYP1A1*2A*, *CYP1A1*2C* and *NQO1 609T* alleles were 0.39, 0.23 and 0.38, respectively, and genotype frequencies of the three polymorphisms were all in agreement with the Hardy-Weinberg equilibrium (P = 0.62 for *CYP1A1*2A*; P = 0.32 for *CYP1A1*2C*; and P = 0.76 for *NQO1* C609T). The *CYP1A1*2A* and *CYP1A1*2C* polymorphisms were in complete linkage disequilibrium except for two cases; the deviation of these two was probably due to error in genotyping.

None of the five polymorphisms showed a significant association with colorectal adenomas, nor was the combination of *GSTM1* and *GSTT1* (Table 2). The gene-gene interaction was examined for the combina-

Table 1 Selected characteristics of the study subjects

Characteristics	Cases (n = 455)	Controls (n = 1052)
Age (yr), mean ± SD	52.4 ± 0.8	52.4 ± 0.9
Body mass index (kg/m^2), mean ± SD	24.1 ± 2.8	23.7 ± 2.5
MET-h/wk, median (IQR)[1]	12 (3-24)	14 (5-24)
Smoking (cigarette-yr)		
0	20.90%	33.70%
1-399	14.10%	18.80%
400-799	45.50%	33.70%
⩾ 800	19.60%	13.70%
Alcohol use (mL/d)		
Never	11.20%	13.80%
Past	2.90%	3.10%
< 30	21.10%	30.70%
30-59	34.10%	28.70%
⩾ 60	30.80%	23.70%

[1]Leisure-time physical activity. IQR: Interquartile range; MET: Metabolic equivalent.

Table 2 Associations between genetic polymorphisms and colorectal adenomas n (%)

Genotype	Cases	Controls	Crude OR (95%CI)	Adjusted[3] OR (95%CI)
*CYP1A1*2A*				
0[1]	174 (38.2)	388 (36.9)	1.00 (referent)	1.00 (referent)
1	219 (48.1)	508 (48.3)	0.96 (0.76-1.22)	0.97 (0.76-1.24)
2	62 (13.6)	156 (14.8)	0.89 (0.63-1.25)	0.86 (0.60-1.23)
*CYP1A1*2C*				
0[1]	281 (61.8)	611 (58.1)	1.00 (referent)	1.00 (referent)
1	152 (33.4)	389 (37.0)	0.85 (0.67-1.07)	0.81 (0.64-1.03)
2	22 (4.8)	52 (4.9)	0.92 (0.55-1.54)	0.94 (0.55-1.60)
GSTM1				
Non-null	200 (44.0)	506 (48.1)	1.00 (referent)	1.00 (referent)
Null	255 (56.0)	546 (51.9)	1.18 (0.95-1.47)	1.19 (0.94-1.49)
GSTT1				
Non-null	258 (56.7)	552 (52.5)	1.00 (referent)	1.00 (referent)
Null	197 (43.3)	500 (47.5)	0.84 (0.68-1.05)	0.87 (0.70-1.10)
GSTM1 + *GSTT1*				
0[2]	118 (25.9)	273 (26.0)	1.00 (referent)	1.00 (referent)
1	222 (48.8)	512 (48.7)	1.00 (0.77-1.31)	1.04 (0.79-1.37)
2 (both null)	115 (25.3)	267 (25.4)	1.00 (0.73-1.35)	1.04 (0.76-1.42)
NQO1 C609T				
CC	161 (35.4)	412 (39.2)	1.00 (referent)	1.00 (referent)
CT	220 (48.4)	489 (46.5)	1.15 (0.90-1.47)	1.18 (0.92-1.52)
TT	74 (16.3)	151 (14.4)	1.25 (0.90-1.75)	1.31 (0.93-1.84)

[1]Number of variant alleles; [2]Number of null genotypes; [3]Adjusted for age, hospital, rank in the Self-Defense Forces, body mass index, cigarette smoking, alcohol use, leisure-time physical activity and parental history of colorectal cancer.

tions of the *CYP1A1* polymorphisms and the *GST* or *NQO1* polymorphism (Table 3). As regards *CYP1A1*2A*, *CYP1A1*2C* and *NQO1*, the homozygous variant genotype was combined with the heterozygous genotype because variant homozygotes were relatively few. A borderline significant interaction was observed for the combination of *CYP1A1*2C* and *NQO1* (P = 0.051). The OR associated with *CYP1A1*2C* was significantly lower than unity among individuals with the *NQO1* 609CC genotype. Although the interaction was far from statistical significance, the OR for individuals carrying the *CYP1A1*2C* allele and *GSTT1* null genotype significantly decreased compared with those who had neither the *CYP1A1*2C* allele nor the *GSTT1* null genotype.

Table 3 Associations between combinations of genetic polymorphisms and colorectal adenomas

Genotype 1	Genotype 2	n[1]	OR (95%CI)[2]	Interaction (P)
*CYP1A1*2A*	*GSTM1*			0.94
0[3]	Non-null	75/182	1.00 (referent)	
0	Null	99/206	1.20 (0.83-1.74)	
⩾ 1	Non-null	125/324	0.96 (0.67-1.35)	
⩾ 1	Null	156/340	1.12 (0.80-1.58)	
*CYP1A1*2A*	*GSTT1*			0.08
0[3]	Non-null	92/210	1.00 (referent)	
0	Null	82/178	1.14 (0.79-1.65)	
⩾ 1	Non-null	166/342	1.15 (0.83-1.58)	
⩾ 1	Null	115/322	0.85 (0.61-1.19)	
*CYP1A1*2A*	*GSTM1* + *GSTT1*			0.44
0[3]	0[4]	41/102	1.00 (referent)	
0	1	85/188	1.19 (0.75-1.88)	
0	2 (both null)	48/98	1.35 (0.81-2.27)	
⩾ 1	0	77/171	1.17 (0.74-1.86)	
⩾ 1	1	137/324	1.12 (0.73-1.72)	
⩾ 1	2 (both null)	67/169	1.03 (0.64-1.66)	
*CYP1A1*2A*	*NQO1* C609T			0.09
0[3]	CC	63/133	1.00 (referent)	
0	CT + TT	111/255	0.92 (0.63-1.36)	
⩾ 1	CC	98/279	0.73 (0.49-1.07)	
⩾ 1	CT + TT	183/385	1.02 (0.71-1.47)	
*CYP1A1*2C*	*GSTM1*			0.77
0[3]	Non-null	127/294	1.00 (referent)	
0	Null	154/317	1.16 (0.86-1.55)	
⩾ 1	Non-null	73/212	0.79 (0.56-1.12)	
⩾ 1	Null	101/229	0.98 (0.71-1.36)	
*CYP1A1*2C*	*GSTT1*			0.24
0[3]	Non-null	154/321	1.00 (referent)	
0	Null	127/290	0.97 (0.73-1.31)	
⩾ 1	Non-null	104/231	0.93 (0.68-1.27)	
⩾ 1	Null	70/210	0.69 (0.49-0.97)	
*CYP1A1*2C*	*GSTM1* + *GSTT1*			0.37
0[3]	4	74/156	1.00 (referent)	
0	1	133/303	0.96 (0.67-1.37)	
0	2 (both null)	74/152	1.13 (0.75-1.69)	
⩾ 1	0	44/117	0.79 (0.50-1.25)	
⩾ 1	1	89/209	0.92 (0.63-1.35)	
⩾ 1	2 (both null)	41/115	0.72 (0.45-1.15)	
*CYP1A1*2C*	*NQO1* C609T			0.05
0[3]	CC	102/220	1.00 (referent)	
0	CT + TT	179/391	0.99 (0.73-1.34)	
⩾ 1	CC	59/192	0.61 (0.42-0.91)	
⩾ 1	CT + TT	115/249	0.98 (0.71-1.37)	

[1]Number of cases/controls; [2]Adjusted for age, hospital, rank in the Self-Defense Forces, body mass index, cigarette smoking, alcohol use, leisure-time physical activity and parental history of colorectal cancer; [3]Number of variant alleles; [4]Number of null genotypes.

Smoking was positively associated with colorectal adenomas; the multivariate-adjusted ORs for the smoking categories of 0, 1-399, 400-799 and ⩾ 800 cigarette-years were 1.00 (referent), 1.18 (95%CI: 0.82-1.71), 2.11 (95%CI: 1.58-2.82) and 2.11 (95%CI: 1.47-3.03), respectively. However, smoking did not modify the associations of the *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* polymorphisms with colorectal adenomas (Table 4). The ORs associated with heavy smoking were consistently increased,

Table 4 Associations between genetic polymorphisms and colorectal adenomas with stratification by smoking category

Genotype	< 20 pack-years		⩾ 20 pack-years		Interaction (*P*)
	n[1]	OR (95%CI)[2]	*n*[1]	OR (95%CI)[2]	
*CYP1A1*2A*					
0[3]	59/207	1.00 (referent)	115/181	2.22 (1.52-3.24)	0.45
⩾ 1	100/346	1.05 (0.73-1.53)	181/318	1.95 (1.37-2.76)	
*CYP1A1*2C*					
0[3]	106/329	1.00 (referent)	175/282	1.90 (1.41-2.56)	0.59
⩾ 1	53/224	0.76 (0.52-1.11)	121/217	1.65 (1.20-2.27)	
GSTM1					
Non-null	69/264	1.00 (referent)	131/242	1.99 (1.41-2.82)	0.96
Null	90/289	1.19 (0.83-1.71)	165/257	2.35 (1.68-3.29)	
GSTT1					
Non-null	89/268	1.00 (referent)	169/284	1.74 (1.27-2.38)	0.26
Null	70/285	0.74 (0.52-1.06)	127/215	1.69 (1.21-2.36)	
GSTM1 + *GSTT1*					
0[4]	39/134	1.00 (referent)	79/139	1.87 (1.18-2.96)	0.63
1	80/264	1.03 (0.67-1.61)	142/248	1.91 (1.26-2.91)	
2 (both null)	40/155	0.89 (0.54-1.48)	75/112	2.14 (1.34-3.43)	
NQO1 C609T					
CC	52/221	1.00 (referent)	109/191	2.23 (1.51-3.29)	0.46
CT + TT	107/332	1.35 (0.93-1.97)	187/308	2.51 (1.75-3.60)	

[1]Number of cases/controls; [2]Adjusted for age, hospital, rank in the Self-Defense Forces, body mass index, cigarette smoking, alcohol use, leisure-time physical activity and parental history of colorectal cancer; [3]Number of variant alleles; [4]Number of null genotypes.

regardless of genotype of the polymorphism. There was no measurable effect modification of smoking even regarding the combination of the genetic polymorphisms of the phase I and phase Ⅱ enzymes (Table 5). The OR was lowest for the combination of the *CYP1A1*2C* and *NQO1* 609CC genotypes among the four composite genotypes in each stratum of smoking. The OR varied according to the combination of *CYP1A1*2C* and the composite genotypes of *GSTM1* and *GSTT1* within each stratum of smoking. The OR for the combination of the *CYP1A1*2C* allele and the non-null genotypes of both *GSTM1* and *GSTT1* was significantly lower than unity in the category of < 20 pack-years, and the OR increased significantly among heavy smokers without the *CYP1A1*2C* allele who had null genotypes for both *GSTM1* and *GSTT1*.

DISCUSSION

According to recent meta-analyses[32,33], the *CYP1A1*2C* polymorphism, but not *CYP1A1*2A* polymorphism, was significantly associated with a modest increase in the risk of colorectal cancer. Neither *CYP1A1*2A* nor *CYP1A1*2C* polymorphism was related to colorectal adenomas individually in the present study. The findings are consistent with the previous observation regarding colorectal adenomas[16,26]. Further investigation is needed to clarify whether the associations with the *CYP1A1*2C* polymorphism is differential for colorectal cancer and adenomas.

The null effects of the *GSTM1* and *GSTT1* polymorphisms in the present study are in agreement with previous observations regarding colorectal adenomas[23,34]. Some of the previous studies showed an increased risk of colorectal cancer among individuals with the *GSTM1* null genotype[11,21,22], the *GSTT1* null genotype[21], or both the *GSTM1* and *GSTT1* null genotypes[21,22]. However, these findings were not replicated in other studies on colorectal cancer[14,15,35]. Previous studies found no effect of smoking on the association with *GSTM1* and *GSTT1*, either singly or in combination, in relation to colorectal adenomas[23] and cancer[15,22,33]. The *GSTM1* and *GSTT1* non-null genotypes as determined by gel electrophoresis include heterozygous genotypes (*i.e.*, one active and one inactive allele). One study differentiated the heterozygous genotype from the homozygous non-null genotype for *GSTM1* and *GSTT1* by TaqMan assay[36]. Both heterozygous and homozygous null genotypes of *GSTM1* were associated with a decreased risk of colorectal adenomas irrespective of smoking status, while adenoma risk was increased in association with both heterozygous and homozygous null genotypes of *GSTT1* among ever-smokers, but not among never-smokers[36]. However, it is unclear whether heterozygosity in either *GSTM1* or *GSTT1* affects enzyme activity[18].

Few studies have addressed the combined effect of the *CYP1A1* polymorphisms and the *GSTM1* and/or *GSTT1* null genotype in relation to colorectal adenomas and cancer. The combination of *CYP1A1*2A* and *GSTM1* null genotype was shown to be unrelated to colorectal adenomas[16]. There was no interaction between the two *CYP1A1* polymorphisms and either *GSTM1* or *GSTT1* null genotype on the risk of colorectal cancer[14]. A Japanese study showed a decreased risk of colorectal cancer for the combination of *CYP1A*2C* and *GSTT1* non-null genotype[15]. The present study indicated a decreased risk of colorectal adenomas for the combination

Table 5 Associations between combinations of genetic polymorphisms and colorectal adenomas with stratification by smoking category

Genotype 1	Genotype 2	< 20 pack-years		⩾ 20 pack-years		Interaction (*P*)
		n[1]	OR (95%CI)[2]	*n*[1]	OR (95%CI)[2]	
*CYP1A1*2A*	*GSTM1*					0.44
0[3]	Non-null	25/87	1.00 (referent)	50/95	1.78 (1.00-3.14)	
0	Null	34/120	0.94 (0.52-1.69)	65/86	2.53 (1.45-4.43)	
⩾ 1	Non-null	44/177	0.86 (0.49-1.50)	81/147	1.82 (1.07-3.10)	
⩾ 1	Null	56/169	1.19 (0.69-2.05)	100/171	1.92 (1.14-3.22)	
*CYP1A1*2A*	*GSTT1*					0.56
0[3]	Non-null	29/98	1.00 (referent)	63/112	1.94 (1.15-3.28)	
0	Null	30/109	0.95 (0.53-1.71)	52/69	2.53 (1.44-4.44)	
⩾ 1	Non-null	60/170	1.26 (0.75-2.11)	106/172	2.07 (1.27-3.39)	
⩾ 1	Null	40/176	0.81 (0.47-1.40)	75/146	1.70 (1.02-2.82)	
*CYP1A1*2A*	*GSTM1* + *GSTT1*					0.40
0[3]	0[4]	14/42	1.00 (referent)	27/60	1.34 (0.62-2.89)	
0	1	26/101	0.76 (0.36-1.62)	59/87	2.02 (1.00-4.07)	
0	2 (both null)	19/64	0.87 (0.39-1.94)	29/34	2.46 (1.11-5.46)	
⩾ 1	0	25/92	0.83 (0.39-1.78)	52/79	1.89 (0.93-3.86)	
⩾ 1	1	54/163	1.01 (0.51-2.01)	83/161	1.52 (0.78-2.98)	
⩾ 1	2 (both null)	21/91	0.73 (0.33-1.59)	46/78	1.66 (0.81-3.40)	
*CYP1A1*2A*	*NQO1* C609T					0.76
0[3]	CC	20/73	1.00 (referent)	43/60	2.55 (1.34-4.86)	
0	CT + TT	39/134	1.04 (0.56-1.94)	72/121	2.15 (1.20-3.86)	
⩾ 1	CC	32/148	0.81 (0.43-1.54)	66/131	1.69 (0.94-3.04)	
⩾ 1	CT + TT	68/198	1.29 (0.72-2.29)	115/187	2.23 (1.28-3.89)	
*CYP1A1*2C*	*GSTM1*					0.78
0[2]	Non-null	47/150	1.00 (referent)	80/144	1.74 (1.13-2.69)	
0	Null	59/179	1.04 (0.67-1.63)	95/138	2.15 (1.40-3.31)	
⩾ 1	Non-null	22/114	0.63 (0.36-1.11)	51/98	1.59 (0.98-2.57)	
⩾ 1	Null	31/110	0.94 (0.55-1.58)	70/119	1.77 (1.13-2.78)	
*CYP1A1*2C*	*GSTT1*					0.11
0[2]	Non-null	60/151	1.00 (referent)	94/170	1.39 (0.93-2.07)	
0	Null	46/178	0.66 (0.42-1.03)	81/112	1.80 (1.18-2.75)	
⩾ 1	Non-null	29/117	0.65 (0.39-1.09)	75/114	1.62 (1.06-2.48)	
⩾ 1	Null	24/107	0.59 (0.34-1.01)	46/103	1.06 (0.66-1.69)	
*CYP1A1*2C*	*GSTM1* + *GSTT1*					0.22
0[2]	0[4]	29/71	1.00 (referent)	45/85	1.26 (0.71-2.23)	
0	1	49/159	0.73 (0.42-1.26)	84/144	1.41 (0.84-2.37)	
0	2 (both null)	28/99	0.69 (0.38-1.28)	46/53	2.03 (1.12-3.69)	
⩾ 1	0	10/63	0.39 (0.17-0.87)	34/54	1.46 (0.79-2.72)	
⩾ 1	1	31/105	0.75 (0.41-1.36)	58/104	1.31 (0.76-2.26)	
⩾ 1	2 (both null)	12/56	0.53 (0.25-1.15)	29/59	1.09 (0.58-2.05)	
*CYP1A1*2C*	*NQO1* C609T					0.34
0[2]	CC	34/127	1.00 (referent)	68/93	2.64 (1.60-4.36)	
0	CT + TT	72/202	1.33 (0.83-2.12)	107/189	2.10 (1.33-3.31)	
⩾ 1	CC	18/94	0.75 (0.39-1.41)	41/98	1.41 (0.83-2.41)	
⩾ 1	CT + TT	35/130	1.03 (0.60-1.77)	80/119	2.47 (1.53-4.00)	

[1]Number of cases/controls; [2]Adjusted for age, hospital, rank in the Self-Defense Forces, body mass index, cigarette smoking, alcohol use, leisure-time physical activity and parental history of colorectal cancer; [3]Number of variant alleles; [4]Number of null genotypes.

of *CYP1A1*2C* and *GSTT1* null genotype, although the interaction was not significant. Inconsistent findings regarding the combination of *CYP1A1*2C* and *GSTT1* are probably ascribed to random fluctuation, although further studies are needed.

The risk of adenomas was lowest for the combination of *CYP1A1*2C* and the *GSTM1* and *GSTT1* non-null genotypes among never-smokers or light smokers, and an increased risk associated with heavy smoking was most evident among men without *CYP1A1*2C* who had the *GSTM1* and *GSTT1* null genotypes. Caution is required in interpreting the findings because of the small number of each combination of the three polymorphisms when stratified by smoking. Nonetheless, further investigation is warranted because the association of the three-polymorphism combination with colorectal cancer or adenomas has not been investigated previously.

The present study did not corroborate an increased risk of colorectal adenomas associated with the combination of *CYP1A1*2C* and *NQO1 609T* alleles reported among non-Hispanic whites[26], but showed a decreased risk among men harboring the *CYP1A1*2C* allele and the *NQO1* 609CC genotype. In that study[26], however, there were only a few subjects with both the *CYP1A1*2C* and *NQO1 609T* alleles (12 cases and 26 controls), accounting for only 4.0% of the cases and 1.8% of the controls,

because these variant alleles are much less frequent in Caucasians than in Asians[10,31]. In a study of Caucasians[26], the OR for the combination of *CYP1A1*2C* allele and *NQO1* 609CC genotype was 0.6 (95%CI: 0.3-1.2), as compared with the same referent combination as used in the present study. This finding is thus compatible with the present observation. It should be noted that the borderline significant interaction between *CYP1A1*2C* and *NQO1* C609T resulted from the eight statistical tests (Table 3). The probability of detecting at least one statistically significant result is 0.34, even when none of the eight interactions are present. The combined effect of these two polymorphisms requires careful interpretation, but requires further studies for mechanistic plausibility. Exposure to activated carcinogens may be lowered in individuals with both the *CYP1A1*2C* allele and the *NQO1* 609CC genotype for faster activation and detoxification.

The advantages of our study were that colonoscopy was done non-selectively in a defined population and that the absence of polyp lesions was confirmed in the control subjects by complete colonoscopy. Ethnic homogeneity was another advantage. There were several limitations. Statistical adjustment was not made for dietary factors because such data were not available. Only men were included, and the findings may not be applicable to women. Smoking is much less prevalent in women than in men in Japan[15]. The study subjects were not representative of Japanese men in the general population, but selection was unlikely to have occurred with regard to the genetic polymorphisms under study. The allele and genotype frequencies in the present study population were almost the same as those observed among Japanese individuals elsewhere. Among community controls (n = 778) in a Japanese case-control study of colorectal cancer[15], the frequencies of the *CYP1A1*2A*, *CYP1A1*2C* and *NQO1* 609T alleles were 37%, 23% and 38%, respectively, and the *GSTM1* and *GSTT1* null genotypes accounted for 54% and 44%, respectively. In a random sample of approximately 300 Japanese adults[37], the frequencies of the *CYP1A1*2C* and *NQO1* 609T alleles were 21% and 38%, respectively. Finally, the study size was not sufficiently large to detect a moderately increased risk for the variant homozygote. With two-sided a = 0.05, the power of detecting a 1.5-fold increase in the risk for the variant homozygote in the additive model was 0.66 for *CYP1A1*2A*, 0.39 for *CYP1A1*2C* and 0.65 for *NQO1* C609T.

In conclusion, the combination of *CYP1A1*2C* and *NQO1* 609CC genotype was associated with a decreased risk of colorectal adenomas, regardless of smoking status, in Japanese men. Future studies are needed to clarify the biological mechanisms involved.

COMMENTS

Background

Cigarette smoking has consistently been related to an increased risk of colorectal adenomas, and possibly of colorectal cancer, but the biological mechanisms remain unknown.

Research frontiers

Tobacco smoke contains various types of carcinogens, which are activated by phase Ⅰ enzymes and/or detoxified by phase Ⅱ enzymes. It is a matter of interest whether or not functional genetic polymorphisms of the metabolic enzymes are related to colorectal adenomas and cancer.

Innovations and breakthroughs

Few studies have examined the association of genetic polymorphisms of phase Ⅰ and phase Ⅱ enzymes in combination with colorectal adenomas or cancer. Adenoma risk differed by the combination of genetic polymorphisms of CYP1A1 (phase Ⅰ enzyme) and NAD(P)H-quinone oxidoreductase 1 (NQO1) (phase Ⅱ enzyme), and the association was not modified by smoking.

Applications

The findings confer clues to understanding the biological mechanisms of the association between smoking and colorectal adenomas and cancer.

Terminology

CYP1A1 is responsible for bioactivation of tobacco carcinogens. *CYP1A1*2A* and *CYP1A1*2C* polymorphisms are putatively functional, and have been related to increased risk of tobacco-related cancers; Glutathione *S*-transferases are involved in detoxification of chemical carcinogens, and individuals with the *GSTM1* and/or *GSTT1* null genotype may be susceptible to increased risk of cancer; NQO1 also acts as a phase Ⅱ enzyme, and the 609C>T polymorphism results in loss of NQO1 activity.

Peer review

This was a good study. The study subjects were all male officials in the Self-Defense Forces. Besides smoking, they may have risk factors, such as alcohol drinking and dietary habits, similar to those in the general population.

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BRIEF ARTICLE

Clinical and pathological differences between serum immunoglobulin G4-positive and -negative type 1 autoimmune pancreatitis

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Author contributions: All authors were involved in recruitment of patients and diagnosis of autoimmune pancreatitis; Paik WH and Park JM performed the research and wrote the paper; Ryu JK designed the research, analyzed the data and contributed to the preparation, editing, and final approval of the manuscript; Park JK and Song BJ wrote the paper; Kim YT contributed to the preparation and final approval of the manuscript and conceived the project; Lee KB was involved in re-evaluation of the histology.
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Abstract

AIM: To identify clinical and pathological differences between serum immunoglobulin G4 (IgG4)-positive (SIP) and IgG4-negative (SIN) type 1 autoimmune pancreatitis (AIP) in South Korea.

METHODS: AIP was diagnosed by the international consensus diagnostic criteria. The medical records and pathology were retrospectively reviewed and IgG4-positive cells were counted in a high power field (HPF). Type I AIP was defined as a high serum level of IgG4 or histological finding. SIN type 1 AIP was defined as a histological evidence of type 1 AIP and a normal serum IgG4 level. The clinical and pathological findings were compared between the two groups. The analysis was performed using Student's *t* test, Fischer's exact test and Mann-Whitney's *U* test. A *P* value of < 0.05 was considered statistically significant. As repeated comparison was made, *P* values of less than 5% ($P < 0.05$) were considered significant.

RESULTS: Twenty five patients with definite type 1 AIP (19 histologically and six serologically diagnosed cases) were enrolled. The mean tissue IgG4 concentrations were significantly higher in SIP than SIN group (40 cells per HPF *vs* 18 cells per HPF, $P = 0.02$). Among eight SIN patients, the tissue IgG4 concentrations were less than 15 cells per HPF in most of cases, except one. The sensitivity of serum IgG4 was 68% (17 SIP and eight SIN AIP). Other organ involvement was more frequently associated with SIP than SIN AIP (59% *vs* 26%, $P = 0.016$). However, the relapse rate and diffuse swelling of the pancreas were not associated with serum IgG4 level. The concentrations of IgG4-positive cells per HPF were higher in SIP than SIN AIP (40 *vs* 18, $P = 0.02$).

CONCLUSION: The sensitivity of serum IgG4 was 68% in type 1 AIP. High serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration but did not affect the relapse rate in type 1 AIP.

Key words: Autoimmunity; Chronic pancreatitis; Immunoglobulin G4-related systemic disease; Lymphoplasmacytic sclerosing pancreatitis; Immunoglobulin G4

Core tip: Type 1 autoimmune pancreatitis (AIP) is one

of the immunoglobulin G4 (IgG4)-related diseases and serum IgG4 is known as a useful diagnostic marker. However, the sensitivity of serum IgG4 is variable. The sensitivity of serum IgG4 was not sufficient (68%) in definite type 1 AIP. The demographic findings were not different between SIP and SIN type 1 AIP, but other organ involvement was significantly more common in SIP than in SIN type 1 AIP. High serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration, but did not affect the relapse rate in type 1 AIP.

Paik WH, Ryu JK, Park JM, Song BJ, Park JK, Kim YT, Lee K. Clinical and pathological differences between serum immunoglobulin G4-positive and -negative type 1 autoimmune pancreatitis. *World J Gastroenterol* 2013; 19(25): 4031-4038 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4031.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4031

INTRODUCTION

Autoimmune pancreatitis (AIP) is a type of chronic pancreatitis with irregular narrowing of the pancreatic duct and systemic fibroinflammatory disease. AIP is characterized by a remarkable response to steroid therapy. According to a multicenter nationwide study in Korea, the prevalence of AIP was 2.0% among 814 patients with chronic pancreatitis[1]. An early report from Japan that proposed the term lymphoplasmacytic sclerosing pancreatitis (LPSP) described some specific morphological features of AIP, such as diffuse lymphoplasmacytic infiltration with marked interstitial fibrosis and obliterative phlebitis[2].

The Japan Pancreas Society proposed diagnostic criteria for the first time in 2002, and the characteristic features of AIP were defined as the elevation of serum immunoglobulin G4 (IgG4) and LPSP on pathology[3]. However, emerging evidence suggests the presence of two AIP types that have different clinical profiles and outcomes. In 2003, a Mayo clinic group found two distinct histological patterns, which were designated LPSP and idiopathic duct-centric chronic pancreatitis (IDCP)[4]. IDCP was characterized by inflammatory infiltrates that were denser in the lobules than in interlobular fibrotic areas.

Recently, the expert panel in the international consensus study has agreed that there are two distinct histopathological types of AIP[5]. Type 1 AIP has dense periductal lymphoplasmacytic infiltrate with storiform fibrosis and obliterative phlebitis, whereas type 2 is distinguished from type 1 by granulocyte epithelial lesions, less prominent lymphoplasmacytic infiltrate, and less prominent storiform fibrosis. Recently, international consensus diagnostic criteria (ICDC) for AIP were developed based on the agreement of an international panel of experts and ICDC include both types 1 and 2 AIP[6].

According to the ICDC, the radiological imaging and the response to steroids are common features of both types 1 and 2 AIP. However, typical serological abnormalities, such as serum IgG4 elevation and other organ involvement, can be seen only in type 1. Thus, for a definitive diagnosis of type 2 AIP, histological confirmation is always necessary. Type 2 AIP is associated with inflammatory bowel disease and affects younger patients without a gender predilection[7]. Both types of AIP respond to steroid very well, but type 2 AIP has a lower relapse rate than type 1 AIP[7].

Although elevation of serum IgG4 is the one of the characteristic features in type 1 AIP, the sensitivity of serum IgG4 is variable. The initial Japanese study reported that the sensitivities of IgG4 were 90.9%[8]. However, other studies reported the sensitivity of IgG4 as approximately 70.0%[9-12]. The problem of the previous studies was that there was no clear classification of AIP type because the study was performed before the concept of type 2 AIP was established. If the study population had included more type 2 AIP, the sensitivity of IgG4 would have been low. However, a recent multicenter study also showed that the sensitivity was only 63.0% among histologically proven type 1 AIP[13]. Type 1 AIP is considered as the pancreatic manifestation of IgG4-related systemic disease in which tissue infiltration of IgG4-positive plasma cells is a characteristic feature[14-16]. However, the reason for the variable level of serum IgG4, the relation between serum level and tissue concentration of IgG4, and clinical significance of serum IgG4 level in type 1 AIP is unknown and remains an interesting issue.

The aim of this study was to find clinical and pathological differences between serum IgG4-positive (SIP) and serum IGg4-negative (SIN) type 1 AIP in Korea.

MATERIALS AND METHODS

Patients

From January 2005 to May 2011, all patients with AIP were retrospectively reviewed at the Seoul National University Hospital. The diagnosis of AIP was based on the ICDC[6] and patients without available serum IgG4 level were excluded. Patients with definite AIP were enrolled. The institutional review board of Seoul National University Hospital approved the study.

Definition of AIP type

The histology was obtained before steroid therapy in all cases. If the histology was available, type 1 AIP was defined as LPSP and type 2 as IDCP. The serum IgG4 level was obtained before steroid therapy and tissue acquisition. If tissue was not obtained, type 1 AIP was also defined if the serum IgG4 level was higher than upper limit of normal value (134 mg/dL). If the patients had no or unclear pathological findings and serum IgG4 level was normal, the patients were classified as indeterminate type and excluded in this study.

Radiological analysis

Pancreatic imaging was categorized as diffuse or segmen-

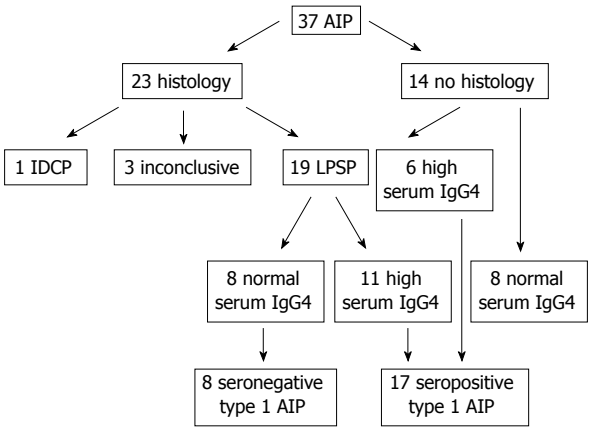

Figure 1 Enrolled patients and classification of autoimmune pancreatitis. Among 37 patients with autoimmune pancreatitis (AIP), one case was type 2 AIP and 19 patients were type 1 AIP by histology. The pathological diagnosis was inconclusive in three cases among 23 tissue samples. Among 14 patients without histology, eight patients were excluded because of normal serum immunoglobulin G4 (IgG4) levels. Ultimately, 25 patients with definite type 1 AIP (19 histologically and six serologically diagnosed cases) were enrolled in this study. LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP: Idiopathic duct-centric chronic pancreatitis.

tal swelling by computed tomography (CT) scan. The presence of extrapancreatic lesions included sclerosing cholangitis, sclerosing sialoadenitis, lymphadenopathy, retroperitoneal fibrosis, and ulcerative colitis. Sclerosing cholangitis was defined as the presence of benign stricture of the bile duct on cholangiography. The stricture of only lower bile duct was not included in sclerosing cholangitis. The presence of sialoadenitis, lymphadenopathy and retroperitoneal fibrosis was determined based on CT findings.

Steroid therapy and relapse

Steroid therapy was done at 0.6 mg/kg per day of prednisolone for one month and gradually tapered to a maintenance dose over three months. Steroid maintenance therapy (5 mg/d) was administered for 6 mo to prevent relapse. Relapse was defined as a recurrence of symptoms with the development of pancreatic or extrapancreatic abnormal findings on imaging studies.

Histological examination

Surgically resected or core biopsied specimens were reviewed by a specialist pathologist without any clinical information. Fine needle aspiration specimens were not considered as available histology and not reviewed. All specimens were stained with anti-IgG4 antibody for immunohistochemical examination. The number of IgG4-positive plasma cells was counted in a high power field (HPF). In surgical specimens, LPSP was defined with at least three of the four characteristic features which are (1) dense infiltration of plasma cells and lymphocytes, particularly periductal; (2) peculiar storiform fibrosis; (3) venulitis with lymphocytes and plasma cells often leading to obliteration of the affected veins; and (4) abundant (> 10 cells per HPF) IgG4-positive plasma cells. In biopsy specimens, AIP was considered with lymphoplasmacytic infiltration with fibrosis and abundant (> 10 cells per HPF) IgG4-positive plasma cells.

Statistical analysis

Statistical analysis was done with statistical software (SPSS version 19.0 for Windows, SPSS Inc, Chicago, IL, United States; MedCalc version 11.5.0.0, MedCalc Software, Mariakerke, Belgium). The data were compared between two groups. The analysis was performed using Student's *t* test, Fischer's exact test and Mann-Whitney's *U* test. A *P* value of < 0.05 was considered statistically significant. As repeated comparisons were made, *P* values of less than 5% ($P < 0.05$) were considered significant.

RESULTS

Enrolled patients and classification of AIP

Thirty seven patients with AIP were enrolled and histology was available for 23 patients (Figure 1). Among 23 patients with histology, 19 patients showed typical finding of type 1 AIP and were confirmed as type 1 AIP. Only one patient was histologically confirmed as a type 2 AIP and had a history of ulcerative colitis. The pathological diagnosis was inconclusive in three cases among eight core biopsies. One type 2 and three SIN AIP patients with inconclusive pathology were excluded from this study. Among 19 patients with type 1 AIP, 11 patients had high serum IgG4 level and eight patients had normal levels. Among 14 patients without histology, six patients had elevated serum IgG4 levels (146, 213, 250, 279, 300 and 4000 mg/dL) and were included in type 1 AIP. Another eight patients with normal serum IgG4 levels were classified as indeterminate AIP and excluded from this study. Ultimately, 17 patients with SIP type 1 AIP and eight patients with SIN type 1 AIP were enrolled in this study. The median age was 61 years (range, 33-84 years) and males were predominant (72%). The sensitivity of serum IgG4 was 68.0%.

Comparison of SIP and SIN type 1 AIP

The mean age of the two groups was similar (62 years *vs* 60 years in SIP and SIN type 1 AIP) and there was no difference in sex between two groups (Table 1). The diffuse type of AIP seemed to be more common in the SIP than in the SIN group (47% *vs* 31%) but the difference was not significant ($P = 0.39$). The median serum IgG4 level was 312 mg/dL (normal range, 145-4000 mg/dL) in the SIP group and was 33 mg/dL (normal range, 6-75 mg/dL) in the SIN group and the difference was significant ($P = 0.03$). The patients of the SIP group were more likely to have other organ involvement than those of the SIN group (59% *vs* 26%, $P = 0.016$). Among the SIP group, sclerosing cholangitis was the most common (four cases) and sialoadenitis was also common (three cases) as other organ involvement. Retroperitoneal fibrosis, mediastinal lymphadenitis and lacrimal gland also represented other organ involvements. Among the SIN group, one patient

Table 1 Comparison of clinical characteristics of serum immunoglobulin G4-positive and negative type 1 autoimmune pancreatitis patients *n* (%)

Variables	SIP	SIN	*P* value
Patients	17	8	
Mean age, yr	62 (33-84)	60 (42-72)	0.359
Sex (M/F)	13:4	5:3	0.172
Diffuse type	8 (47)	3 (31)	0.390
Median serum IgG4 (mg/dL)	312 (145-4000)	33 (6-75)	0.030
Other organ involvement	10 (59)	1 (26)	0.016
Histologic examination			
Resection	5 (26)	6 (75)	0.018
Biopsy	6 (32)	2 (25)	
Not done	6 (32)		
Mean follow up, mo	30	16	0.075
Relapse	6 (35)	2 (25)	0.850

SIP: Serum immunoglobulin G4 (IgG4)-positive; SIN: Serum IgG4-negative; F: Female; M: Male.

had retroperitoneal fibrosis. Only one patient with sclerosing cholangitis was pathologically confirmed as an other organ involvement and other patients were diagnosed with only image and steroid responsiveness. The surgical resection rate was higher in the SIN than in the SIP group (75% *vs* 26%, $P = 0.018$). The mean follow up duration was not different between the two groups (30 mo *vs* 16 mo in SIP and SIN groups, $P = 0.075$). All patients, except those who received surgical resection, received steroid treatment and the response rate was 100% in both SIP and SIN groups. The relapse rate was not different between the two groups (36% *vs* 25% in SIP and SIN group, $P = 0.80$). The mean interval from steroid treatment and relapse was not different between the two groups (14 mo *vs* 11 mo in SIP and SIN groups, $P = 0.82$).

Correlation between serum and tissue IgG4 concentrations

Among the 25 patients with type 1 AIP, 19 patients had tissue specimens, which included 11 SIP and 8 SIN groups. The mean tissue IgG4 concentrations were significantly higher in the SIP than the SIN group (40 cells per HPF *vs* 18 cells per HPF, $P = 0.02$). Among eight SIN patients, the tissue IgG4 concentrations were less than 15 cells per HPF in most of cases, except one (Figure 2). Among 11 SIP patients, the tissue IgG4 concentrations were more than 25 cells per HPF, except for one case (15 cells per HPF). However, there was no linear correlation between serum and tissue IgG4 concentration among the 11 SIP patients.

Clinical features of eight patients with SIN type 1 AIP

The clinical features of eight patients with SIN type 1 AIP are summarized in Table 2. Three cases were typical diffuse type AIP. However, surgical resection was done in two cases because serum IgG4 was normal and the possibility of malignancy could not be excluded in the early period (2005). For four cases with segmental type, surgical resections were performed because the possibility of malignancy could not be excluded by imaging at that time. Only one patient had retroperitoneal fibrosis and experienced disease relapse. Six patients who received surgical resection could be confirmed as type 1 AIP with LPSP (level 1 criterion) and level 1/2 parenchymal imaging. One patient (case 3) had level 1 parenchymal imaging and level 2 histology. The other patient (case 4) could be diagnosed as type 1 AIP with level 1 ductal imaging, level 2 histology and response to steroids.

Table 2 Clinical features of eight patients with serum immunoglobulin G4-negative type 1 autoimmune pancreatitis

Patients	Age/sex	Tissue	Image	OOI	Serum IgG4 (mg/dL)	Tissue IgG4 in HPF	Relapse
Case 1	72/F	Resection	Diffuse	No	75	5	No
Case 2	42/M	Resection	Diffuse	No	26	5	Yes
Case 3	71/F	Biopsy	Diffuse	RF	33	15	Yes
Case 4	61/M	Biopsy	Tail	No	39	11	No
Case 5	61/M	Resection	Body	No	43	80	No
Case 6	51/M	Resection	Tail	No	21	5	No
Case 7	66/M	Resection	Head	No	6	12	No
Case 8	53/F	Resection	Body	No	11	12	No

OOI: Other organ involvement; RF: Retroperitoneal fibrosis; HPF: High power field; F: Female; M: Male; IgG4: Immunoglobulin G4.

One patient had a relatively high tissue IgG4 concentration (80 cells per HPF) despite a low serum IgG4 level (43 mg/dL). He was 61-year-old male and a mass was detected incidentally at the body of the pancreas. Magnetic resonance image (MRI) findings also showed a slightly exophytic mass of iso-attenuation at the body of the pancreas with distal parenchymal atrophy and an abrupt cutting of the pancreatic duct was noticed with upstream ductal dilatation (Figure 3). Image findings were compatible with pancreatic cancer and a distal pancreatectomy was performed. Gross pathological finding showed a 1.3 cm × 1.2 cm × 3 cm sized white solid mass with uncertain margins. Microscopy showed dense periductal lymphoplasmacytic infiltration, storiform fibrosis and obliterative phlebitis (Figure 4A). The IgG4 immunohistochemistry also showed dense infiltration (80 cells per HPF) (Figure 4B). After the operation, he did not develop any symptoms or signs of recurrence for 3 years of follow-up.

DISCUSSION

IgG4-related disease was recognized as a systemic disease since 2003[17] and AIP was proposed as one of the IgG4-related sclerosing diseases in 2006[18]. Since then, two histopathological subtypes, LPSP and IDCP, have been recognized[19]. Type 1 AIP is now considered as the pancreatic manifestation of an IgG4-related systemic fibroinflammatory diseases involving the salivary gland, bile duct, and retroperitoneum. Thus, serum IgG4 is a useful marker for the diagnosis of type 1 AIP and most diagnostic criteria of AIP include serum IgG4 elevation as one of the criteria[6,9,20]. However, the sensitivity of serum IgG4 is variable and different among countries. If the

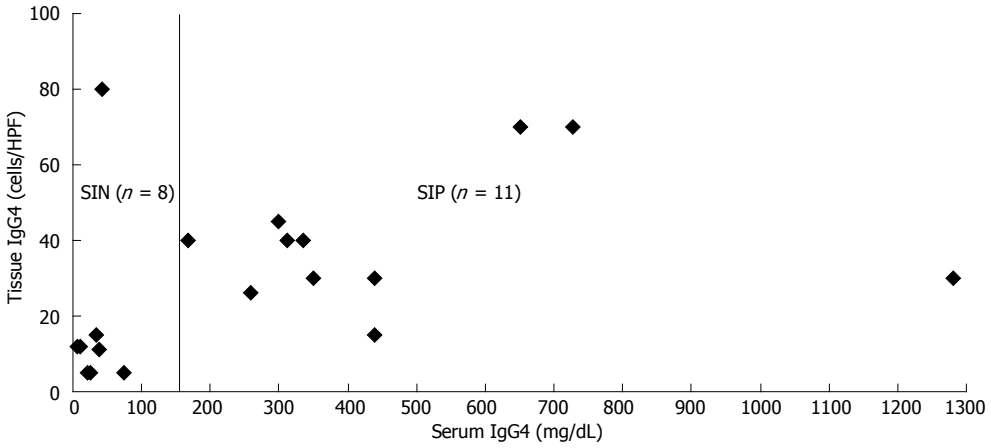

Figure 2 Correlation between serum and tissue immunoglobulin G4 concentrations. Among eight serum immunoglobulin G4 (IgG4)-negative (SIN) patients, the tissue IgG4 concentrations were less than 15 cells per high power filed (HPF) in most of cases, except one. Among 11 serum IgG4-positive (SIP) patients, the tissue IgG4 concentrations were more than 25 cells per HPF, except one case (15 cells per HPF). There was no linear correlation between serum and tissue IgG4 concentration among the 11 SIP patients.

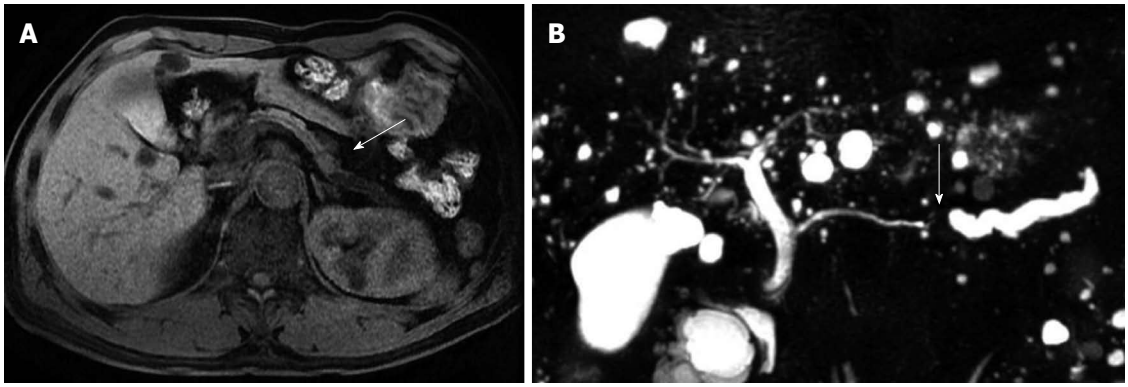

Figure 3 Magnetic resonance image of 61-year-old male patient with normal serum immunoglobulin G4. A: Magnetic resonance image shows slightly exophytic mass of iso-attenuation at the body of pancreas; B: Distal parenchymal atrophy and abrupt cutting of pancreatic duct with upstream ductal dilatation.

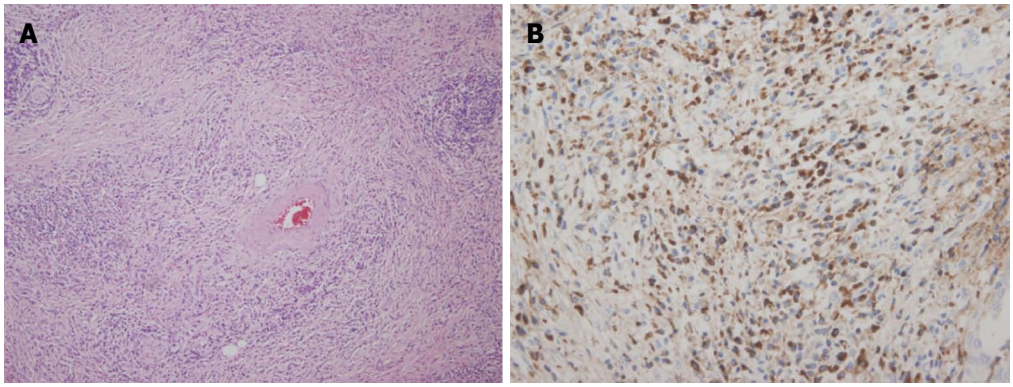

Figure 4 Histology and immunoglobulin G4 immunohistochemical staining. A: Hematoxylin and eosin staining shows typical finding of lymphoplasmacytic sclerosing pancreatitis (× 200); B: Immunoglobulin G4 (IgG4) staining shows dense infiltration of IgG4 positive cells (× 400).

study population includes more type 2 AIP, the sensitivity of IgG4 may be low because serum IgG4 is not usually elevated in type 2 AIP. A recent international multicenter study, which enrolled 713 patients with AIP from eight countries, reported that sensitivity of serum IgG4 was only 63% among 204 patients with histologically proven type 1 AIP[13]. The relatively low sensitivity of serum IgG4 can make the diagnosis of AIP in the clinical setting confusing. In our study, four patients with segmental type AIP underwent unnecessary surgical resection.

We questioned why the serum IgG4 test was not sufficiently sensitive if type 1 AIP is a type of IgG4-related

systemic disease. Therefore, we conducted our study to analyze the clinical and pathological differences between SIP and SIN type 1 AIP. Unfortunately, there have been few studies concerning the normal serum IgG4 AIP[21,22]. One study included 58 AIP patients including 13 normal serum IgG4 AIP[21] but histology was available in only 14 cases (six cases among 13 SIN AIP). Another study included 27 patients with AIP, including seven SIN AIP[22]. Histology was not available in any cases because endoscopic ultrasonography guided fine needle aspiration was performed in 26 cases using 22 gauge needle, not to diagnose AIP, but to exclude pancreatic malignancy. Thus, it could not be determined that all of the enrolled patients were really type 1 AIP in both studies. To exclude possible type 2 AIP, our study enrolled 19 patients with histologically proven type 1 AIP and six patients who were clinically diagnosed as type 1 AIP with elevated serum IgG4 levels. Of course, there is the possibility of type 2 AIP despite the elevated serum IgG4 level among six patients, because serum IgG4 elevation was detected in 23% among 47 patients with histologically proven type 2 AIP according to a recent study[13]. However, the possibility might be very low, because the serum IgG4 level was relatively high (213, 250, 279, 300, 4000 mg/dL), except in one case (146 mg/dL) and type 2 AIP was reported to be relatively rare in Asian countries, including South Korea[13]. In addition, five patients had other organ involvement, which is rarely be seen in type 2 AIP[7].

The surgical resection rate was higher in the SIN than the SIP group. One reason could be a difficult diagnosis of AIP. If the lesion is in the body/tail and serum IgG4 is normal, the clinicians would not suspect the possibility of AIP and would not hesitate to perform a surgical resection. Another reason might be selection bias of this study, because we excluded eight patients with normal serum IgG4 and no histology. The eight patients received steroid treatment and their steroid responsiveness was 100%. One patient experienced a relapse.

In this study, the clinical profiles of type 1 AIP were similar to another recent multicenter study including 327 Asian patients[12]. The higher mean age (over 60 year), male predominance, common other organ involvement, especially sclerosing cholangitis and frequent relapse, are common features of Asian patients of AIP that are similar to our study. Interestingly, the important clinical difference between SIP and SIN type 1 AIP was the frequency of other organ involvement. Other organ involvement was significantly more common in SIP than SIN type 1 AIP (59% *vs* 26%). Only one patient among the SIN group had retroperitoneal fibrosis. This result implies that other organ involvement can affect the serum IgG4 level. Mikulicz's disease refers to idiopathic symmetrical swelling of the lacrimal, submandibular gland and is an IgG4-related systemic disease. A recent study reported that the serum IgG4 level is very high (894 mg/dL) in Mikulicz's disease and significantly higher in patients with extrasalivary gland involvement[23]. More frequent other organ involvement in our SIP type 1 AIP is similar to the results of previous studies[21,22,24].

Another reason for variable serum IgG4 levels may be the number of IgG4-positive plasma cells in the tissue. As expected, the mean tissue IgG4 concentration was significantly low in SIN compared with SIP type 1 AIP. All patients in the SIP group had high IgG4 concentrations (over 25 cells per HPF), except one case (15 cells per HPF). However, the patients in the SIN group had very low IgG4 concentrations (below 15 cells per HPF), except one case (80 cells per HPF). The data might lead us to conclude that the IgG4 concentration of pancreatic tissue can influence the sensitivity of serum IgG4 in type 1 AIP. However, the serum level of IgG4 had no correlation with tissue IgG4 concentration in SIP type 1 AIP. Thus the serum level may be influenced by not only tissue concentration, but also other factors, such as the size of the involved pancreas and other organ involvement. We think that this is the first study to investigate the correlation between serum and tissue IgG4 concentration in type 1 AIP.

Other possible clinical roles of serum IgG4, other than as a diagnostic marker, are uncertain and are a interesting issue in type 1 AIP. The clinical use of serum IgG4 may be relevant in three settings: monitoring of therapy, monitoring for disease relapse and prediction of relapse. The large multicenter study in Japan reported that IgG4 levels failed to normalize in 115/182 (63%) of the patients treated with steroids[25]. The study suggested that serial IgG4 levels are helpful in identifying early relapse. However, only 30% of patients with persistent IgG4 elevation relapsed, whereas relapse was also seen in 10% of patients with normal IgG4 levels. The results regarding the value of initial serum IgG4 levels in predicting relapse vary among studies, some reporting higher relapse rate in patients with elevated serum IgG4 levels[22,26], whereas others failed to observe any association[7,27-29]. To clarify the role of serum IgG4 in predicting relapse, type 2 AIP should be excluded in the normal serum IgG4 group, because type 2 AIP is known for rare relapse[7]. The positive study might include some patients with type 2 AIP. In our study, the relapse rate was similar between the two groups of type 1 AIP. Thus, our data supports the view that initial serum IgG4 levels cannot predict relapse in type 1 AIP.

In conclusion, the sensitivity of serum IgG4 was not sufficient (68%) for definite diagnosis of type 1 AIP. The demographic findings were similar between SIP and SIN type 1 AIP, but other organ involvement was significantly more common in SIP than SIN type 1 AIP. High serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration, but did not affect the relapse rate in type 1 AIP.

COMMENTS

Background

Type 1 autoimmune pancreatitis (AIP) is one of the immunoglobulin G4 (IgG4)-related diseases and serum IgG4 is known as a useful diagnostic marker.

However, the sensitivity of serum IgG4 is variable. AIP is a type of chronic pancreatitis with irregular narrowing of the pancreatic duct and systemic fibroinflammatory disease and is characterized by a remarkable response to steroid therapy.

Research frontiers

IgG4-related disease was recognized as a systemic disease in 2003 and AIP was proposed as one of the IgG4-related sclerosing diseases in 2006. Two histopathological subtypes, lymphoplasmacytic sclerosing pancreatitis and idiopathic duct-centric chronic pancreatitis, have been recognized, and type 1 AIP is now considered as the pancreatic manifestation of an IgG4-related systemic fibroinflammatory diseases involving the salivary gland, bile duct, and retroperitoneum. Thus, serum IgG4 is a useful marker for the diagnosis of type 1 AIP and most diagnostic criteria of AIP include serum IgG4 elevation as one of the criteria. However, the sensitivity of serum IgG4 is variable and different among countries.

Innovations and breakthroughs

The sensitivity of serum IgG4 was not sufficient (68%) for defining type 1 AIP. The demographic findings were similar between serum IgG4-positive (SIP) and serum IgG4-negative (SIN) type 1 AIP, but other organ involvement was significantly more common in SIP than SIN type 1 AIP. High serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration, but did not affect the relapse rate in type 1 AIP.

Peer review

The authors compared the clinical and pathological differences between serum IgG4-positive and IgG4-negative type 1 autoimmune pancreatitis and demonstrated that the sensitivity of serum IgG4 was 68% in type 1 AIP. The high serum IgG4 level was associated with other organ involvement and tissue IgG4 concentration, but did not affect the relapse rate in type 1 AIP.

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BRIEF ARTICLE

Massive presacral bleeding during rectal surgery: From anatomy to clinical practice

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Abstract

AIM: To investigate control of two different types of massive presacral bleeding according to the anatomy of the presacral venous system.

METHODS: A retrospective review was performed in 1628 patients with middle or low rectal carcinoma who were treated surgically in the Department of Colorectal Surgery, Changhai Hospital, Shanghai, China from January 2008 to December 2012. In four of these patients, the presacral venous plexus (n = 2) or basivertebral veins (n = 2) were injured with massive presacral bleeding during mobilization of the rectum. The first two patients with low rectal carcinoma were operated upon by a junior associate professor and the source of bleeding was the presacral venous plexus. The other two patients with recurrent rectal carcinoma were both women and the source of bleeding was the basivertebral veins.

RESULTS: Two different techniques were used to control the bleeding. In the first two patients with massive bleeding from the presacral venous plexus, we used suture ligation around the venous plexus in the area with intact presacral fascia that communicated with the site of bleeding (surrounding suture ligation). In the second two patients with massive bleeding from the basivertebral veins, the pelvis was packed with gauze, which resulted in recurrent bleeding as soon as it was removed. Following this, we used electrocautery applied through one epiploic appendix pressed with a long Kelly clamp over the bleeding sacral neural foramen where was felt like a pit Electrocautery adjusted to the highest setting was then applied to the clamp to "weld" closed the bleeding point. Postoperatively, the blood loss was minimal and the drain tube was removed on days 4-7.

CONCLUSION: Surrounding suture ligation and epiploic appendices welding are effective techniques for controlling massive presacral bleeding from presacral venous plexus and sacral neural foramen, respectively.

Key words: Massive presacral bleeding; Rectal surgery; Suture ligation; Welding

Core tip: Massive presacral bleeding is an uncommon but potentially life-threatening complication of rectal surgery. It is difficult to control the bleeding and several alternative techniques for hemostasis have been proposed. We described the use of two simple and effective techniques for controlling two different types of massive presacral bleeding, classified according to the anatomy of the presacral venous system.

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INTRODUCTION

Massive presacral bleeding is a potentially life-threatening complication of rectal surgery and remains one of the most challenging intraoperative emergencies to colorectal surgeons[1,2]. The incidence and the mortality have been reported as high as 9.4% and 4.3%, respectively[3,4]. Total mesorectal excision was introduced in 1982 and is considered the gold standard with an acceptable intraoperative risk for rectal carcinoma[5]. However, massive presacral bleeding remains inevitable, especially in recurrent rectal carcinoma or in operations performed by junior colorectal surgeons. Several hemostatic techniques for controlling this intraoperative emergency have been proposed, such as the use of thumbtacks, bone wax, balloon tamponade, and endoscopic stapling[6-8]. However, some techniques fail to arrest the bleeding[9], resulting in shock and even death.

Based on the anatomy of presacral venous system, massive presacral bleeding can be divided into two different types. In our opinion, the key feature in controlling massive presacral bleeding is correct judgment of the bleeding type. Here, we report our experience with massive presacral bleeding and describe the use of two simple and effective techniques (surrounding suture ligation and epiploic appendices welding) for controlling two different types of massive presacral bleeding according to the anatomy of the presacral venous system. To the best of our knowledge, there are no reports of this hemostatic strategy in the literature.

MATERIALS AND METHODS

This was a retrospective review of 1628 patients with middle or low rectal carcinoma who were treated surgically in the Department of Colorectal Surgery, Changhai Hospital, Shanghai, China from January 2008 to December 2012 (Table 1). All the patients who sustained massive presacral bleeding during mobilization of the rectum were recorded.

In four of these patients, the presacral venous plexus (n = 2) or basivertebral veins (n = 2) were injured. The first two patients (a 63-year-old woman and a 58-year-old man) with low rectal carcinoma were operated upon by a junior associate professor and the source of bleeding was the presacral venous plexus. The other two patients with recurrent rectal carcinoma were both women (aged 69 and 72 years, respectively). The rectal stumps were found to be densely adherent to the surrounding structures and the source of bleeding was the basivertebral veins. Two different techniques were used to control the bleeding.

RESULTS

In the first patient with massive bleeding from the presacral venous plexus, suture ligation was used initially to control the bleeding, which exacerbated the bleeding. As an alternative, the pelvis was packed with gauze, which resulted in recurrent bleeding as soon as the packing was removed. Following this, we tried to perform suture ligation around the venous plexus in the area with intact presacral fascia that communicated with the bleeding site (surrounding suture ligation). The bleeding stopped after 11 attempts at suture ligation. The patient underwent a super-low anterior resection with protective ileostomy. The estimated blood loss was 2000 mL. In the second patient with massive bleeding from the presacral venous plexus, we used the same technique to control bleeding with eight attempts at surrounding suture ligation. The estimated blood loss was 800 mL.

Table 1 Adjuvant therapy for low rectal dissection in 1628 patients *n* (%)

Adjuvant therapy	Patients	Patients with bleeding
Rectal carcinoma	1606	2 (0.12)
Without neoadjuvant therapy	1463	2 (0.14)
With neoadjuvant radiotherapy	89	0 (0.00)
With neoadjuvant chemotherapy	29	0 (0.00)
With neoadjuvant radiochemotherapy	25	0 (0.00)
Recurrent rectal carcinoma	22	2 (9.00)
With preoperative radiotherapy	6	0 (0.00)
With preoperative chemotherapy	1	0 (0.00)
With preoperative radiochemotherapy	3	0 (0.00)
Without preoperative radiotherapy	12	2 (16.7)

In the following two patients with massive bleeding from the basivertebral veins, the pelvis was packed with gauze, which resulted in recurrent bleeding as soon as it was removed. In the first of these patients, we tried to control bleeding by surrounding suture ligation initially. However, this technique was unsuccessful. Following this, we used electrocautery applied through one epiploic appendix pressed with a long Kelly clamp over the bleeding sacral neural foramen where was felt like a pit. First, fingertip pressure was applied directly to the sacral neural foramen to control the bleeding. Then, one epiploic appendix, 1-2 cm in diameter, was excised and mounted on a long Kelly clamp. The finger was rapidly withdrawn and the epiploic appendices pressed directly over the sacral neural foramen. Electrocautery adjusted to the highest setting was applied to the clamp to "weld" closed the bleeding point. The bleeding stopped after 3 min. In this patient, blood loss was 6000 mL. In the last patient with massive bleeding from the basivertebral veins, we used the same technique to control bleeding within 10 min. The estimated blood loss was 600 mL. Intraoperative data are shown in Table 2.

Postoperatively, the blood loss was minimal and the drain tube was removed on days 4-7. Patients were discharged on day 8 and they all returned for ileostomy reversal 3 mo later.

DISCUSSION

Massive presacral bleeding is considered to be an intraoperative emergency during rectal surgery. The anatomy of the presacral venous system makes it vulnerable to serious bleeding that can often be difficult to control[10]. The

Table 2 Patients with massive presacral bleeding

Patient	Sex	Age (yr)	TNM stage	Surgical procedure	Blood loss (mL)	Procedures used to control bleeding	Postoperative complication
1	Female	63	T4N0M0	AR + ileostomy	2000	Surrounding suture ligation	None
2	Male	58	T4N1M0	AR + ileostomy	800	Surrounding suture ligation	None
3	Female	69	Recurrent	AR + ileostomy	6000	Epiploic appendices welding	None
4	Female	72	Recurrent	APR	600	Epiploic appendices welding	None

TNM: Tumor-node-metastasis; AR: Anterior resection; APR: Abdominoperineal resection.

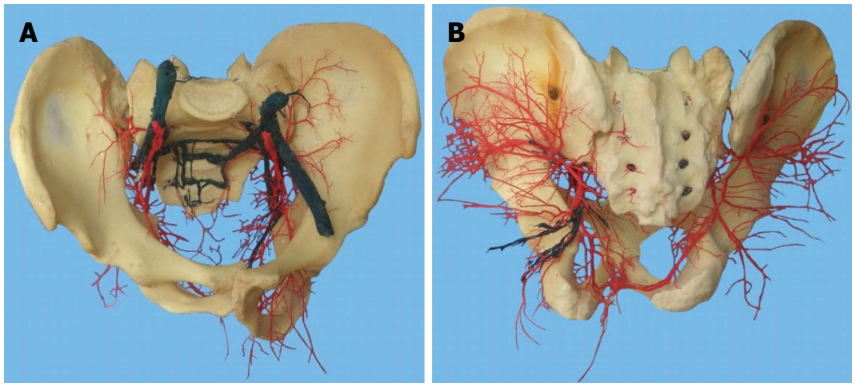

Figure 1 Presacral vascular cast. A: Front view; B: Dorsal view.

presacral venous plexus runs into the pelvic fascia that covers the anterior aspect of the sacrum. It is formed by the two lateral sacral veins, the middle sacral vein, and the in-between communicating veins. These veins are avalvular and communicate *via* the basivertebral veins with the internal vertebral venous system (Figure 1)[11]. Massive presacral bleeding can be divided into two different types according to the anatomy. The first type of bleeding arises from the presacral venous plexus. It may be massive, but can be stopped by suture ligation. The other type is massive, high-pressure bleeding that can be controlled only by pressing the sacrum with the finger or gauze. This type of bleeding originates from the sacral neural foramen where the basivertebral vein is injured[12]. When the patient is in the lithotomy position, the hydrostatic pressure is increased 2-3 times above the pressure in the inferior vena cava[13]. This avalvular system communicates with the vertebral veins, which explains why it is difficult to stop the bleeding.

Intraoperative massive bleeding may be more common during difficult operations in patients with large and fixed tumors, neoadjuvant radiotherapy, and recurrent rectal carcinoma[14]. The rate of massive presacral bleeding was higher in patients with recurrent rectal carcinoma than in those with rectal carcinoma (9.0% *vs* 0.12%, P = 0.001). The higher incidence of this emergency in patients with recurrent rectal carcinoma might be related to the more difficult dissection that results from fibrosis and anatomical disruption in this area. The expectation that resection of locally advanced tumors carries a higher risk of presacral vessel lesions was not confirmed in our study because we studied a small number of cases in a single institution.

Incorrect pelvic contraction or inappropriate manipulation is the most common cause of injury to the presacral venous plexus in patients without the above common contributing factors. In this series, two operations with massive presacral bleeding were performed by a junior associate professor. Initial suture ligation was performed incorrectly, which caused more massive bleeding and blood loss was estimated at 2000 mL. Massive bleeding was eventually controlled using or surrounding suture ligation technique. Blood loss was likely related to the extent and site of intraoperative vessel injury, the specific management of the bleeding, and the expertise of the surgeon[1].

The main clinical characteristics of massive presacral bleeding include: (1) bleeding that occurs suddenly during mobilization of the rectum, which can quickly lead to hemorrhagic shock and even result in death; (2) gushing of blood from the pelvic floor, which makes the bleeding site undetectable; (3) ligation of the internal iliac vessel is futile; and (4) bleeding does not stop, even in hemorrhagic shock. According to previous reports, blood loss in presacral bleeding ranges between 300 and 7800 mL[12]. Most of these patients need blood transfusion. In our study, blood loss ranged between 600 and 6000 mL (mean, 2350 mL), and three patients needed blood transfusions. In patients with colorectal carcinoma undergoing surgery, blood transfusion is associated with adverse clinical outcomes, including increased mortality[15]. Therefore, it is important to use a simple and effective procedure to control massive presacral bleeding in rectal surgery.

In our experience, whenever massive presacral bleeding occurs, the first step is direct pressure with the finger at the bleeding point. At the same time, surgeons should inform an anesthetist to prepare sufficient blood. When the bleeding point cannot be exposed clearly, gauze should be pressed directly over the presacral area and the pressure maintained for 15-20 min. The blood surround-

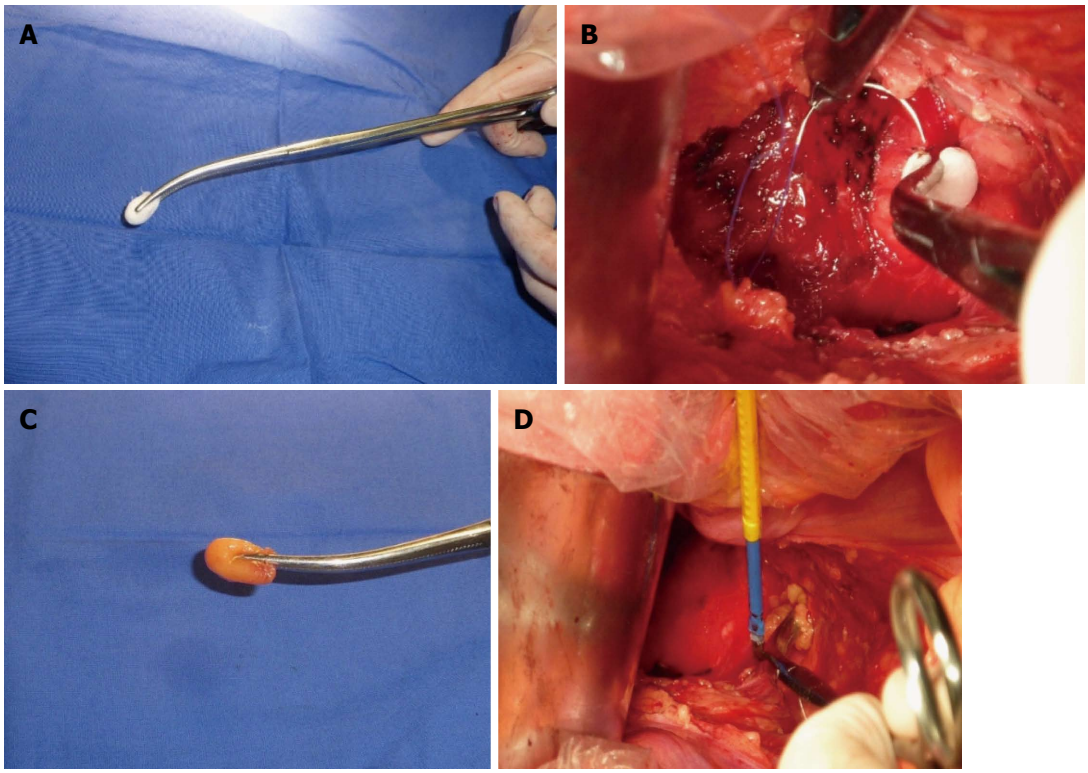

Figure 2 Bleeding point originated from the presacral venous plexus and a sacral neural foramen where the basivertebral veins were injured. A: Continuous pressure over the bleeding site using a gauze nut at the tip of a long Kelly clamp; B: Venous branches surrounding the gauze nut could be identified, and were suture ligated one by one with 3-0 suture thread; C: Continuous pressure over the bleeding site using the epiploic appendices at the tip of a long Kelly clamp; D: Electrocautery applied through the epiploic appendices pressed with a long Kelly clamp over the bleeding vessel.

ing the gauze should be removed by suction. If possible, the specimen should be removed to achieve better exposure. Next, the surgeon should remove the packing gauze, slowly exposing the bleeding point. The bleeding type should been distinguished as soon as possible and an appropriate hemostatic technique can be deployed.

In the first type of massive presacral bleeding, the bleeding point originates from the presacral venous plexus. In our experience, appropriate suture ligation remains an effective method to control this type of bleeding. It should be performed by an experienced surgeon, maintaining continuous pressure over the bleeding site using a gauze nut at the tip of a long Kelly clamp. Surgeons should continue to mobilize the rectum to achieve better exposure if possible. In cases in which the venous branches surrounding the gauze nut can be identified, they are suture ligated one by one with suture thread (VCP772D; Ethicon). Importantly, the suture-ligated tissues should include the presacral fascia, presacral veins, and deep connective tissues. Suture ligation should be performed where the presacral fascia is intact. Jiang *et al*[16] have reported that circular suture ligation of the venous plexus in the area with intact presacral fascia that surrounds the bleeding site is an effective and simple technique to control presacral venous bleeding. In the present study, this type of bleeding was successfully controlled in two patients by the surrounding suture ligation technique (Figure 2A and B).

However, there are several limitations to the surrounding suture ligation technique. First, it can be difficult for bleeding occurring at the bottom of a narrow pelvis, which is typical in patients with obesity[16]. Second, previous rectal surgery can lead to fibrosis of the presacral area, which increases the difficulty in identification of presacral vein distribution and suture ligation. Assessment of the vein locations according to the typical pattern of vein distribution could be wrong, leading to failure of bleeding control. We performed surgery for recurrent rectal carcinoma in two cases. Surrounding suture ligation technique was ineffective because vessel distribution was difficult to identify. Lastly, for bleeding coming from a retracted vein inside the sacrum, other techniques may be used to control the massive bleeding.

In the second type of massive presacral bleeding, the bleeding point originates from a sacral neural foramen where the basivertebral veins are injured.

Harrison *et al*[17] have reported a technique of muscle fragment welding to control presacral bleeding during rectal mobilization. We performed this technique in two cases whose bleeding points originated from a sacral neural foramen during surgery for recurrent rectal carcinoma. This type of bleeding was effectively controlled using electrocautery applied through the epiploic appendices pressed with a long Kelly clamp over the bleeding vessel (Figure 2C and D). Compared with the technique of muscle fragment welding, it is easier to excise one epiploic appendix than a muscle fragment. Because of the round shape of the epiploic appendices, it is easier to fill

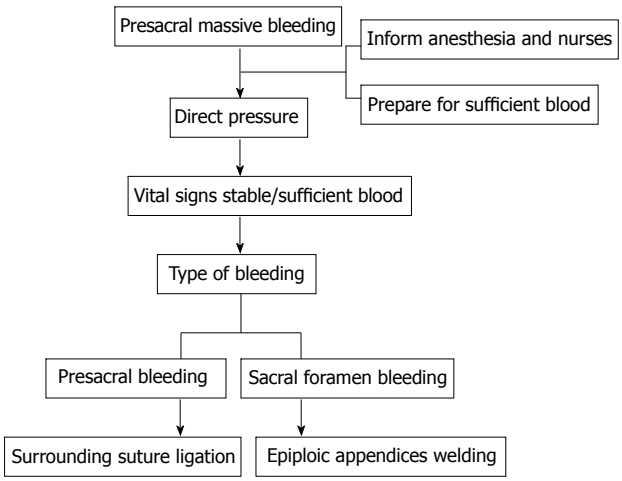

Figure 3 Process in the management of massive presacral bleeding.

the sacral neural foramen. The cauterized epiploic appendices usually adhere to the presacral tissue as a charred coagulum. Hemostasis was immediate and permanent, and no major complications were noted. This technique is intended to deliver heat energy through the forceps to the epiploic appendices. The epiploic appendices act primarily as a fluid-containing electrode that allows conduction of energy and heat to the basivertebral veins. The temperature increases gradually and coagulation is achieved. As in a previous study, necrosis and subsequent abscess development were not seen in our patients, and this may be related to the hypervascular nature of the presacral area and revascularization of the small segment of the epiploic appendices[18].

Alternative methods have been described in the literature. Pelvic packing effectively controls massive presacral bleeding. Intra-abdominal packing should be familiar to colorectal surgeons because when other attempts to provide hemostasis fail, it can be the last resort to control life-threatening bleeding[19]. Packing gauze must be carefully removed at a planned second laparotomy when the patient has stabilized hemodynamically. However, there is a risk of infection or secondary complication from foreign bodies.

Nowadays hemostatic agents are readily available. Some authors have reported that they are effective in stopping bleeding from presacral veins[20-30]. However, in our experience, they are ineffective in stopping massive presacral bleeding. Hemostatic agents may be considered in cases of little bleeding when other techniques have failed.

In conclusion, surrounding suture ligation and epiploic appendices welding are safe, readily available, and highly effective techniques for controlling massive presacral bleeding from the presacral venous plexus and sacral neural foramen, respectively (Figure 3).

COMMENTS

Background

Massive presacral bleeding is a potentially life-threatening complication of rectal surgery and remains one of the most challenging intraoperative emergencies. The incidence and mortality have been reported to be as high as 9.4% and 4.3%, respectively. Total mesorectal excision was introduced in 1982 and is considered a gold standard with an acceptable intraoperative risk during surgery for rectal carcinoma. However, massive presacral bleeding remains inevitable, especially in surgery for recurrent rectal carcinoma or during operations performed by junior colorectal surgeons.

Research frontiers

Several hemostatic techniques for controlling this intraoperative emergency have been proposed, such as the use of thumbtacks, bone wax, balloon tamponade, and endoscopic stapling. However, some techniques fail to arrest the bleeding, resulting in shock and even death.

Innovations and breakthroughs

Based on the anatomy of the presacral venous system, massive presacral bleeding can be divided into two different types. The key factor in controlling massive presacral bleeding is correct assessment of the bleeding type. This article reports two simple and effective techniques for controlling two different types of massive presacral bleeding, classified according to the anatomy of the presacral venous system. According to the authors, there are no reports of this hemostatic strategy in the literature.

Applications

Surrounding suture ligation and epiploic appendices welding are safe, readily available, and highly effective techniques for controlling massive presacral bleeding from the presacral venous plexus and sacral neural foramen, respectively.

Terminology

Presacral bleeding is considered to be an intraoperative emergency in rectal surgery. The anatomy of the presacral venous system makes it vulnerable to serious bleeding that can often be difficult to control.

Peer review

The literature review indicates a large body of work on presacral bleeding already. Often a greater number of techniques described equates to a lack of a gold standard of care, which can be problematic in whatever field. This article outlines two useful and effective techniques to deal with this severe, although not frequent, complication of rectal surgery. The results are interesting and suggest that surrounding suture ligation and epiploic appendices welding for controlling two different types of massive presacral bleeding are simple and effective techniques.

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BRIEF ARTICLE

Recurrent abdominal liposarcoma: Analysis of 19 cases and prognostic factors

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Abstract

AIM: To evaluate the clinical outcome of re-operation for recurrent abdominal liposarcoma following multidisciplinary team cooperation.

METHODS: Nineteen consecutive patients who had recurrent abdominal liposarcoma underwent re-operation by the retroperitoneal sarcoma team at our institution from May 2009 to January 2012. Patient demographic and clinical data were reviewed retrospectively. Multidisciplinary team discussions were held prior to treatment, and re-operation was deemed the best treatment. The categories of the extent of resection were as follows: gross total resection (GTR), palliative resection and partial resection. Surgical techniques were divided into discrete lesion resection and combined contiguous multivisceral resection (CMR). Tumor size was determined as the largest diameter of the specimen. Patients were followed up at approximately 3-monthly intervals. For survival analysis, a univariate analysis was performed using the Kaplan-Meier method, and a multivariate analysis was performed using the Cox proportional hazards model.

RESULTS: Nineteen patients with recurrent abdominal liposarcoma (RAL) underwent 32 re-operations at our institute. A total of 51 operations were reviewed with a total follow-up time ranging from 4 to 120 (47.4 ± 34.2) mo. The GTR rate in the CMR group was higher than that in the non-CMR group (P = 0.034). CMR was positively correlated with intra-operative bleeding (correlation coefficient = 0.514, P = 0.010). Six cases with severe postoperative complications were recorded. Patients with tumor sizes greater than 20 cm carried a significant risk of profuse intra-operative bleeding (P = 0.009). The ratio of a highly malignant subtype (de-differentiated or pleomorphic) in recurrent cases was higher compared to primary cases (P = 0.027). Both single-factor survival using the Kaplan-Meier model and multivariate analysis using the Cox proportional hazards model showed that overall survival was correlated with resection extent and pathological subtype (P < 0.001 and P = 0.02), however, relapse-free interval (RFI) was only correlated with resection extent (P = 0.002).

CONCLUSION: Close follow-up should be conducted in patients with RAL. Early re-operation for relapse is preferred and gross resection most likely prolongs the RFI.

Key words: Overall survival; Recurrent abdominal liposarcoma; Relapse-free interval

Core tip: Recurrent abdominal liposarcoma (RAL) is an intractable disease encountered by both general surgeons and surgical oncologists. RAL commonly affects multiple organs, and re-operation for RAL is often difficult and is associated with significant risk, even when debulking is imminent. The high likelihood of postoperative complications and a lower survival outcome are

detractors for repeat operations. A multidisciplinary team approach, realistic risk stratification, and careful management may help increase the success rate of gross total resection, lower these complication rates, improve survival, and increase the quality of life of these patients. Overall survival, relapse-free interval and other clinical follow-up data are also presented in detail in this study.

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INTRODUCTION

Liposarcoma is the most common retroperitoneal sarcoma[1,2]. It accounts for more than 20% of all sarcomas in adults and up to 41% of all retroperitoneal sarcomas[3,4]. Liposarcomas also originate from the mesentery, gastrointestinal wall, and even from solitary organs, which has been reported sporadically[4-11]. Complete surgical resection is the only effective treatment method for retroperitoneal liposarcomas[3,12,13].

However, liposarcomas are associated with a high local recurrence rate[14-16]. Re-operation is the only effective treatment for recurrent abdominal liposarcoma (RAL)[17]. For those who are not amenable to complete radical resection, debulking resection should be performed to relieve symptoms, reduce complications, and increase the life span[18]. However, there is no consensus concerning the utility of repeat debulking resections. RAL commonly affects multiple organs, and re-operation for RAL is often difficult and is associated with significant risk, even when debulking is imminent. The high likelihood of post-operative complications and a lower survival outcome are detractors for repeat operations.

A multidisciplinary team approach, realistic risk stratification, and careful management may help lower these complication rates, improve survival, and increase the quality of life of these patients. We have treated 19 RAL patients over the past 3 years using a multidisciplinary team approach. The clinical and follow-up data of these patients were retrospectively analyzed and summarized.

MATERIALS AND METHODS

Patient enrollment and operation selection

Between May 2009 and Jan 2012, 19 consecutive patients with RAL were treated by the retroperitoneal sarcoma team at our institution. Patients were identified by reviewing a database that accrued data prospectively. Histology was reviewed and classified according to the World Health Organization classification[19,20]. The multidisciplinary team were involved in case discussions which were held prior to treatment, and repeat resection was deemed the best treatment. The multidisciplinary team members included general surgeons, a pathologist, radiologist, oncologist, radiologist, urologist and gynecologist. Multivisceral resection was recommended only in cases of expected gross tumor resection. The operative plan was explained to the patient in detail, and informed consent was obtained before surgery.

Extent of resection

The categories of the extent of resection were as follows: gross total resection (GTR), whether the margin was histologically free or not; palliative resection; and partial resection. Palliative resections were performed when the gross disease could not be completely removed and less than a 1 cm rim of tumor remained. Partial resections were defined as visually more than a 1 cm rim of remaining tumor. Surgical techniques were divided into discrete lesion resection (DLR) and combined contiguous multivisceral resection (CMR). Tumor size was determined as the largest diameter of the specimen.

Clinical data

Patients' demographic and clinical data were reviewed retrospectively and included age, gender, disease onset date, combined resected organ, pathology subtype, tumor size, intra-operative bleeding, post-operative complications, disease relapse date and survival time in order to analyze prognostic factors.

Follow up

Patients were followed-up at approximately 3-mo intervals. The relapse-free interval (RFI) was defined as the time between initial surgery and confirmation of clinical recurrence.

Statistical analysis

The median and standard error were used to present continuous variables. Fisher's test or a crosstab analysis was performed to compare variables between groups. For survival analysis, a univariate analysis was performed using the Kaplan-Meier method, and a multivariate analysis was performed using the Cox proportional hazards model. $P < 0.05$ was considered statistically significant.

RESULTS

Patient clinical characteristics

Nineteen patients with RAL underwent 32 re-operations at our institute. The patient demographic, surgical, and pathological data are summarized in Table 1. A total of 51 operations were reviewed. The recurrences were tracked from Mar 2002 to Aug 2011, with a total follow-up time ranging from 4 to 120 (47.4 ± 34.2) mo.

Surgical treatment

The surgical methods and resection extent are summarized in Table 2. Five of the nineteen patients underwent the primary operation at our institute. The resected or-

Table 1 Patient demographics and clinical data *n* (%)

Variables	Mean/median
Age (yr)	
mean ± SD	55 ± 10.8
Median (range)	58 (34-84)
Gender	
Male	12 (63.2)
Female	7 (36.8)
No. of operations	
Two	11 (57.9)
Three	4 (21.1)
Four	3 (15.9)
Five	1 (5.3)
Follow-up time (mo)	
mean ± SD	48.9 ± 34.8
Range	4-120
Primary tumor location	
Retroperitoneum	13 (68.4)
Mesentery	3 (15.8)
Omentum	1 (5.3)
Small intestine	1 (5.3)

Table 2 Surgical methods and resection extent of primary and recurrent liposarcomas *n* (%)

Variables	DLR	CMR	Total
Primary tumor			
GTR	11 (57.89)	4 (21.05)	15 (78.94)
Palliative resection	3 (15.79)	0 (0.00)	3 (15.79)
Partial resection	1 (5.26)	0 (0.00)	1 (5.26)
Total	15 (78.95)	4 (21.05)	19 (100.00)
Recurrent tumor			
GTR	5 (15.63)	15 (46.88)	20 (62.50)
Palliative resection	2 (6.25)	6 (18.75)	8 (25.00)
Partial resection	3 (9.38)	1 (3.13)	4 (12.50)
Total	10 (31.25)	22 (68.75)	32 (100.00)

DLR: Discrete lesion resection; CMR: Contiguous multivisceral resection; GTR: Gross total resection.

gans included the small intestine (n = 14), colon (n = 11), kidney (n = 8), spleen (n = 7), pancreas (n = 5), stomach, appendix, ovary (n = 3 each), and liver, bladder, testicle, and abdominal wall (n = 1 each). The GTR rate in the CMR group was higher than that in the non-CMR group (P = 0.034). Only one CMR case underwent partial resection. This patient had a spontaneous enterobrosis and therefore required an emergency operation. He lived for 3 mo after this salvage treatment. The median intra-operative blood loss was 500 mL. Thirteen cases had bleeding ranging from 500-4000 (1300 ± 1100) mL; bleeding in 12 of these 13 cases occurred during CMR. CMR was positively correlated with intra-operative bleeding (correlation coefficient = 0.514, P = 0.010). Six cases with severe postoperative complications were recorded. Two cases experienced anastomotic leakage, and the other four experienced either pleural effusion, subdiaphragmatic effusion, abdominal abscess, or an abdominal wall wound infection.

Pathology data

The primary tumor size was recorded in nine patients, including one patient with multiple lesions; the other eight tumors ranged in size from 13-38 (22.6 ± 9.9) cm. A total of 24 relapse cases were observed who had measurable specimens with tumor sizes ranging from 4-46 (27.2 ± 14.5) cm, and 8 cases had multiple lesions. The median size was 20 cm for all specimens. The resection extent, surgical approach, and operative blood loss were compared according to tumor size. The relapse cases were subgrouped by median tumor size when comparing the clinical data with the number of cases. Patients with tumor sizes greater than 20 cm carried a significant risk of profuse intra-operative bleeding (P = 0.009), as detailed in Table 3.

Table 3 Comparison of clinical data according to recurrent tumor size

Tumor size	< 20 cm	> 20 cm	Total
GTR	4	10	14
Palliative resection	3	5	8
Partial resection	2	0	2
DLR	4	2	6
CMR	6	12	18
Bleeding (< 500 mL)	4	6	10
Profuse bleeding (≥ 500 mL)[1]	1	13	14

[1]P = 0.009 between different recurrent tumor size group. DLR: Discrete lesion resection; CMR: Contiguous multivisceral resection; GTR: Gross total resection.

The pathological subtypes were significantly different between recurrent and primary tumors. The subtype frequently changed with each recurrence within the same patient. In this series, well-differentiated and myxoid liposarcomas were more commonly found within the primary tumor; however, dedifferentiated liposarcomas were more common in recurrent tumors. The ratio of a highly malignant subtype (dedifferentiated or pleomorphic) in the recurrent cases was higher compared to the primary cases (5/9 *vs* 23/9, P = 0.027).

Follow-up and survival analysis

Survival was tracked during the follow-up period. Six patients died of their disease after an overall survival (OS) of 8-90 (33.7 ± 29.7) mo. Single-factor survival was analyzed according to surgical method, resection extent, tumor location, tumor size, and pathological subtype of the primary disease. Patients with a GTR of the primary tumor had a longer survival than those with a palliative or partial resection (P = 0.001, Figure 1A). Patients who underwent a CMR at first operation had a slightly longer survival (P = 0.081, Figure 1B). Patients with a primary retroperitoneal liposarcoma had a worse survival than liposarcoma at any other site (mesentery, omentum and small intestine, P = 0.054, Figure 1C). Patients with a less malignant subtype of primary liposarcoma (well differentiated and myxoid cell type) tended to live longer than those with a more highly malignant subtype (dedifferentiated and pleomorphic cell type, P = 0.002, Figure 1D). Multivariate analysis using the Cox proportional hazards model showed that OS correlated with resection extent and pathological subtype (P < 0.001 and P = 0.02).

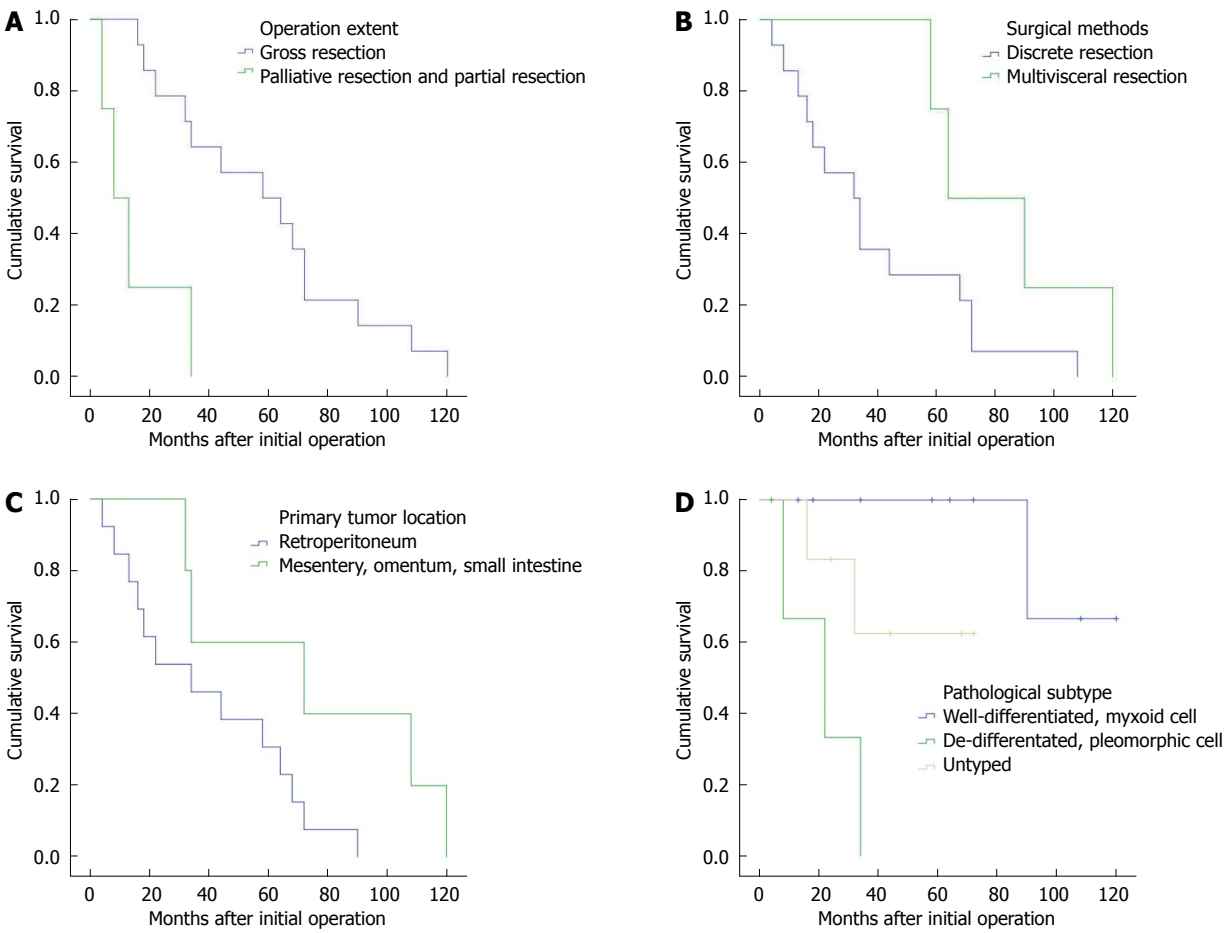

Figure 1 Relationship between overall survival and operation extent (A), surgical methods (B), tumor origin (C), and pathological subtype (D) in patients who underwent resection of a primary abdominal liposarcoma.

The RFI of the primary surgical treatment ranged from 2-84 (22.0 ± 21.2) mo. The RFI differed between GTR patients (6-84/27.0 ± 21.2 mo) and patients who underwent partial or palliative resections (2-4/3.3 ± 1.0 mo, P = 0.001). Eighteen recurrences were observed after a gross or palliative resection for recurrent tumor, and the RFI was 1-28 (8.3 ± 7.4) mo. Of these, 11 were post-GTR (RFI = 4-28/12.5 ± 7.4 mo) and seven were post-palliative resection (RFI = 3-6/4 ± 1.3 mo). Eight post-GTR cases had a follow-up of 3-30 (10.3 ± 10.1) mo with no relapse. Patients who underwent GTR had a longer RFI than those who underwent palliative resection (P = 0.01).

The RFI was compared according to the revision operation time, surgical method, resection extent, primary tumor location, tumor size, simultaneous tumor number, and pathological subtype. The RFI was shorter in patients who underwent more than 2 operations (P = 0.035, Figure 2A). No significant differences in RFI were found between CMR and DLR (P = 0.599, Figure 2B). However, there was a significant difference between GTR cases and non-GTR cases (P < 0.001, Figure 2C). Patients with well-differentiated liposarcomas had a longer RFI compared to those with other liposarcoma subtypes (P = 0.007, Figure 2D). When grouped by median tumor size (20 cm) or simultaneous tumor number (solitary or multiple), no significant difference was observed (P = 0.54, Figure 2E and P = 0.33, Figure 2F). A multivariate analysis using a Cox proportional hazards model showed that the RFI only correlated with resection extent (P = 0.002).

DISCUSSION

Liposarcoma is the most common mesenchymal tumor in the abdomen. To date, surgical resection is the only effective treatment for liposarcoma. Unfortunately, these tumors are almost always very large at the time of diagnosis due to their slow growth and often vague symptoms[11], which make GTR difficult. These tumors are known for their frequent local recurrence and expansive growth with contiguous organ infiltration, which are the main causes of death from this disease. There is no strong evidence that chemotherapy or radiotherapy is curative[21,22]. Re-operation is still the mainstay of treatment, but is associated with significant risk. Using a multidisciplinary team approach, the surgical management of RAL has been improved at our institute. Very few studies have focused on the re-operative treatment of RAL.

In this series, we reviewed 19 patients with RAL who

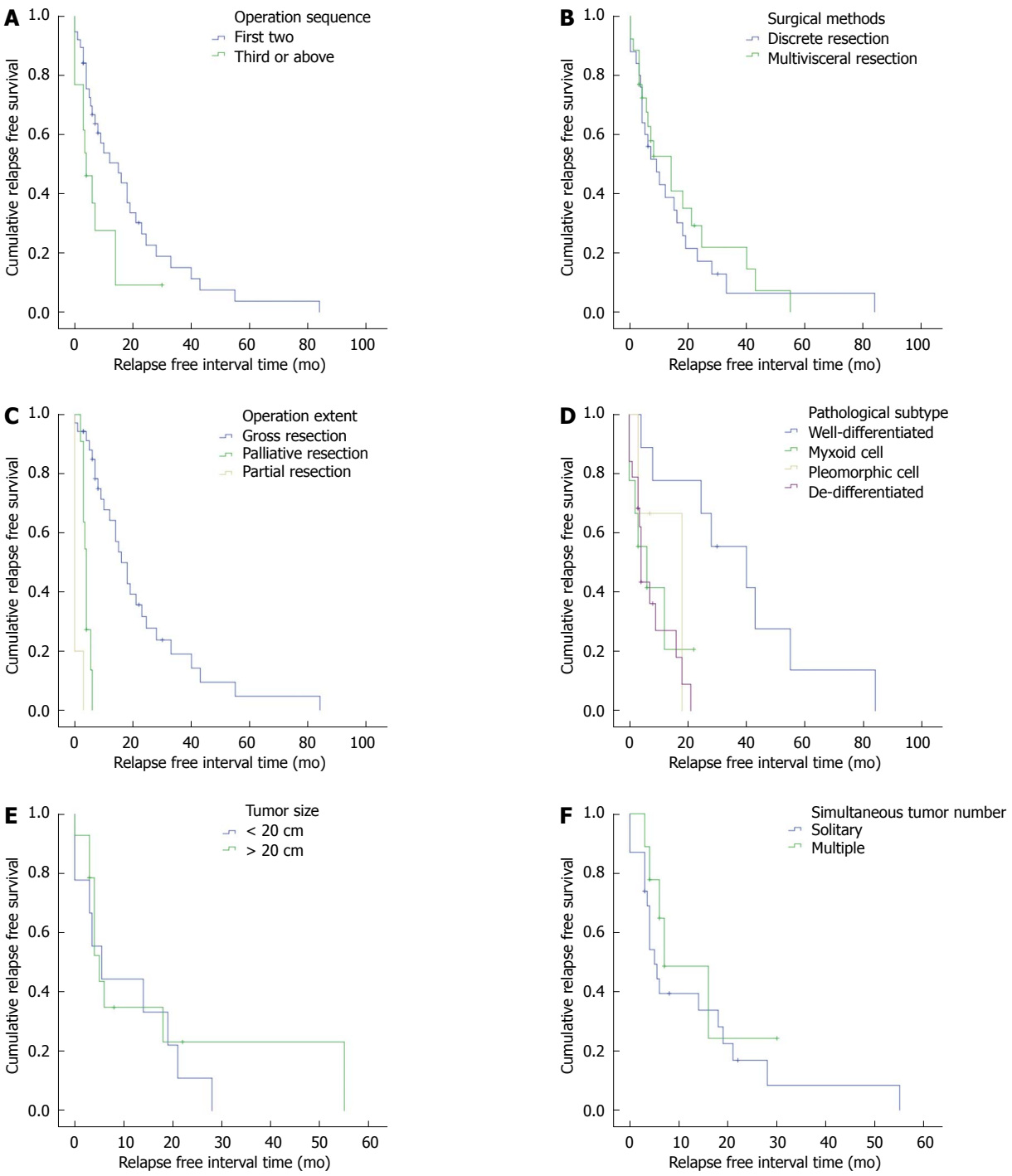

Figure 2 Relationship between the relapse-free interval and operation sequence (A), surgical method (B), operation extent (C), pathological subtype (D), tumor size (E), and simultaneous tumor number (F) in patients who underwent resection of an abdominal liposarcoma.

underwent 32 re-operations. All 19 patients had a successful re-operation with no intra-operative mortalities. However, the surgical treatment of RAL was associated with intra-operative bleeding and postoperative complications. These were most notable in cases where CMR was anticipated. The most common postoperative complications were anastomotic leak and effusion/infection.

There is no consensus in the literature regarding the guiding principles of surgical treatment for RAL. A large series of 177 primary retroperitoneal liposarcoma patients demonstrated that the pathological subtype on gross resection was the most significant prognostic factor[16]. In our multi-disciplinary team, the benefits and risks of re-operation were evaluated, and plans were formulated for all the RALs we encountered. GTR is the preferred approach for patients with RAL when CMR is necessary. If there was no possibility of gross resection, palliative resection was performed without multivisceral resection. Partial resections for RAL should only be performed in patients with intolerable symptoms (*e.g.*, extreme increas-

ing intra-abdominal pressure, grave complications, and in some emergency conditions). CMR should be avoided in patients who have undergone partial resection because this does not result in cure and incurs greater morbidity. One partial resection included an enterectomy due to spontaneous perforation caused by the RAL.

In this study, 75% (15/20) of patients who underwent GTR involved CMR. There is no similar study from our institute or similar data in the literature. It is unknown whether CMRs increase the GTR rate for RAL. However, the GTR rate was higher in CMR cases than in non-CMR cases for RALs. In operations for the primary tumor, there were more non-combined resections in GTR patients (57.9% *vs* 21.1%). The tumor size was 4-46 (median 20) cm, which is similar to that in another retrospective study of 21 cases of primary retroperitoneal liposarcoma[25]. The most frequently combined resected organ was the small intestine, which is in contrast to another study reporting the kidney[24]. In our study, the small intestine was associated with a risk of anastomotic leak. Tumor size was also correlated with intra-operative profuse bleeding (> 20 cm, $P = 0.009$). Additionally, a pathologic subtype change was observed in the RALs compared to the primary tumors or previous relapsed tumors. A pathologic subtype change predicted deterioration in repeat relapse cases[25]. Dedifferentiated liposarcomas were more commonly found as recurrent tumors[14,26-28].

There have been no studies that have focused on recurrent abdominal liposarcomas or retroperitoneal liposarcomas. Most reported studies are single cases or include less than 3 cases in a report. However, several studies have described primary and recurrent retroperitoneal liposarcoma, with more than 10 cases reported since 1991[23,25,29-32], but no primary mesentery or omental liposarcomas have been described. In our study, patients with primary retroperitoneal liposarcoma had a poorer survival, however, this was not statistically significant ($P = 0.054$). It is generally recognized that complete or gross total resection at the initial operation is very important, resulting in a more favorable prognosis[33]. In our study, patients who underwent gross resection of the primary tumor had a longer survival than those who underwent a palliative or partial resection. CMR for retroperitoneal sarcoma was recommended for the initial operation in a study of 77 patients due to an infiltrative tumor pattern[34]. Dedifferentiated tumors tend to present more often as a recurrence[35,36], frequently require multi-organ resection, and carry a shorter disease-free interval when compared to well-differentiated subtypes[25]; a similar result was observed for well-differentiated tumors in this study. OS was correlated with the resection extent and pathological subtype ($P < 0.001$ and $P = 0.02$). CMRs may increase the chance of complete resection.

Macroscopic complete resection for recurrent retroperitoneal liposarcoma has been recommended[37]. It is believed that palliative resection is worthwhile for treating the troublesome symptoms of recurrence in patients who have little chance of gross resection[32]. Repeat operations were performed in our study, and the RFI was shorter in patients who underwent more than two operations. GTR was a significant prognostic factor for the RFI ($P < 0.001$). Tumor subtype in a well-differentiated liposarcoma resulted in a significantly longer RFI compared to other types, according to the Kaplan-Meier analysis. The surgical extent was the only significant prognostic factor, as demonstrated by the Cox regression model. This showed that GTR was the major factor affecting the relapse time regardless of whether the tumor was a primary or recurrent tumor. Our results show that surgical management is the key factor in the successful treatment of abdominal liposarcoma. Multidisciplinary team cooperation has the advantage of a well-designed surgical management plan. Whether tumor size affects OS in addition to the relapse-free interval is controversial. Some authors have reported that large tumor size is negatively associated with prognosis[23,29] as large tumors require more difficult operations. However, other reports have shown no obvious difference in OS or relapse-free interval according to tumor size[25,37]. Tumor size did not affect the RFI in our study. However, it was one of the factors associated with the GTR rate, which indirectly affected OS. Multidisciplinary team approaches and multivisceral resections used in the surgical management of these cases reduced the risk of tumor residue when operating on larger abdominal liposarcomas.

Most abdominal liposarcomas are asymptomatic in the early stages. As the tumor grows patients may experience abdominal distention or other symptoms related to the tumor compressing contiguous organs, vessels, or even the ureter. Some tumors were large when the patients presented to the hospital, and it was difficult to completely resect these tumors at the time of surgery. The abdominal liposarcomas were often recurrent, particularly those with a highly malignant subtype. It is important that such patients have appropriate follow-up. However, to date, follow-up has not been standardized. The relapse time after the initial operation has been reported to vary due to the surgical extent and pathologic subtype. Postoperative adjuvant chemotherapy or radiotherapy was also not recommended as there is little evidence of benefit[38,39]. Proactive re-operation for RAL is strongly recommended. In such cases, close follow-up is necessary to identify relapse early.

RAL is a difficult disease to treat. The surgical treatment of RALs can be particularly challenging for surgical oncologists. GTR is the most important positive prognostic factor for these patients, and proactive surgical treatment is recommended. A multidisciplinary team approach most likely increases the chance of GTR, and CMR is frequently required to achieve gross tumor clearance. Palliative or partial resections are indicated in patients with recurrent disease and insufferable symptoms.

COMMENTS

Background

Abdominal liposarcomas are associated with a high local recurrence rate. Re-operation is the only effective treatment for recurrent abdominal liposarcoma

(RAL). For those who are not amenable to complete radical resection, debulking resection may relieve symptoms, reduce complications, and increase the life span. However, RAL commonly affects multiple organs, and re-operation for RAL is often difficult and is associated with significant risk, even when debulking is imminent, There is no consensus concerning the utility of repeat debulking resections. The high likelihood of post-operative complications and a lower survival outcome are detractors for repeat surgery.

Research frontiers

Re-operation is widely accepted as the treatment for recurrent abdominal liposarcoma. However, repeat re-operation for RAL is associated with high risk and a high complication rate. There are no recommended general criteria regarding when or how the re-operation should be performed. A multidisciplinary team approach, realistic risk stratification, and careful management may help lower the complication rate and improve survival.

Innovations and breakthroughs

Recurrent abdominal liposarcoma is an intractable disease encountered by general surgeons or surgical oncologists. It is generally believed that chemotherapy or radiotherapy provide minor help for patients with abdominal liposarcoma. Macroscopic complete resection or gross total resection is still the only treatment that correlates with overall survival or disease-free survival. However, recurrent lesions involve several adjacent organs in most cases. Multiple contiguous organ resections should be carried out under such conditions, however, this is associated with significant risks of failing to resect the lesion completely, multiple complications and even intra- or post-operative death. With the advantage of a multidisciplinary approach, the surgical oncologist can prepare for the treatment of this difficult disease, enhance the successful rate of gross resection and lower the morbidity and mortality related to the operation. This preliminary study summarized the outcome of multidisciplinary team cooperation in the treatment of abdominal liposarcoma which can be subsequently improved.

Applications

The study results suggest that repeat re-operation for recurrent abdominal liposarcoma with multidisciplinary team cooperation may help lower the complication rates, improve survival, and increase the quality of life of these patients.

Terminology

Recurrent abdominal liposarcoma: Recurrent abdominal liposarcoma is a disease where the liposarcoma relapses mainly in the peritoneal cavity, whether the liposarcoma originated from the retroperitoneal area or another region. Gross total resection: is the same as macroscopic complete resection, and means that the tumor is totally resected whether the pathological margin is negative or positive.

Peer review

This is a good retrospective study in which authors analyze the clinical outcome of repeated re-operation on recurrent abdominal liposarcoma. The results are interesting and suggest that repeated re-operation on recurrent abdominal liposarcoma under multidisciplinary team cooperation gain satisfactory clinical outcome.

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BRIEF ARTICLE

Probiotics improve survival of septic rats by suppressing conditioned pathogens in ascites

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Abstract

AIM: To investigate the benefits of probiotics treatment in septic rats.

METHODS: The septic rats were induced by cecal ligation and puncture. The animals of control, septic model and probiotics treated groups were treated with vehicle and mixed probiotics, respectively. The mixture of probiotics included *Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. We observed the survival of septic rats using different amounts of mixed probiotics. We also detected the bacterial population in ascites and blood of experimental sepsis using cultivation and real-time polymerase chain reaction. The severity of mucosal inflammation in colonic tissues was determined.

RESULTS: Probiotics treatment improved survival of the rats significantly and this effect was dose dependent. The survival rate was 30% for vehicle-treated septic model group. However, 1 and 1/4 doses of probiotics treatment increased survival rate significantly compared with septic model group (80% and 55% *vs* 30%, $P < 0.05$). The total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group (5.20 ± 0.57 *vs* 9.81 ± 0.67, $P < 0.05$). The total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group (33.3% *vs* 100.0%, $P < 0.05$). The population of *Escherichia coli* and *Staphylococcus aureus* in ascites of probiotics treated group were decreased significantly compared with that of septic model group (3.93 ± 0.73 *vs* 8.80 ± 0.83, $P < 0.05$; 2.80 ± 1.04 *vs* 5.39 ± 1.21, $P < 0.05$). With probiotics treatment, there was a decrease in the scores of inflammatory cell infiltration into the intestinal mucosa in septic animals (1.50 ± 0.25 *vs* 2.88 ± 0.14, $P < 0.01$).

CONCLUSION: *Escherichia coli* and *Staphylococcus aureus* may be primary pathogens in septic rats. Probiotics improve survival of septic rats by suppressing these conditioned pathogens.

Key words: Sepsis; Probiotics; Pathogens; *Escherichia coli*; *Staphylococcus aureus*

Core tip: We observed the survival of septic rats treated with different amounts of mixed probiotics. The data indicated that conditioned pathogens such as *Escherichia coli* and *Staphylococcus aureus* may be primary pathogens of septic rats in our study. Probiotics improve the survival of septic rats by suppressing the conditioned pathogens.

Liu DQ, Gao QY, Liu HB, Li DH, Wu SW. Probiotics improve survival of septic rats by suppressing conditioned pathogens in ascites. *World J Gastroenterol* 2013; 19(25): 4053-4059 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4053.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4053

INTRODUCTION

Sepsis is the systemic inflammatory response to infection and one of the most common causes of death in critically-illed patients[1]. Each year, more than 750000 clinical cases of death occur due to sepsis, and the mortalities from severe sepsis were 20%-30% in the period of 1979-2000 in the United States[2,3]. Microbial infection initiates and promotes systemic inflammatory responses by increasing cytokines release and neutrophils recruitment in target organs and inducing systemic inflammatory response syndrome and multiple organ dysfunction syndrome[4]. It has been demonstrated that intestinal microbes play an important role in sepsis[5]. Cecum is a pouch of large intestines connecting the terminal ileum to the ascending colon and home to a large number of anaerobic and aerobic microbes[6]. Cecal ligation and puncture (CLP) of rats produce cecal ischemia and polymicrobial infection[7]. The bacteria of colonic contents will spill into the abdomen, and produce severe peritonitis and bacteremia[8]. So the CLP has been used as a classic animal model of sepsis[9-11].

There is a complex microbial population in intestinal tract, some of which are probiotics. When administered in adequate amounts, probiotics confer a health benefit to the host[12]. The products of probiotics include mucin, organic acids, branched chain fatty acids, H_2, CO_2, ammonia, amines and vitamins. These products regulate host health through different pathways such as regulating energy, gene expression and cell differentiation, producing anti-inflammatory agents and keeping gut homeostasis[13,14]. The probiotics include *Bifidobacteria, Lactobacilli, Enterococci, Streptococci, Propionibacteria, Bacillus,* and yeasts. A variety of species of probiotics have been shown to benefit human gastrointestinal health[15-17]. However, the mechanisms of probiotics in improving survival in sepsis are unclear. In this study, we sought to address this question in a septic model of Wistar rats.

MATERIALS AND METHODS

Animal experiments

Male Wistar rats (8-10 wk old, Animal Center of Academy of Malitary Medical Sciences, China) were housed on a 12:12 h light-dark cycle under pathogen-free conditions with free access to food and water. We performed CLP, a clinically relevant animal model for human sepsis[18,19]. The animals were anesthetized by 10% chloral hydrate (3 mL/kg *via* intraperitoneal injection). After a midline incision was made in the abdomen, we isolated the cecum gently and placed a ligature 2.0 cm from the cecal tip using 2-0 silk suture. Ligated cecal stump was punctured by a 12-gauge needle. Colonic contents were extruded into abdominal cavity. We put back the cecum into its normal position and closed the abdomen by suturing muscle and skin, respectively. For control animals, the cecum was isolated without ligating and puncturing. The probiotic mixture consisted of three different viable strains. One dose of the probiotic mixture contained 1×10^7 CFU *Bifidobacterium longum* (ATCC 15697), 1×10^6 CFU *Lactobacillus bulgaricus* (ATCC 11842) and 1×10^6 CFU *Streptococcus thermophilus* (ATCC 19987). Before administration, the probiotic mixture was reconstituted in sterile water for 10 min at 37 ℃. We gave probiotics to animals of treated groups through intragastric administration. The animals of control and septic model groups were treated with vehicle (sterile water). The first administration of probiotics or vehicle was started 6 h after surgery. Thereafter, it was administered once a day for 3 d.

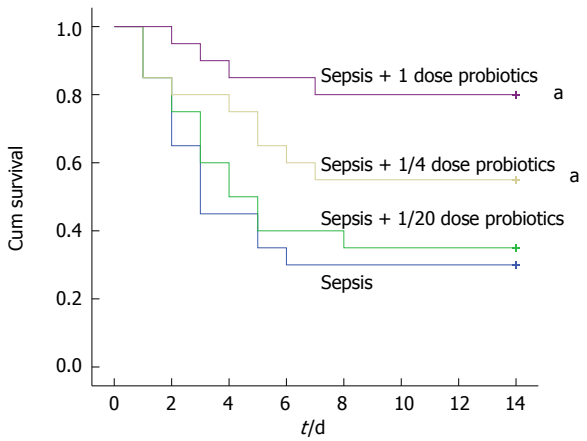

Figure 1 Survival in experimental sepsis. Survival was analysed in Wistar rats subjected to cecal ligation and puncture. Probiotics (1, 1/4 or 1/20 doses) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. All animals were observed for two weeks to compare their survival rates (n = 20; $^aP < 0.05$ *vs* septic model group).

Samples collection

All surviving animals were anaesthetised by 10% chloral hydrate (3 mL/kg, *via* intraperitoneal injection) after a 72 h period of CLP. Samples of blood and ascites were harvested for both anaerobic and aerobic microbial analysis immediately. Another portion of ascites was stored at -80 ℃ for DNA extraction. Then rats were killed by cervical dislocation, and colonic tissues were collected in neutral buffered formalin for histological analysis.

Microbial analysis of blood and ascites

Serial 10-fold dilutions were made in 0.9% sterile saline. We spread 20 μL of 10^0-10^{-7} dilutions on the nonselective blood-agar (Jinzhang Co, Ltd., Tianjin, China) surface. For anaerobic incubation, the anaerobic blood-agar dishes (Jinzhang Co, Ltd., Tianjin, China) were placed in anaerobic bags (bioMérieux, France) immediately. The time of aerobic incubation was shorter (24 h) than anaerobic (48 h) at 37 ℃. The colonies were determined in appropriate dilution, and total viable counts of original samples were calculated. Different colonies were separated and isolated for 2-3 times. We identified bacterial species using colony morphology and Gram's stain. Microstation microbe analysis system (Biolog, Winooski, VT, United States) was used for advanced identification.

Table 1 Comparison of bacterial spectrum and total viable count in ascites between septic model group and probiotics treated group

Group	Bacterial spectrums in ascites	Total viable counts (Log_{10} cells/mL ascites)
Septic model group	*Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Enterococcus avium, Streptococcus viridans, Streptococcus agalactiae, Micrococcus luteus, Enterococcus gallinarum, Enterococcus durans, Enterococcus malodoratus, Streptococcus ferus, Morganella morganii ss morganii, Acinetobacter radioresistens, Streptococcus criceti, Lactobacillus reuteri, Veillonella criceti\ratti, Desulfovibrio fructosivorans, Clostridium oroticum, Lactobacillus bifermentans*	9.81 ± 0.67
Probiotics treated group	*Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Enterococcus malodoratus, Morganella morganii ss morganii, Enterococcus durans, Streptococcus viridans, Prevotella dentioola, Desulfovibrio fructosivorcms, Bacterorides ovatus, Prevotella nigrescens*	5.20 ± 0.57[a]

Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of ascites were harvested for both anaerobic and aerobic culture. The bacterial spectrum of ascites was lower in probiotics treated group than in septic model group. The total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group (n = 18; [a]P < 0.05 *vs* septic model group).

Quantitative real-time polymerase chain reaction

Bacterial genomic DNA was extracted from ascites of rats using the QIAamp DNA mini kit (Qiagen, Hilden, United States) according to the manufacturer's protocol. We obtained 16S rRNA sequences of bacteria from the Ribosomal Database: (http://rdp.cme.msu.edu/), and designed primers for the specific bacterial strain using Primer 5.0 software package. The genomic DNA was used as template for the amplification of specimen and control standard bacterial strain through real-time polymerase chain reaction (PCR). PCR cycles were as follows: initial denaturation at 94 ℃ for 4 min, followed by 40 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 40 s. PCR primers were: *Escherichia coli* forward: 5'-CATGCCGCGTGTATGAAGAA-3' and reverse: 5'-CGGGTAACGTCAATGAGCAAA-3'; *Enterococcus faecalis* forward: 5'-CAGCAGTAGGGAATCTTCGGCAATG-3' and reverse: 5'-AGCCTCAGCGTCAGTTACAGACCAG 3'; *Staphylococcus aureus* forward: 5'-CGTCAGCTCGTGTCGTGAGATGTTG-3' and reverse: 5'-GCGGTTTCGCTACCCTTTGTATTGT-3'. The real-time PCR was performed using FastStart SYBR Green Master (Roche, Basel, Switzerland) and IQ5 PCR system (BIO-RAD, Hercules, CA, United States).

Table 2 Comparison of bacterial spectrum and total positive rate of hemoculture between septic model group and probiotics treated group

Group	Bacterial spectrums of hemoculture	Total positive rate of hemoculture
Septic model group	*Escherichia coli, Staphylococcus aureus, Curtobacterium pusillum, CDC group II-E subgroup A*	100%
Probiotics treated group	*Escherichia coli, Staphylococcus aureus*	33.3%[a]

Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of blood were harvested for both anaerobic and aerobic culture. The bacterial spectrum of hemoculture was lower in probiotics treated group than in septic model group. The total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group (n = 18; [a]P < 0.05 *vs* septic model group).

Histological examination of intestinal inflammation

The colonic tissues of at least four rats in each group were fixed in neutral buffered formalin, and processed for histological analysis. The sections of colonic tissues were stained by haematoxylin-eosin. Colonic sections were assessed for the severity of mucosal inflammation based on the following: infiltration of neutrophils and mononuclear cells into the intestinal mucosa (0, scant to normal; 1, minimal to mild; 2, mild to moderate; 3, moderate to severe; 4, severe inflammation)[20,21], and four fields of each sample were assessed. Moreover, epithelial thickness was measured under microscope (Leica, Frankfurt, Germany).

Statistical analysis

Data were expressed as the mean ± SD. All statistical analyses were performed using SPSS 17.0 software package. Survival analysis was shown in Kaplan-Meier survival curves. Survival comparisons between two subgroups were performed by the log-rank test. Differences between two groups were analysed using unpaired t test for continuous variables and the χ^2 test for nominal variables. A P value of less than 0.05 was considered statistically significant.

RESULTS

Probiotics improve the survival of rats with experimental sepsis

One hundred male Wistar rats were divided into five groups (control group, septic model group and three sepsis plus treatment groups) for survival analysis. We gave probiotic mixture (1, 1/4 or 1/20 doses) to animals in three treated groups by intragastric administration (once a day for 3 d). The animals of control and septic model

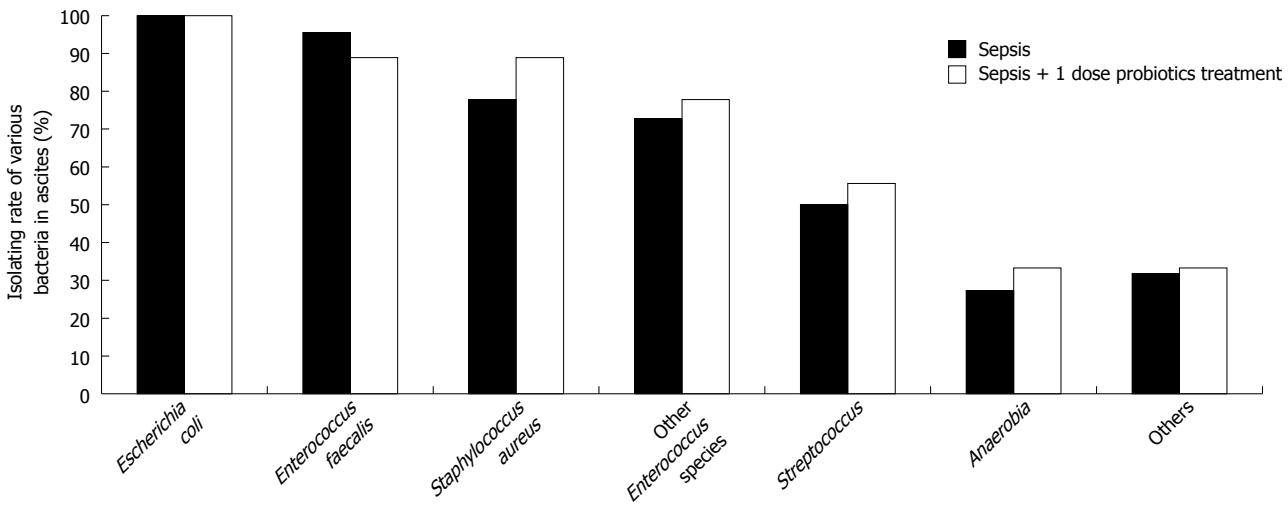

Figure 2 Comparison of isolating rate of various bacteria in ascites between sepsis and probiotics treated group. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of ascites were harvested for both anaerobic and aerobic culture. There was no statistical significance in isolating rates between two groups for all bacterial species ($P > 0.05$). In addition, "other *Entertrococcus* species" include *Enterococcus avium, Enterococcus gallinarum, Enterococcus durans* and *Enterococcus malodoratus.* "Anaerobia" include *Lactobacillus reuteri, Veillonella cricetiratti, Desulfovibrio fructosivorans, Clostridium oroticum, Lactobacillus bifermentans, Prevotella dentioola, Bacterorides ovatus* and *Prevotella nigrescens.* "Others" include *Micrococcus luteus, Morganella morganii ss morganii* and *Acinetobacter radioresistens.*

groups were treated with vehicle only. We observed all animals for two weeks. The animals in control group survived normally. The majority of rats who had CLP showed clear signs of sepsis such as piloerection, lethargy, malaise and forming ascites. Probiotics attenuated the clinical manifestations of sepsis. Probiotics treatment also improved survival significantly and this effect was dose dependent. The survival rate was the lowest (30%, 6/20 rats) in the vehicle-treated septic model group. There was no protective effect using 1/20 dose probiotics (survival rate was 35%, 7/20 rats). However, 1 and 1/4 doses of probiotics treatment increased survival rate significantly (80%, 16/20 rats and 55%, 11/20 rats) compared with vehicle treated septic model group ($P < 0.05$) (Figure 1).

Probiotics inhibit bacteria in blood and ascites of rat experimental sepsis

The consequence of survival analysis indicated that 1 dose probiotics treatment was more effective than other doses. Therefore, we divided 80 male Wistar rats into three groups (control group, 8 rats; septic model group, 50 rats; and sepsis plus 1 dose probiotics treated group, 22 rats). Probiotics or vehicle were given to the animals through intragastric administration (once a day for 3 d), respectively. All animals (8 rats) in the control group survived normally. Forty-four percent of animals (22 rats) were alive in septic model group, and 81.8% of animals (18 rats) were alive in probiotics treated group. We harvested samples after a 72 h period of CLP. The microbial composition of blood and ascites were analysed. No bacteria were determined in blood and ascites of control group. The bacterial spectrum of ascites (Table 1) and blood (Table 2) was lower in probiotics treated group than in septic model group. There was no statistical significance in isolating rates between two groups for all bacterial species ($P > 0.05$, Figure 2). However, the total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Table 1). Similarly, the total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Table 2).

The consequence of bacterial cultivation indicated that *Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus* were predominant microbial population in ascites of sepsis. For this reason, we detected the population of these bacteria in ascites using quantitative real-time PCR. The data indicated that all population of these bacteria decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Figure 3).

Probiotics improve colonic mucosal inflammation of experimental sepsis

With probiotics treatment, there was a decrease in the infiltration of neutrophils and mononuclear cells into the intestinal mucosa in septic animals ($P < 0.05$, Figure 4). No apparent differences of epithelial cell hyperplasia were found between the rats in probiotics treated group and septic model group (data not shown).

DISCUSSION

Despite the development of antibiotics and other intensive care treatment, sepsis has a high mortality. CLP of rats is one of animal models of human sepsis. Because colonic contents are extruded into abdominal cavity, various microbes proliferate in ascites immediately. Therefore, the bacteria from feces cause polymicrobial infection, bacteremia and lethal peritonitis[22,23]. In this study, we treated the experimental septic rats with mixture of

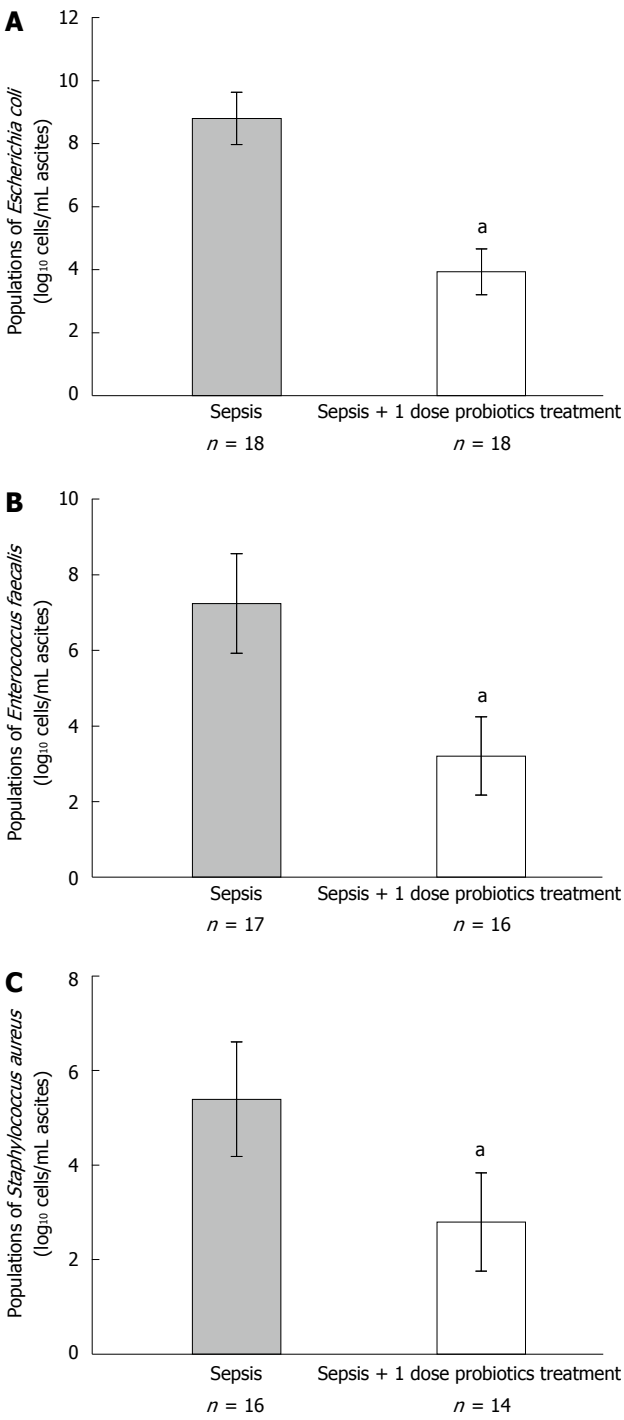

Figure 3 Comparison of predominant bacterial populations in ascites between sepsis and probiotics treated groups. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Bacterial genomic DNA was extracted and analysed by quantitative real-time PCR as described previously. The population of *Escherichia coli* (A), *Enterococcus faecalis* (B) and *Staphylococcus aureus* (C) were compared between two groups ($^aP < 0.05$ *vs* septic model group).

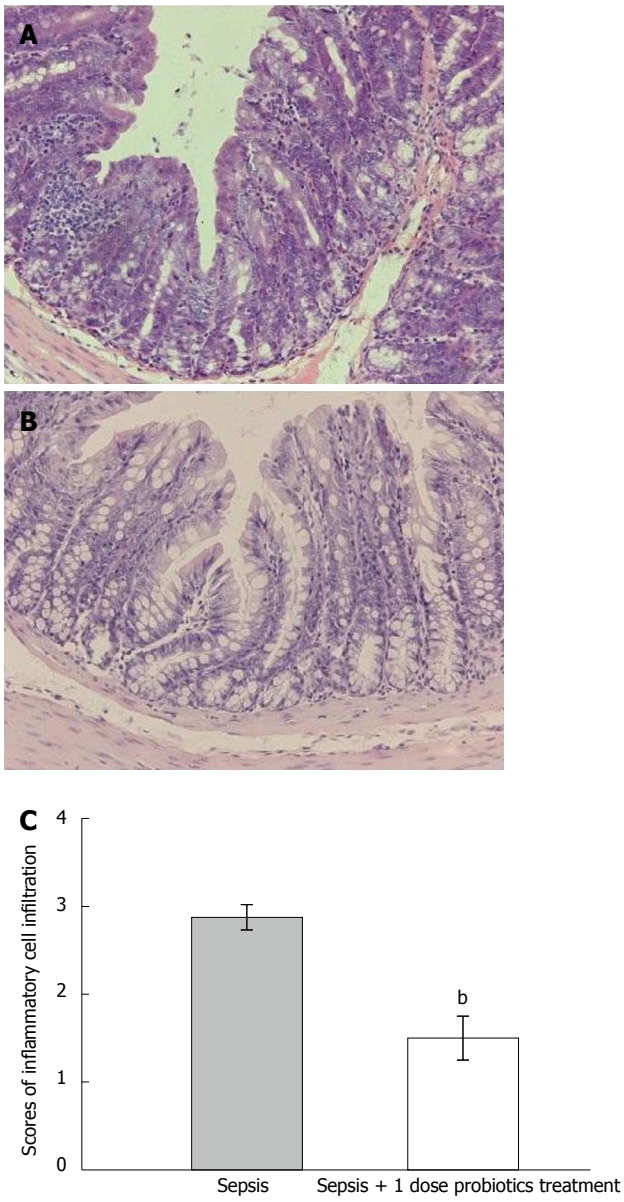

Figure 4 Colonic mucosal inflammation of experimental sepsis. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. We harvested colonic tissues after a 72 h period of cecal ligation and puncture. The sections of colonic tissues were stained by haematoxylin-eosin (× 400). Four fields of each sample were assessed. More neutrophils and mononuclear cells infiltrated into the intestinal mucosa in septic model group (A) than probiotics treatment group (B). We also compared scores of inflammatory cell infiltration between two groups (C) ($^bP < 0.01$ *vs* septic model group).

three live probiotics. We also analysed the survival of probiotics treated septic animals. It was demonstrated that probiotics improved the survival of rats with experimental sepsis and this effect was dose dependent. No protective effect was observed using the lowest concentration of probiotics (1/20 dose). However, 1 and 1/4 doses of probiotics treatment increased survival significantly compared with septic model group. Therefore, we treated septic animals in subsequent experiments using 1 dose of probiotics all the time.

Ascites culture data indicates that more pathogens grew in septic model group (10^9-10^{10} cells/mL) than in probiotic treated group (10^4-10^5 cells/mL). Both aerobes and anaerobes were detected in ascitic samples, although the majority of microbes were aerobes. Cecum contained anaerobes, facultative aerobes and aerobes. Furthermore, the amounts of anaerobes were greater than those of aerobes[24,25]. However, in our study, aerobes had been

isolated frequently from septic ascitic samples such as *Escherichia coli* (isolating rate was 100%), *Enterococcus faecalis* (95.5%) and *Staphylococcus aureus* (77.8%). The total isolating rate of anaerobes was less than 30%. The main reason for this phenomenon was "oxygen". When the operation of CLP was performed in experimental sepsis, the anaerobes were exposed to oxygen directly. Furthermore, some oxygen was stored in abdominal cavity of animal after operation. For these reasons, the majority of anaerobes were killed by oxygen. Thereafter, the aerobes which were minority in original colonic contents proliferated immediately. When we gave probiotics to septic rats, the bacterial spectrum of ascites and blood was lower than in the septic model group. Meanwhile, probiotics decreased total viable counts of pathogens in septic ascites significantly. In addition, the data of hemoculture showed that *Escherichia coli* and *Staphylococcus aureus* usually were detected in septic model group. Probiotics decreased the positive rate of hemoculture in septic rats.

It seemed that *Escherichia coli* and *Staphylococcus aureus* are the primary pathogens of CLP rats in septic model in our study. On one hand, we detected the population of these bacteria in ascites by quantitative real-time PCR. All population of these bacteria decreased significantly in probiotics treated group compared with septic model group. On the other hand, inflammatory response of intestinal mucosa was lessened in probiotics treated group compared with septic model group. All these data indicated that the mixture of probiotics improved the survival in a murine model of polymicrobial sepsis by suppressing the conditioned pathogens. However, the reasons for this suppression are not clear. There are two potential reasons: first, the decreased bacterial number may result from the inhibition of bacterial proliferation; second, a less bacteria infiltration or promoted bacterial killing[26-30].

Based on what had been mentioned above, we draw a conclusion that conditioned pathogens (*Escherichia coli* and *Staphylococcus aureus*) may be primary pathogens of CLP rats in septic model in our study. Probiotics (*Bifidobacterium longum, Lactobacillus bulgaricus* and *Streptococcus thermophilus*) contribute to improving the survival in an animal sepsis model by suppressing the conditioned pathogens.

COMMENTS

Background

Sepsis is the systemic inflammatory response to infection. Microbial infection initiates and promotes systemic inflammatory responses. A variety of species of probiotics have been shown to benefit human gastrointestinal health. However, the mechanisms of probiotics in improving sepsis are unclear. In this study, the authors sought to address this question in the septic model of Wistar rats.

Research frontiers

Recently, more and more gastrointestinal diseases have been treated using probiotics. The probiotics and their products keep gastrointestinal tract homeostasis and regulate immune responses. For example, probiotics can regulate IgE production level and maturation of T cell in the gut.

Innovations and breakthroughs

To study the benefits of probiotics for sepsis, the authors observed the survival of cecal ligation and puncture (CLP) rats using different amounts mixed probiotics. The mixture of probiotics included *Bifidobacterium longum, Lactobacillus bulgaricus* and *Streptococcus thermophilus*. They also detected bacterial populations in ascites and blood of CLP rats using cultivation and real-time polymerase chain reaction. The data suggested that *Escherichia coli* and *Staphylococcus aureu* may be primary pathogens in the septic model. Probiotics improve survival in the septic model by suppressing the conditioned pathogens.

Applications

The results of this study suggest that probiotics improve survival in the septic model by suppressing the conditioned pathogens. This study helps to know whether probiotics can improve the clinical course of sepsis.

Terminology

CLP of rats produces cecal ischemia and polymicrobial infection. The bacteria of fecal contents will spill into the abdomen, and produce severe peritonitis and bacteremia. So CLP has been used as a classic animal model of sepsis. There are complex microbial populations in intestinal tract. Some of them are probiotics. When administered in adequate amounts, probiotics confer a health benefit to the host. The products of probiotics include mucin, organic acids, branched chain fatty acids, H_2, CO_2, ammonia, amines and vitamins. These products regulate host health through different pathways such as regulating energy, gene expression and cell differentiation, producing anti-inflammatory agents and keeping gut homeostasis.

Peer review

This study investigated the potential benefit of probiotic supplement in preventing septic death by studying the survival rates in rats treated with different doses of a probiotic mixture and then further investigated the effects of probiotics administration on the bacteria proliferation in blood and ascites in a cecal ligation and puncture sepsis model. This study shows the importance of knowing whether probiotics can improve the survival of experimental sepsis.

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BRIEF ARTICLE

Impact of postoperative complications on long-term survival after radical resection for gastric cancer

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Abstract

AIM: To investigate the potential impact of complications in gastric cancer patients who survive the initial postoperative period.

METHODS: Between January 1, 2005 and December 31, 2006, 432 patients who received curative gastrectomy with D2 lymph node dissection for gastric cancer at our department were studied. Associations between clinicopathological factors [age, sex, American Society of Anesthesiologists grade, body mass index, tumor-node-metastases (TNM) stage and tumor grade], including postoperative complications (defined as any deviation from an uneventful postoperative course within 30 d of the operation and survival rates) and treatment-specific factors (blood transfusion, neoadjuvant therapy and duration of surgery). Patients were divided into 2 groups: with (n = 54) or without (n = 378) complications. Survival curves were compared between the groups, and univariate and multivariate models were conducted to identify independent prognostic factors.

RESULTS: Among the 432 patients evaluated, 61 complications occurred affecting 54 patients (12.50%). Complications included anastomotic leakages, gastric motility disorders, anastomotic block, wound infections, intra-abdominal abscesses, infectious diarrhea, bleeding, bowel obstructions, arrhythmias, angina pectoris, pneumonia, atelectasis, thrombosis, unexplained fever, delirium, ocular fungal infection and multiple organ failure. American Society of Anesthesiologists grade, body mass index, combined organ resection and median duration of operation were associated with higher postoperative complications. The 1-, 3- and 5-year survival rates were 83.3%, 53.2% and 37.5%, respectively. In the univariate analysis, the size of lesions, TNM stage, blood transfusion, lymphovascular invasion, perineural invasion, neoadjuvant chemotherapy, and postoperative complications were significant predictors of overall survival. In the multivariate analysis, only TNM stage and the presence of complications remained significant predictors of reduced survival.

CONCLUSION: The occurrence of in-hospital postoperative complications was an independent predictor of worse 5-year overall survival rate after radical resection of gastric cancer.

Key words: Gastric cancer; Perioperative complication; Surgical resection; Complications

Core tip: The concept of perioperative complications as a risk factor for survival is well known in gastric cancer, however, the potential impact of complications for patients who survive the initial postoperative period has not been determined. We showed that the occurrence of in-hospital postoperative complications is an independent predictor of worse 5-year overall survival after radical resection of gastric cancer. In 432 patients evaluated, 61 complications occurred affecting 54 patients (12.50%). American Society of Anesthesiologists grade, body mass index, combined organ resection and median duration of operation were associated with higher post-

operative complications. The 1-, 3- and 5-year survival rates were 83.3%, 53.2% and 37.5%, respectively.

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INTRODUCTION

Globally, gastric cancer ranks fourth and fifth in males and females, respectively, in terms of incidence, and ranks third and fifth in males and females, respectively, in terms of mortality[1]. China is classified as a high incidence area for gastric cancer. Stomach cancer has the third highest incidence and is the second leading cause of death among all cancers[2]. Surgery is the cornerstone in the treatment of gastric cancer. Although postoperative complications after surgical resection of gastric cancer are common, the potential long-term impact of these complications for patients who survive the initial postoperative period is not well understood. Western countries have published complication rates ranging from 35% to 46%, and mortality rates from 4% to 16% after D2 lymph node dissection[3-5]. Major complications include anastomotic leakage, intra-abdominal bleeding, intra-abdominal abscess, intestinal obstruction and wound infection. Previous investigations focused on the immediate effect of postoperative complications and their impact on acute perioperative course and length of hospital stay. The concept of perioperative complications as a risk factor for survival is well known in other cancer surgery, such as esophageal, colorectal cancer liver metastases, hilar cholangiocarcinoma and colorectal cancer[6-9]. To date, few studies have determined the potential impact of early surgical complications on long-term survival for patients with gastric cancer. The aim of this study was to assess the impact of prognostic factors, in particular perioperative complications, on the long-term survival of patients undergoing radical resection for gastric cancer.

MATERIALS AND METHODS

Patients

Patients' medical records and clinicopathological data during the period from January 1, 2005 to December 31, 2006 were studied retrospectively at the Department of Gastrointestinal Surgery, First Clinic Medical School of Yangzhou University, Yangzhou, China. Patients' inclusion criteria were: (1) All patients in the study had histologically confirmed gastric adenocarcinoma and received curative gastrectomy with D2 lymph node dissection; (2) Information regarding postoperative complications and mortality was available for each patient studied. As a result, 432 patients were eligible for analysis. All these patients were followed up for a minimum of 60 mo after gastric resection. These 432 patients comprised 263 men (60.88%) and 169 women (39.12%). Their median age was 64 years (range, 28-83 years). The follow-up of patients after surgery was 5 years. Numbers of subtotal and total gastric resection were 186 and 246, respectively, and gastrointestinal reconstruction comprised 82 Billroth Ⅰ, 68 Billroth Ⅱ and 282 Roux-en-Y anastomoses. This research was in compliance with the Helsinki Declaration and was approved by the ethics committee of the First Clinic Medical School of Yangzhou University. The main characteristics of 432 people included study are summarized in Table 1.

Assessment of complications

Complications were defined as any deviation from an uneventful postoperative course within 30 d of the operation. A recently published standardized complication classification system (Clavien-Dindo classification) was used to grade postoperative complications[10]. Briefly, grade I complications include any deviation from the normal postoperative course not needing specific treatment, as well as wound infections treated topically at the bedside. Grade Ⅱ complications can be treated solely by drugs, blood transfusion, physiotherapy and nutritional support. Grade Ⅲ complications require interventional or surgical treatment, without (Ⅲa) or with (Ⅲb) general anesthesia. Grade Ⅳ complications are life-threatening complications requiring intensive-care unit management (Ⅳa, single organ dysfunction; Ⅳb, multiple organ dysfunction). Grade Ⅴ represents death of the patient. In the present study, if a patient had more than 1 complication, the grade used for analysis was defined by the highest-ranked complication.

Follow-up

Complete follow-up was available for all study patients. Follow-up was calculated from the date of surgery. Follow-up data were obtained by phone, letter, and the outpatient clinical database. The end of the follow-up period was 5 years after surgery.

Statistical analysis

The impact of clinicopathological and therapy-related variables with a potential influence on a postoperative complication were investigated. For this purpose, patients with postoperative complications were compared with those who recovered normally. The two groups were compared by univariate analysis with respect to clinicopathological factors [age, sex, American Society of Anesthesiologists (ASA) grade, body mass index (BMI), tumor-node-metastases (TNM) stage and tumor grade] and treatment-specific (blood transfusion, neoadjuvant therapy and duration of surgery) variables. For the outcome analysis, the patients were divided into two groups: those with complications and those with no postoperative complications. To investigate the impact of such complications on postoperative

Table 1 Intergroup comparison of epidemiological and treatment-related variables in 432 patients

Variable	Postoperative complications (n = 54)	No postoperative complications (n = 378)	P value
Age (yr), median (range)	60 (28-80)	59 (23-76)	0.516[1]
Sex			0.236[2]
Male	37	226	
Female	17	152	
ASA grade			0.000[2]
Ⅰ	24	341	
Ⅱ	18	33	
Ⅲ	11	4	
Ⅳ	1	0	
Body mass index (kg/m^2)			0.040[2]
< 28	42	332	
⩾ 28	12	46	
TNM stage			0.358[2]
Ⅰ	5	49	
Ⅱ	9	94	
Ⅲ	28	174	
Ⅳ	12	61	
Tumor size (cm), mean (range)	4.9 (2.0-12.0)	4.7 (0.5-12.0)	0.338[1]
Combined organ resection			0.000[2]
No	35	346	
Yes	19	32	
Neoadjuvant therapy			0.126[2]
No	50	366	
Yes	4	12	
Median (range) duration of operation (min)	220 (175-310)	195 (160-300)	0.000[1]

[1]t test; [2]Pearson χ^2. ASA: American Society of Anesthesiologists; TNM: Tumor-node-metastases.

outcome, the two groups were compared using Kaplan-Meier survival curves. Postoperative complications are listed in Table 2. Assessment of the oncological relevance of the complication was based on an analysis of the two groups of patients. The 5-year survival rates were first subjected to univariate analysis, followed by multivariate analysis. Three patients who died within 30 d after surgery were excluded from the survival analysis.

RESULTS

Complications

Sixty-one complications occurred, affecting 54 patients (12.50%). Complications were graded into seven categories according to their severity. The details are given in Table 2. Ten patients suffered from gastric resection-related complications. Thirteen patients experienced infectious complications. Eight patients had bleeding complications and six had bowel obstructions. Five patients developed cardiac complications. Eleven patients had pulmonary complications. Two had thrombosis and six patients had other complications. Three patients died during hospitalization within 5 to 26 d after the initial gastrectomy, representing an in-hospital mortality of 0.69%. Two patients died as a result of postoperative sepsis and multiple organ failure. One died from acute respiratory distress syndrome.

Table 2 Post-operative complication types, frequencies and severities

Variables	Number
Gastric resection-related complications	
Anastomotic leakages	5
Gastric motility disorders	4
Anastomotic block	1
Infectious complications	
Wound infection	7
Intra-abdominal abscess	5
Infectious diarrhea	1
Bleeding complications	
Anastomotic bleeding	2
Intra-abdominal bleeding	5
Subcutaneous hematoma surrounding drainage tubes	1
Bowel obstructions	6
Pulmonary complications	
Pneumonia	10
Atelectasis	1
Cardiac complications	
Arrhythmia	4
Angina pectoris	1
Thrombosis	
Deep venous thrombosis	1
Portal venous thrombosis	1
Other complications	
Delirium	2
Unexpected fever	2
Ocular fungal infection	1
Multiple-organ failure	1
Incidence and severity (Clavien-Dindo grade)	
Ⅰ	8
Ⅱ	35
Ⅲa	11
Ⅲb	2
Ⅳa	1
Ⅳb	1
Ⅴ	3

Risk factors for post-operative complication

The variables age, sex, ASA grade, BMI, TNM stage, mean tumor size, combined organ resection, neoadjuvant therapy and median duration of operation were investigated by Pearson χ^2 test or by t test. Only ASA grade, BMI, combined organ resection and median duration of operation had an independent impact on the occurrence of complications ($P < 0.05$).

Survival

The 1-, 3- and 5-year survival rates were 83.3%, 53.2% and 37.5%, respectively. In the univariate analysis, survival was not influenced by gender, age or BMI. In contrast, the size of the lesions, TNM stage, blood transfusion, lymphovascular invasion, perineural invasion, neoadjuvant chemotherapy and postoperative complications were significant predictors of overall survival (Table 3). In the multivariate analysis, perineural invasion, the size of the lesions, blood transfusion, lymphovascular invasion and neoadjuvant chemotherapy were no longer predictive factors for reduced survival. However, the TNM stage and the presence of complications remained significant predictors of reduced survival (Table 4).

Table 3 Univariate survival analysis of gastric cancer patients according to various clinicopathological variables and complications

Variable	Patients	5-yr survival	Log rank χ^2 test	*P* value
Gender			2.847	0.092
Male	262	34.00%		
Female	167	43.70%		
Age (yr)			1.157	0.282
≤ 60	230	40.00%		
> 60	199	35.20%		
BMI (kg/m^2)			0.018	0.893
≤ 26	278	38.50%		
> 26	151	36.40%		
Size of lesions (cm)			8.130	0.004
< 5	269	42.00%		
≥ 5	160	30.60%		
TNM stage			60.453	0.000
Ⅰ	54	72.20%		
Ⅱ	101	54.90%		
Ⅲ	202	28.70%		
Ⅳ	72	12.70%		
Blood transfusion			4.982	0.026
Yes	74	27.00%		
No	355	40.00%		
Lymphovascular invasion			4.673	0.031
Yes	69	21.70%		
No	360	40.80%		
Perineural invasion			5.237	0.022
Yes	36	25.00%		
No	393	38.90%		
Neoadjuvant chemotherapy			7.124	0.008
No	411	38.60%		
Yes	18	16.70%		
Complications			25.946	0.000
Yes	51	21.80%		
No	378	39.90%		

BMI: Body mass index; TNM: Tumor-node-metastases.

Table 4 Predictors of survival: Multivariate analysis

Risk factor	HR (95%CI)	*P* value
Size of lesions	1.2 (0.9-1.5)	0.156
TNM stage	1.6 (1.4-1.9)	0.000
Blood transfusion	0.9 (0.5-1.4)	0.752
Lymphovascular invasion	1.0 (0.6-1.6)	0.841
Perineural invasion	0.7 (0.4-1.0)	0.107
Neoadjuvant chemotherapy	1.5 (0.8-2.5)	0.134
Post-operative complications	2.5 (1.8-3.6)	0.000

Cox regression analysis of patient survival. TNM: Tumor-node-metastases.

DISCUSSION

In the surgical approach for early and selective advanced gastric cancer, gastrectomy with D2 lymphadenectomy is justified[11,12]. Local tumor control and long-term oncological survival are dependent on the quality of the surgical treatment and the surgeon's case-load[13]. The occurrence of postoperative complications is higher in inexperienced hands, and there is a considerable difference in early surgical outcomes among centers[14,15]. Overall survival rate is higher at specialized centers. Therefore, it may be stated that gastric cancer surgery is safe at specialized centers. The postoperative complications at our institution were in the acceptable range: most patients had a smooth recovery and postoperative mortality was not high.

In this study, ASA grade, BMI, combined organ resection and duration of operation were greater in the postoperative complications group than in the no complications group. These findings are in agreement with recent reports showing similar predictors of postoperative complications. ASA grade was reported to affect surgical complications[1], but this was not consistent with prior studies reported by Kawamura *et al*[16]. A negative effect of BMI on perioperative complications of gastrectomy has also been reported[17,18]; elevated BMI was significantly associated with increased weight of the stomach extirpated *en bloc* with omentum and perigastric lymph nodes, which was found to increase operative times. Additional organ resection in surgical therapy for gastric cancer has been associated with increased complications and perioperative mortality in pursuit of a D2 lymphadenectomy[19,20]. A large retrospective study from Japan found no survival difference when patients undergoing gastrectomy alone were compared to patients with additional organ resection, however, the complication rate was greater[21]. The duration of operation was greater in the postoperative complications group than in the no complications group. Patients with advanced TNM stage, combined organ resection and elevated BMI always require a longer operation time than others.

Although this is the first study to identify the independent impact of postoperative complications among patients undergoing surgery for gastric cancer, other investigators have explored the potential relationship between postoperative complications and long-term survival beyond the initial perioperative period in other malignancies. In an analysis of 197 colorectal cancer liver metastases patients, Schiesser *et al*[22] reported 30% perioperative complications, and the median survival time of patients with perioperative complications was 3.2 years, compared to 4.4 years in those patients without complications. Similar results were reported for hilar cholangiocarcinoma[8]. Given these findings, it is logical to fully explore the potential impact of in-hospital, postoperative complications on long-term cancer survival. Mechanisms currently under discussion include more serious immunosuppression and more obvious inflammation associated with postoperative complications. Generally, postoperative complications have been suggested to lead to an extended period of immunosuppression, which permits residual tumor cells to proliferate and survive in the host. One example supporting this hypothesis is based, in part, on the finding that in several other malignancies, perioperative blood transfusions correlated with negative immunomodulatory effects and earlier cancer recurrence[23,24]. Our findings could be explained in a similar manner. Infective complications are the most common complications, including intra-abdominal abscesses, wound infections, pneumonia, infectious diarrhea and anastomotic leakage, which can also cause abdominal infection. Several studies have demonstrated a correlation

between long-term outcomes after curative resections of solid tumors and postoperative infection and sepsis[7,25-27]. Infection and sepsis potentiate proinflammatory cytokine cascades, including tumor necrosis factor-alpha and interleukins 1, 6 and 8. These immune modulators can affect the function and regulation of natural killer cells, cytotoxic T-lymphocytes and antigen-presenting cells[28-30]. Hypothetically, micrometastases may progress rapidly during brief and prolonged periods of relative immunosuppression resulting from postoperative complications. Besides, both sepsis and blood transfusion may stimulate vascular endothelial growth factor release, which is one of the most potent stimulators of metastatic growth[31,32]. This combination of transfusion and sepsis may stimulate cancer recurrence[33].

Our results show a clear association between postoperative complications and long-term survival for patients undergoing resection for gastric cancer. The study highlights the significance not only of appropriate patient selection and surgical technique, but also serves to emphasize the potential impact that postoperative monitoring and hospital care can have on long-term outcomes. Performance of a safe operation with minimal blood loss, careful lymphadenectomy and gastrointestinal reconstruction are important for reducing post-operative complications. Avoidance of complications improves long-term survival.

In summary, our study aimed to evaluate the impact of complications on survival for patients who received radical surgery for gastric cancer. We found that the occurrence of in-hospital postoperative complications was an independent predictor of worse 5-year overall survival rate after radical resection of gastric cancer.

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COMMENTS

Background

The concept of perioperative complications as a risk factor for survival is well known in gastric cancer, however, the potential impact of complications for patients who survive the initial postoperative period has not been determined. This article studies the potential impact of complications on patients who survive the initial postoperative period.

Research frontiers

Perioperative complications are an important factor after surgery. Currently, many experts have focused on lymphatic metastasis for survival; however, perioperative complications have not been unequivocally addressed. In this study, the authors demonstrate that the occurrence of in-hospital postoperative complications was an independent predictor of worse 5-year overall survival rate after radical resection of gastric cancer.

Innovations and breakthroughs

Between January 1, 2005 and December 31, 2006, 432 patients who received curative gastrectomy with D2 lymph node dissection were studied. Associations between clinicopathological factors, including postoperative complications and survival, were studied using univariate and multivariate models.

Applications

To understand survival during the initial postoperative period of gastric cancer, this article studied the potential impact of complications for patients who survive the initial postoperative period. In the univariate analysis, size of lesions, tumor-node-metastases (TNM) stage, blood transfusion, lymphovascular invasion, perineural invasion, neoadjuvant chemotherapy and postoperative complications were significant predictors of overall survival. In the multivariate analysis, only TNM stage and the presence of complications remained significant predictors of reduced survival.

Peer review

The article provides a detailed description of the survival impact of postoperative complications. This data is valuable for the treatment of gastric malignancy.

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BRIEF ARTICLE

Clinical significance of melatonin concentrations in predicting the severity of acute pancreatitis

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Abstract

AIM: To assess the value of plasma melatonin in predicting acute pancreatitis when combined with the acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) and bedside index for severity in acute pancreatitis (BISAP) scoring systems.

METHODS: APACHE Ⅱ and BISAP scores were calculated for 55 patients with acute physiology (AP) in the first 24 h of admission to the hospital. Additionally, morning (6:00 AM) serum melatonin concentrations were measured on the first day after admission. According to the diagnosis and treatment guidelines for acute pancreatitis in China, 42 patients suffered mild AP (MAP). The other 13 patients developed severe AP (SAP). A total of 45 healthy volunteers were used in this study as controls. The ability of melatonin and the APACHE Ⅱ and BISAP scoring systems to predict SAP was evaluated using a receiver operating characteristic (ROC) curve. The optimal melatonin cutoff concentration for SAP patients, based on the ROC curve, was used to classify the patients into either a high concentration group (34 cases) or a low concentration group (21 cases). Differences in the incidence of high scores, according to the APACHE Ⅱ and BISAP scoring systems, were compared between the two groups.

RESULTS: The MAP patients had increased melatonin levels compared to the SAP (38.34 ng/L *vs* 26.77 ng/L) (P = 0.021) and control patients (38.34 ng/L *vs* 30.73 ng/L) (P = 0.003). There was no significant difference inmelatoninconcentrations between the SAP group and the control group. The accuracy of determining SAP based on the melatonin level, the APACHE Ⅱ score and the BISAP score was 0.758, 0.872, and 0.906, respectively, according to the ROC curve. A melatonin concentration ⩽ 28.74 ng/L was associated with an increased risk of developing SAP. The incidence of high scores (⩾ 3) using the BISAP system was significantly higher in patients with low melatonin concentration (⩽ 28.74 ng/L) compared to patients with high melatonin concentration (> 28.74 ng/L) (42.9% *vs* 14.7%, P = 0.02). The incidence of high APACHE Ⅱ scores (⩾ 10) between the two groups was not significantly different.

CONCLUSION: The melatonin concentration is closely related to the severity of AP and the BISAP score. Therefore, we can evaluate the severity of disease by measuring the levels of serum melatonin.

Key words: Pancreatitis; Melatonin concentrations; Predict; Cutoff; Bedside index for severity in acute pancreatitis; Acute physiology and chronic health evaluation Ⅱ

Core tip: It is important to assess the severity and changes in a patient's condition in a timely and accurate manner. Thus, a comprehensive treatment plan for acute pancreatitis patients is critical. Melatonin plays a protective role in the early course of human acute pancreatitis, and melatonin concentration variations

are closely related to the severity of acute pancreatitis and the bedside index for severity in acute pancreatitis score. We can determine the severity of disease in the clinic more objectively, accurately and rapidly by measuring the levels of serum melatonin than by using the standard scoring systems. When the serum concentration of melatonin is below 28.74 ng/L, it is possible that acute pancreatitis patients will develop severe acute pancreatitis.

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INTRODUCTION

Most patients with acute pancreatitis have a favorable prognosis. However, the mortality rate of acute pancreatitis (AP) has been reported as 6%-23%[1]. Effectively treating the disease becomes more difficult as it develops into severe AP (SAP). Therefore, it is important to assess the disease severity in a timely and accurate manner to provide comprehensive treatment to AP patients. Accurate treatment can improve the prognosis and reduce mortality[2]. As a result, there is an urgent need for an objective, accurate, fast and simple method of monitoring changes in AP patients.

Melatonin is best known as the activator of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxydase, or glutathione reductase[3-6]. Melatonin is also well-known as a scavenger of radical oxygen and nitrogen species[7-9]. Melatonin, together with reduced glutathione, vitamins C and E, uric acid, selenium, and creatinine, belongs to the category of nonenzymatic scavengers[6,10,11]. A number of studies have shown that melatonin (MT) plays a protective role in AP. In acute pancreatitis, melatonin was demonstrated to inhibit nuclear binding of nuclear factor kappa B (NF-κB). NF-κB is a transcription factor that controls the expression of genes involved in immunity and inflammation and the production of prostaglandins, cytokines, cell adhesion molecules, nitricoxide (NO), and inhibitors of apoptosis[12,13]. Melatonin has been demonstrated to reduce gene expression and synthesis of proinflammatory cytokines such as tumor necrosis factor α (TNFα) and proinflammatory interleukins such as interleukin (IL)-1β, IL-6, IL-8, and prostaglandins[1,14,15]. In addition, melatonin was also reported to modulate the processes of apoptosis and necrosis by stimulating the production of vascular endothelial growth factor to activate angiogenesis[16-18]. Furthermore, MT plays a protective role in AP-associated organ injuries in animal models[19-21]. For example, Huai *et al*[22] found that melatonin protects rats against acute pancreatitis-associated lung injury through the upregulation of IL-22 and Th22. The upregulation of IL-22 increases innate immunity in tissues and enhances regeneration.

Data on the relationship between the levels of MT in patients with AP and the severity and prognosis of this disease have not been reported. The aims of this study were to assess the value of plasma MT in determining the severity of AP and in predicting SAP. Additionally, we analyzed changes in plasma MT levels and the use of two scoring systems in AP patients.

MATERIALS AND METHODS

Patients

This study enrolled 55 consecutive patients with AP (35 men and 20 women) admitted to department of gastroenterology of our hospital between July 2010 and March 2011 (median age 51 years, range 17-82 years). The diagnosis and classification of AP were based on the diagnosis and treatment guidelines for acute pancreatitis in China (2009)[23]. SAP was diagnosed by the presence of organ failure and (or) local complications. Organ failure included shock (systolic blood pressure < 90 mmHg), pulmonary insufficiency (arterial PO_2 < 60 mmHg at room air or the need for mechanical ventilation), or renal failure (serum creatinine level > 2 mg/dL after rehydration or hemodialysis). Examples of localcomplications included pancreatic necrosis, a pseudocyst, or a pancreatic abscess. According to the diagnosis and treatment guidelines for acute pancreatitis in China, 42 cases were defined as mild AP (MAP), and 13 cases were classified as SAP. Within the population of SAP patients, there were 11 patients (84.6%) with pseudocysts and 2 patients (15.4%) with pancreatic necrosis. There were also 2 patients (15.4%) with acute renal failure. The disease etiology was biliary in 19 cases (34.5%), hyperlipidemic in 14 cases (25.5%), and idiopathic in 14 cases (25.5%). The causes of the remaining 8 cases (14.5%) were hyperlipidemic and biliary, alcoholic and biliary, or alcoholic and pancreatic (duct obstruction). There were no patient deaths during the study period (Table 1). We also analyzed 45 healthy individuals as controls for the study. There were 27 men and 18 women in the control group. The median age of the controls was 44 years (range of 24-64 years).

Monitoring

The study protocol was reviewed and approved by the local ethics committee. The study patients and healthy volunteers were enrolled after providing written informed consent. The patient-acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) and bedside index for severity in acute pancreatitis (BISAP) scores were calculated within the first 24 h after admission in all patients with AP. The APACHE Ⅱ score is the most commonly used scoring system for determining the severity and prognosis of AP. This scoring system contains 12 monitoring indicators, and the final score is composed of an acute

Table 1 Characteristics of 55 patients with acute pancreatitis *n* (%)

Variables	Total (*n* = 55)	Mild (*n* = 42)	Severe (*n* = 13)
Age, yr (range)	51 (17-82)	51 (17-77)	50 (30-82)
Male	35 (63.6)	27 (64.3)	8 (61.5)
Female	20 (36.4)	15 (35.7)	5 (38.5)
Etiology			
Biliary	19 (34.5)	17 (40.5)	2 (15.4)
Hyperlipidemia	14 (25.5)	8 (19)	6 (46.2)
Idiopathic	14 (25.5)	11 (26.2)	3 (23.1)
Other	8 (14.5)	6 (14.3)	2 (15.4)
APACHE Ⅱ (range)	7 (2-22)	6 (2-12)	12 (6-22)
BISAP (range)	2 (0-5)	1 (0-4)	3 (2-5)
Operations	10 (18.2)	9 (21.4)	1 (7.7)
Organ failure	2 (3.6)	0 (0.0)	2 (15.4)
Pancreatic necrosis	2 (3.6)	0 (0.0)	2 (15.4)
Pseudocyst	11 (20.0)	0 (0.0)	11 (84.6)
Mortality	0 (0.0)	0 (0.0)	0 (0.0)

APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; BISAP: Bedside index for severity in acute pancreatitis.

physiology score, an age index and a chronic health index[24]. The BISAP scoring standard consists of five elements: blood urea nitrogen, disturbance of consciousness, systemic inflammatory response syndrome, age and pleural effusion[25]. A 3 mL sample of fasting peripheral venous blood was obtained from all patients on the first morning (6:00 AM) after admission. A blood sample was also collected from the control participants.

Laboratory methods

The blood samples from patients with AP and healthy controls were immediately centrifuged at 2500 *g* for 5 min. The sample supernatants were then stored at -80 ℃ until further investigation. The melatonin levels in serum were measured using an enzyme-linked immunosorbent assay (Changfeng Chemical Company, Shanghai, China).

Statistical analysis

The statistical analysis was performed using the SPSS 13.0 statistical program. The measurement data are expressed as the mean ± SE. Differences in MT between the mean values of various groups of experiments were compared using one-way analysis of variance and SNK post hoc analysis. The incidences of high scores for the APACHE Ⅱ and BISAP scoring systems in the high MT concentration group and the low MT concentration group were compared with a χ^2 test. A difference with a P value of < 0.05 was considered statistically significant. An receiver operating characteristic (ROC) curve was generated to analyze the ability of melatonin and the APACHE Ⅱ and BISAP scoring systems to predict SAP.

RESULTS

There was no significant difference in the age ($P = 0.751$) or sex ratio ($P = 1.000$) between patients with mild pancreatitis and severe pancreatitis. Biliary problems were the main factor in the MAP group. Conversely, most cases of SAP were caused by hyperlipidemia (46.2%). Both the APACHE Ⅱ scores and the BISAP scores in severe pancreatitis were significantly higher than in the mild cases at admission. The APACHE Ⅱ scores in the severe and mild AP cases were 12 points *vs* 6 points ($P < 0.001$), while the BISAP scores were 3 points *vs* 1 point ($P < 0.001$).

The median value of melatonin levels in the MAP group, the SAP group and the control group was 38.34, 26.77 and 30.73 ng/L, respectively. The melatonin level was significantly higher in patients with mild AP compared to patients with severe pancreatitis (38.34 ± 13.76 ng/L *vs* 26.77 ± 11.88 ng/L, $P = 0.021$). A similar trend was also observed in patients with mild disease compared to controls ($P = 0.003$). There was no significant difference in melatonin levels between MAP patients and healthy individuals (38.34 ± 13.76 ng/L *vs* 30.73 ± 2.96 ng/L, $P > 0.05$)

The Youden index of MT, the APACHE Ⅱ score and the BISAP score for predicting severe acute pancreatitis was 0.507, 0.602 and 0.674, respectively. The optimal cut-off value, sensitivity, specificity, Youden index and accuracy of the respective parameters in predicting SAP are shown in Table 2. The ROC curves of MT and the APACHE Ⅱ and BISAP scoring systems are presented in Figure 1.

Table 2 Comparison of the capability to predict severe acute pancreatitis

Variables	Sensitivity	Specificity	Youden index	Accuracy
MT ⩽ 28.74 ng/L	73.80%	76.90%	0.507	0.758
APACHE Ⅱ score ⩾ 9.5	76.90%	83.30%	0.602	0.872
BISAP score ⩾ 2.5	76.90%	90.50%	0.674	0.906

MT: Melatonin; APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; BISAP: Bedside index for severity in acute pancreatitis.

The optimal cutoff concentration of 28.74 ng/L for SAP, as determined from the ROC curve, was used to classify the patients into a high concentration group (34 cases) and a low concentration group (21 cases). The incidence of a high BISAP score (⩾ 3) was significantly greater in patients with alow melatonin concentration (⩽ 28.74 ng/L) compared to patients with a high melatonin concentration (> 28.74 ng/L). The incidence of a high BISAP score was 42.9% in patients with low melatonin compared to 14.7% in patients with high melatonin ($P = 0.02$). There was no significant difference in the incidence of high scores (⩾ 10) according to the APACHE Ⅱ scoring system between patients with high and low melatonin levels ($P > 0.05$) (Table 3).

DISCUSSION

In the clinic, 10%-20% of AP patients will develop severe acute pancreatitis, characterized by longer duration of disease, organ failure, systemic inflammatory response syndrome, and pancreatic necrosis. As a result, the disease pathogenesis is serious and complex. It has been reported

Table 3 Relationship between melatonin concentration and patient scores

Group[1]		APACHE Ⅱ score[2]				BISAP score[3]			
	Total cases	High score cases	Incidence	χ^2 value	*P* value	High score cases	Incidence	χ^2 value	*P* value
Low concentrations	21	8	8/21	0.821	> 0.05	9	9/21	5.422	0.02
High concentrations	34	9	9/34			5	5/34		

[1]⩽ 28.74 ng/L is defined as a low concentration, > 28.74 ng/L is defined as a high concentration; [2]APACHE Ⅱ score ⩾ 10 is defined as a high score, the two groups, $P > 0.05$; [3]BISAP ⩾ 3 is defined as a high score. BISAP ⩾ 3, low melatonin concentration compared to high concentration, $P = 0.02$. APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; BISAP: Bedside index for severity in acute pancreatitis.

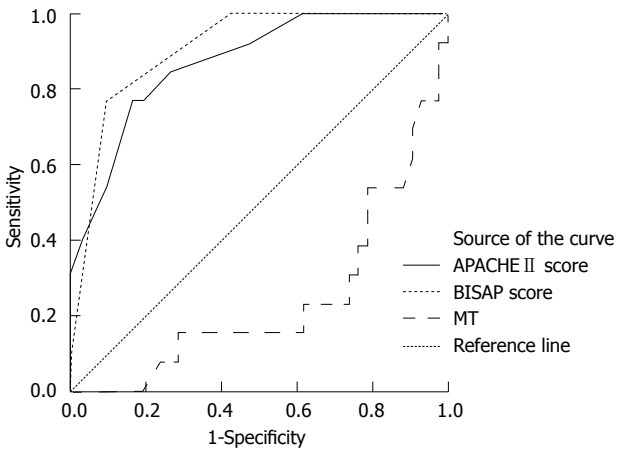

Figure 1 Receiver operating characteristic curves of melatonin, acute physiology and chronic health evaluation Ⅱ score and bedside index for severity in acute pancreatitis score to predict severe acute pancreatitis. MT: Melatonin; APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; BISAP: Bedside index for severity in acute pancreatitis.

that oxidative stress and lipid peroxidation caused by oxygen free radicals cause the destruction of acinar cells and abnormal expression of cytokines during AP pathogenesis[26]. Studies published in the literature concerning MT have demonstrated that it can stabilize cell membranes and protect the cells from oxidative damage. Moreover, MT can also penetrate all of the morphophysiological barriers in the human body and restore acinar cells with their lipophilic and hydrophilic characteristics[27]. Not only is melatonin itself an antioxidant; its metabolites can also reduce oxygen radicals. Additionally, melatonin can strengthen the activity of many antioxidants, such as SOD, Glutathione and CAT, and scavenges both oxygen free radicals and nitrogen free radicals[28,29]. MT has also been reported to have powerful anti-inflammatory and immunomodulatory effects by regulating the production of cytokines[30]. Furthermore, MT was found to promote the spontaneous regeneration process of pancreatic tissue through the activation of stellate cells[31]. MT is potentially capable of limiting pancreatic and associated organ damage produced during AP.

In our study, MT levels in the MAP group were significantly higher than the controls on the first day after admission. This result highlights the importance of the human endocrine system in AP development. The inflammatory response occurs in the early stage of AP prior to the activation of trypsinogen. The organism defense against inflammation occurs primarily through the action of the hypothalamus-pituitary-adrenal axis to increase the secretion of endogenous cortisol. However, the adrenal glands of patients with AP are in a state of relative insufficiency at the onset of disease[32]. Therefore, they may be protected by other mechanisms such as the recruitment of MT to reduce pancreatic damage; thus, an increase in MT will promote a mild disease course. It is well known that inflammation in acute pancreatitis is caused by the imbalance of pro-inflammatory factors and anti-inflammatory factors. This imbalance is more severe in patients with SAP. Perras *et al*[33] showed a clear negative correlation between disease severity and MT levels in patients suffering from severe inflammation. The pineal secretions from patients with profound systemic inflammatory responses were inhibited. This finding could explain why MT concentrations in the SAP group were significantly lower than those in the MAP group in this study. Our results indicate that the MT level is closely related to AP severity. Thus, a lower MT concentration is associated with more severe disease. Conversely, higher MT concentrations are associated with less severe disease. Our data are consistent with the view supported by Belyaev *et al*[32], who indicated that endogenous high levels of serum MT play a protective role in the early course of AP. Our findings have raised the hope that we may be able to control disease severity by using early detection of serum MT concentrations.

The ROC curve is used to compare the accuracy of two or more diagnostic tests. The ROC is considered the most reliable method of evaluating patient prognosis. In this study, we used the ROC curve to assess the relationship between MT and SAP by combining MT values with the APACHE Ⅱ and BISAP scoring systems. As shown in the results, the accuracy of the two scores for SAP were 0.872 and 0.906, respectively. This result indicates that both scoring systems can predict SAP accurately. However, these scoring systems are clinically cumbersome and difficult to remember for clinicians. In addition, it is time-consuming to monitor changes in condition accurately and rapidly using these systems[34,35]. The accuracy of SAP detection using MT was 0.758, and the optimal cut-off concentration was 28.74 ng/L in this study. Our data show that MT levels can predict SAP. Our results further demonstrate that the severity of disease can be determined objectively and accurately by early measurement of serum MT levels. Patients with AP may develop SAP when their MT concentration is below 28.74 ng/L.

Singh *et al*[36] reported that a BISAP score ≥ 3 was associated with an increased risk of developing organ failure. Thus, a BISAP score of 3 was used to divide the patients into the high score and low score groups. A key result of this study was the observation that MT levels were closely related to the BISAP score. The incidence of high score (≥ 3) was significantly increased in patients with low melatonin concentration (≤ 28.74 ng/L) compared to patients with high melatonin concentration (> 28.74 ng/L). Our results clearly demonstrate that a high BISAP score reflects a more severe AP condition and is associated with reduced MT concentration. Conversely, patients with higher MT concentrations had fewer incidences of a high BISAP score. Thus, our results agree with previously published data[36].

Chatzicostas *et al*[37] reported that SAP and its complications could be predicted accurately when the patient had an APACHE Ⅱ score ≥ 10. Therefore, patients were classified into high score and low score groups, with a dividing score of 10 between the groups. However, in our study there was no significant difference in the high score incidence between the low MT concentration group and the high MT concentration group. The reasons for this result include the following: (1) the APACHE Ⅱ score requires knowledge of the patient history, which may not be available if the patient is unconscious, intubated, or transferred from an outside hospital lacking detailed records, thus resulting in an incorrect number of points; and (2) the APACHE Ⅱ score includes a chronic health index, which is not directly correlated with AP. Thus, the relationship between MT levels and the APACHE Ⅱ score will require further studies.

In conclusion, the results of the present study reveal that exogenous melatonin may prevent the damage caused during acute pancreatitis due to its antioxidant, anti-inflammatory, and immunomodulatory properties. The variations of MT concentration might reflect the degree of AP severity to some extent. As a result, we can determine the severity of disease more objectively, accurately and rapidly by measuring the levels of serum melatonin. In addition, a melatonin concentration ≤ 28.74 ng/L was associated with an increased risk of developing SAP. The current clinical study was performed in a single center, and this research had some limitations. Therefore, large sample investigations will be needed to explore the value of serum melatonin in determining the severity of AP.

COMMENTS

Background

Acute pancreatitis (AP) includes both severe AP (SAP) and mild AP (MAP). SAP has a high reported mortality rate. It is important to assess the severity and changes in a patient's condition in a timely and accurate manner to provide comprehensive treatment. This approach could improve the prognosis and reduce mortality. Therefore, authors assessed the predictive value of plasma melatonin in identifying acute pancreatitis in combination with the acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) and bedside index for severity in acute pancreatitis (BISAP) scoring systems.

Research frontiers

A number of studies have showed that melatonin (MT) plays a protective role in AP and its associated organ injuries using animal models. The research objective herein was to assess the value of plasma MT in determining the severity of AP. Additionally, authors predicted SAP and analyzed the changes of plasma MT levels and the use of two scoring systems.

Innovations and breakthroughs

The authors have evaluated the ability of melatonin and the APACHE Ⅱ and BISAP scoring systems to predict SAP by using a receiver operating characteristic (ROC) curve. The optimal cutoff concentration for SAP from the ROC curve was used to classify the patients into a high concentration group and a low concentration group. The differences in the incidence of high scores for the APACHE and BISAP scores scoring systems were compared between the two groups. In the present study, melatonin was shown to play a protective role in the early course of human acute pancreatitis, and concentration variations were closely related to the severity of AP and the BISAP score. The authors can determine the severity of AP more objectively, accurately and rapidly by measuring the levels of serum melatonin. When the melatonin concentration is at or below 28.74 ng/L, AP patients may develop SAP.

Applications

The study results suggest that exogenous melatonin may prevent the damage caused by AP due to its antioxidant, anti-inflammatory, and immunomodulatory properties. Variations of MT concentration might reflect the degree of severity of AP to some extent.

Terminology

The APACHE Ⅱ score contains 12 monitoring indicators, and the final score is composed of an acute physiology score, an age index and a chronic health index. The APACHE Ⅱ scoring system is the most commonly used scoring system for determining the severity and prognosis of AP. The BISAP scoring standard consists of five elements: blood urea nitrogen, disturbance of consciousness, systemic inflammatory response syndrome, age and pleural effusion.

Peer review

The authors focus on the clinical significance of melatonin concentrations in predicting the severity of AP. The results suggest that melatonin concentration variations are closely related to the severity of AP and the BISAP score. The serum melatonin level can be used to evaluate the severity of disease objectively, accurately and rapidly.

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META-ANALYSIS

Safety and efficacy of single-incision laparoscopic surgery for appendectomies: A meta-analysis

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Abstract

AIM: To compare single incision laparoscopic surgery for an appendectomy (SILS-A) with conventional laparoscopic appendectomy (C-LA) when implemented by experienced surgeons.

METHODS: Studies and relevant literature regarding the performance of single-incision laparoscopic surgery *vs* conventional laparoscopic surgery for appendectomy were searched for in the Cochrane Central Register of Controlled Clinical Trials, MEDLINE, EMBASE and World Health Organization international trial register. The operation time (OR time), complications, wound infection and postoperative day using SILS-A or C-LA were pooled and compared using a meta-analysis. The risk ratios and mean differences were calculated with 95%CIs to evaluate the effect of SILS-A.

RESULTS: Sixteen recent studies including 1624 patients were included in this meta-analysis. These studies demonstrated that, compared with C-LA, SILS-A has a similar OR time in adults but needs a longer OR time in children. SILS-A has similar complications, wound infection and length of the postoperative day in adults and children, and required similar doses of narcotics in children, the pooled mean different of -0.14 [95%CI: -2.73-(-2.45), $P > 0.05$], the pooled mean different of 11.47 (95%CI: 10.84-12.09, $P < 0.001$), a pooled RR of 1.15 (95%CI: 0.72-1.83, $P > 0.05$), a pooled RR of 1.9 (95%CI: 0.92-3.91, $P > 0.05$), a pooled RR of 1.01 (95%CI: 0.51-2.0, $P > 0.05$) a pooled RR of 1.86 (95%CI: 0.77-4.48, $P > 0.05$), the pooled mean different of -0.25 (95%CI: -0.50-0, $P = 0.05$) the pooled mean different of -0.01 (95%CI: -0.05-0.04, $P > 0.05$) the pooled mean different of -0.13 (95%CI: -0.49-0.23, $P > 0.05$) respectively.

CONCLUSION: SILS-A is a technically feasible and reliable approach with short-term results similar to those obtained with the C-LA procedure.

Key words: Single incision; Laparoscopy; Appendicitis; Children; Adult

Core tip: Single incision laparoscopic surgery for an appendectomy (SILS-A) is widely accepted and has become the best option for treatment of appendicitis. Compared with conventional laparoscopic appendectomy, the safety and efficacy of SILS-A is not known. This study clarified that SILS-A has a similar operation time in adults but needs more time in children, has similar complications, wound infection and length of the postoperative day in adults and children, and needs similar doses of narcotics in children.

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INTRODUCTION

Today, approximately 8% of the population will undergo appendectomy for acute appendicitis over their lifetime in Europe. An appendectomy comprises the surgical resection of the appendix and is frequently performed as an emergency process in the management of a patient suffering from acute appendicitis, a condition in which the appendix becomes inflamed and putrescent. The operation can be performed with minimally invasive surgery or as an open procedure.

Laparoscopic surgery was first used about 100 years ago, and the concept of minimally invasive surgery has significantly affected the field of traditional surgery. The first laparoscopic appendectomy was performed by the gynecologist Semm[1]. In a classic laparoscopic appendectomy, three to four incisions are required for the placement of multiple trocars. Driven by a quest toward less abdominal trauma in surgery, improved cosmesis, the potential reduction in postoperative pain, and a shorter hospital stay, specialty cameras, ports, and instruments have been developed, and minimal access surgery has undergone an accelerated process of evolution. A recent development in appendectomy has been the introduction of less invasive methods.

Single incision laparoscopic surgery applies a single multi-luminal port, or multiple mono-luminal ports, through a single skin incision. With the appearance of natural orifice translumenal endoscopic surgery, single incision laparoscopic surgery for an appendectomy (SILS-A) can be used to perform advanced[2-10], as well as preliminary procedures[11-24]. While this technique has been embraced by surgeons worldwide, the procedures and instruments used are still in the basic stages of investigation. Currently, two different methods exist for single-incision access. One involves the application of traditional, low profile laparoscopic ports that are clustered within a single skin incision, but penetration the peritoneal cavity through separate fascial incisions. The other involves the adoption of specialized ports created to provide multiple channels through a single port for one larger fascial incision. Both of methods have a good cosmetic effect. Despite its ameliorating effects, conventional laparoscopic appendectomy (C-LA) still requires three to four abdominal incisions for completion of the procedure. Each incision adds to potential morbidity risks, including bleeding, hernia, or internal organ damage[25,26]. There is little published data on the feasibility, safety, and clinical advantage of the procedure. Therefore, this study will analyze and compare the short-term surgical results of SILS-A and C-LA. The primary aim of this meta-analysis was to evaluate SILS-A *vs* C-LA; the secondary aims were to determine the difficulties, limitations or advantages of SILS-A.

MATERIALS AND METHODS

Publication search

Four bibliographic databases (Cochrane Central Register of Controlled Clinical Trials, MEDLINE, EMBASE and the World Health Organization international trial register) were searched for all relevant literature, including articles referenced in the publications. The medical subject headings (MeSH) and keywords searched for individually and in combination were as follows: "single-incision laparoscopic surgery" "multiport laparoscopic surgery" or "conventional laparoscopic" and "appendectomies". The last search was done on January 20, 2013.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) patients should be at least 1 year of age; (2) suspected acute appendicitis on clinical and radiographic (computed tomography) grounds; (3) male or female (excluding pregnant females); (4) patients with American Society of Anesthesiology score < 3; (5) patients informed about the study, and will have read, understood and signed the patient informed; and (6) studies that provided information on at least one of the outcome measures. When a study reporting the same patient cohort was included in several publications, only the most recent or complete study was selected.

The exclusion criteria were as follows: (1) prior open laparotomy with incision through the umbilicus; (2) mental illness, dementia, or inability to provide informed consent; (3) chronic pain requiring daily medication (including opiates and NSAIDs); (4) pregnancy; (5) case reports; (6) articles that were not full text, or non-comparative studies; and (7) open operations.

Data extraction

For identified eligible studies, two reviewers using a standard form containing pre-specified outcomes would have undertaken data extraction independently. Clarification was sought where there was potential data collection but not reported. Any differences of opinion were resolved among the reviewers, and where necessary referred to a fourth party for arbitration.

Statistical analysis

Statistical analysis was performed using Review Manager (RevMan) software version 5.0.0 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark). A pooled RR and a pooled Mean Different with 95%CI were used to assess outcomes of the studies; statistical heterogeneity was tested by the χ^2 test. According to the Forest plot, heterogeneity was limited, so we used the Mantel-Haenszel fixed effect model. The significance of the pooled RR was determined by the Z test and statis-

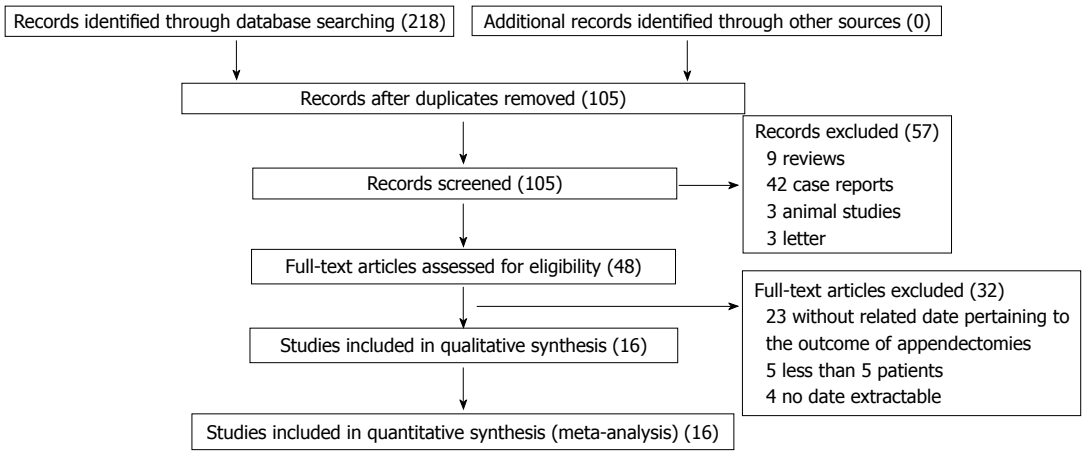


Figure 1 Flow chart for the selection of the studies.

Table 1 Main characteristics of the 10 included studies in adults

Ref.	Year	SILS-A (*n*)	C-LA (*n*)	Age (yr)		M:F	
				SILS-A	C-LA	SILS-A	C-LA
Lee *et al*[27]	2009	72	108	30.3 ± 16.4	33.6 ± 18.6	24:46	56:52
Cho *et al*[28]	2011	23	20	44.7	39.2	14:9	11:9
Teoh *et al*[29]	2011	30	60	32.97 ± 13.31	34.88 ± 11.45	19:11	38:22
Park *et al*[30]	2012	42	62	23.9 ± 11.9	29.9 ± 12.2	14:28	42:21
Kim *et al*[31]	2011	17	33	21.0	28.0	1:10	21:12
Vilallonga *et al*[32]	2012	46	41	34.2 (13.3)	37.7 (13.2)	19:27	22:19
Raakow *et al*[39]	2011	20	20	27.75 ± 8.26	31.75 ± 9.30	8:12	10:10
Amos *et al*[40]	2011	27	17	37.74 ± 18.85	33.71 ± 12.50	6:21	6:11
Chow *et al*[41]	2010	40	33	31.65 ± 15.36	29.85 ± 14.93	18:22	15:18
Kang *et al*[42]	2010	15	25	35.5 ± 13.2	37.9 ± 14.5	8:07	14:11

SILS-A: Single-incision laparoscopic surgery for appendectomy; C-LA: Conventional laparoscopic appendectomy; M: Male; F: Female.

tical significance was considered at $P < 0.05$. Publication bias was estimated using a funnel plot with an Egger's linear regression test, and funnel plot asymmetry on the natural logarithm scale of the RR was measured by a linear regression approach.

RESULTS

Study characteristics

In total, 16 studies were included in the meta-analysis[27-42]. All of these studies were published after 2009 and comprised 751 adult patients, of whom 332 were operated on using SILS-A and 419 were operated on using C-LA. The sample size of the trials ranged from 15 to 108. Eight hundred and seventy three of the patients were children, of whom 428 were operated on using SILS-A and 445 were operated on using C-LA. The sample size of the trials ranged from 8 to 180. Moreover, some studies reported single-incision laparoscopic surgery for appendicitis, but did not report information regarding C-LA and were therefore not compared in this meta-analysis. Other studies did not provide any information about SILS-A *vs* C-LA, and were excluded in present meta-analysis (Figure 1). Tables 1-4 list the main characteristics of the 16 studies included in this analysis.

Meta-analysis results

The present meta-analysis demonstrated the pooled mean different of [-0.14, 95%CI: -2.73-(-2.45), $P > 0.05$, Figure 2A], the pooled RR of 1.15 (95%CI: 0.72-1.83), $P > 0.05$, Figure 3A) a pooled RR of 1.01 (95%CI: 0.51-2.0), $P > 0.05$, Figure 4A, a pooled mean different of -0.25 (95%CI: -0.5-0.0), $P = 0.05$, Figure 5A, a pooled mean different of 11.47 (95%CI: 10.84-12.09), $P < 0.001$, Figure 2B, a pooled RR of 1.9 (95%CI: 0.92-3.91), $P > 0.05$, Figure 3B, the pooled RR of 1.86 (95%CI: 0.77-4.48), $P > 0.05$, Figure 4B, the pooled mean different of -0.01 (95%CI: -0.05-0.04), $P > 0.05$, Figure 5B, the pooled mean different of -0.13 (95%CI: -0.49-0.23), $P > 0.05$, Figure 6, respectively. It revealed that SILS-A is feasible, and appears to have results similar to C-LA in our comparisons. But, in children, SILS-A needs more operative time than C-LA.

Operation time (min): Nine studies (701 patients) provided data on operation time for adults. The pooled results indicated that SILS-A has similar results to C-LA [weighted mean differences (WMD), -0.14 (95%CI: -2.73-2.45), $P > 0.05$]. The χ^2 and I^2 were 21.57 ($P = 0.0006$) and 63%, respectively, indicating heterogeneity among the studies (Figure 2A). Six studies (873 patients)

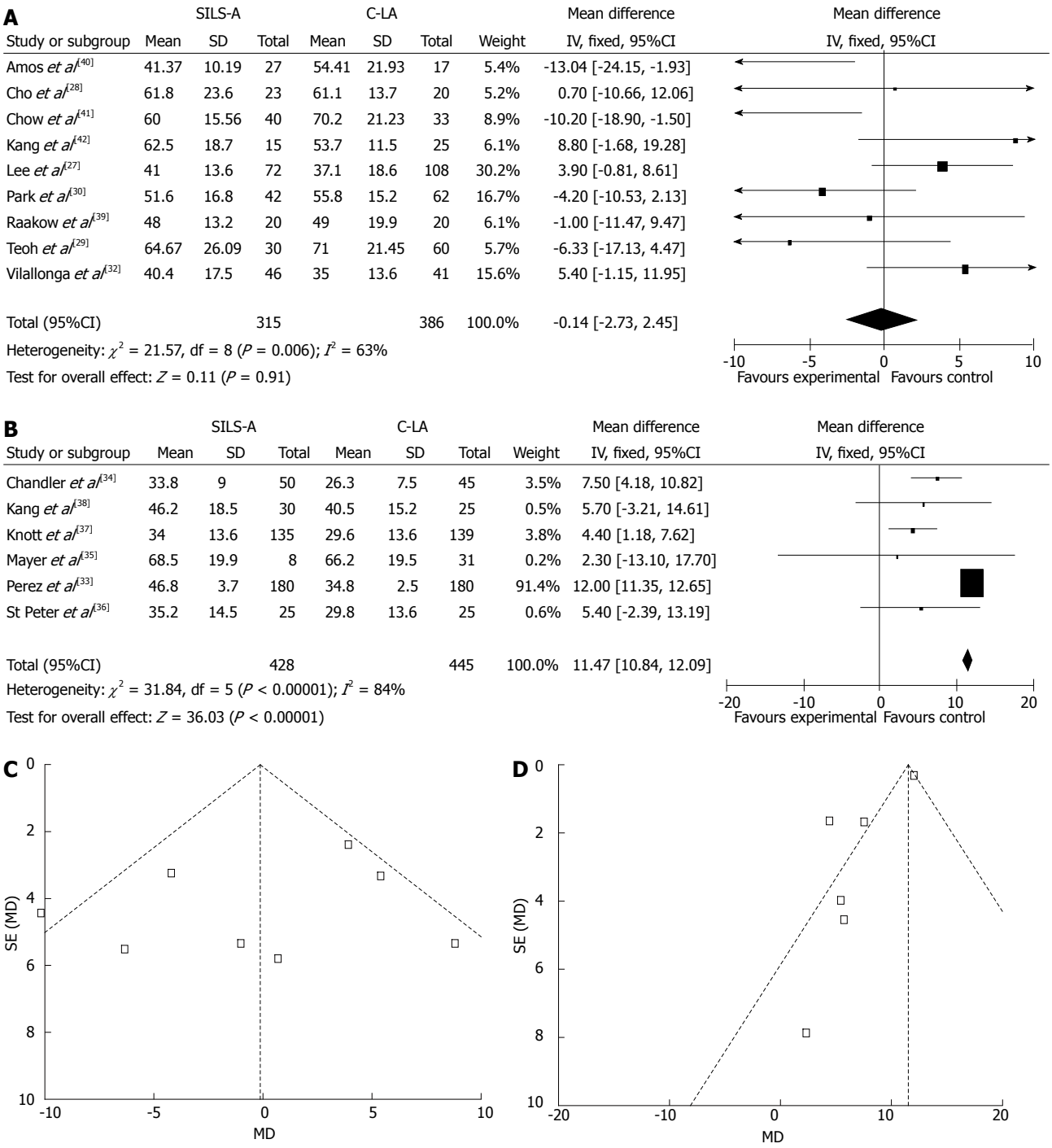


Figure 2 Forest plot of the comparison of single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: operation time (min). A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for adult; B: SILS-A *vs* C-LA in terms of short-term results for children; C: SILS-A *vs* C-LA in terms of short-term results for adult, mean differences (MDs); D: SILS-A *vs* C-LA in terms of short-term results for children, MDs. MDs are shown with 95%CI.

provided data on operation time for children. The pooled the indicated that SILS-A requires more time than C-LA [WMD, 11.47 (95%CI: 10.84-12.09), $P < 0.001$]. The χ^2 and I^2 were 31.84 ($P < 0.001$) and 84%, respectively, indicating heterogeneity among the studies (Figure 2B).

Complications: Ten studies (751 patients) provided data on complications in adults. Complications occurred in 27 of 332 (8.1%) patients after SILS-A and in 33 of 419 (7.8%) after C-LA. Pooling the results indicated that SILS-A had slightly, but not significantly, more complications than C-LA [WMD 1.15 (95%CI: 0.72-1.83), $P > 0.05$]. The χ^2 and I^2 were 6.16 ($P = 0.72$) and 0%, which excluded heterogeneity in the studies (Figure 3A). Six studies (873 patients) provided data on complications in children. Complications occurred in 18 of 428 (4.2%) patients after SILS-A and in 11 of 445 (2.4%) patients after C-LA. Pooling the results indicated that SILS-A and C-LA

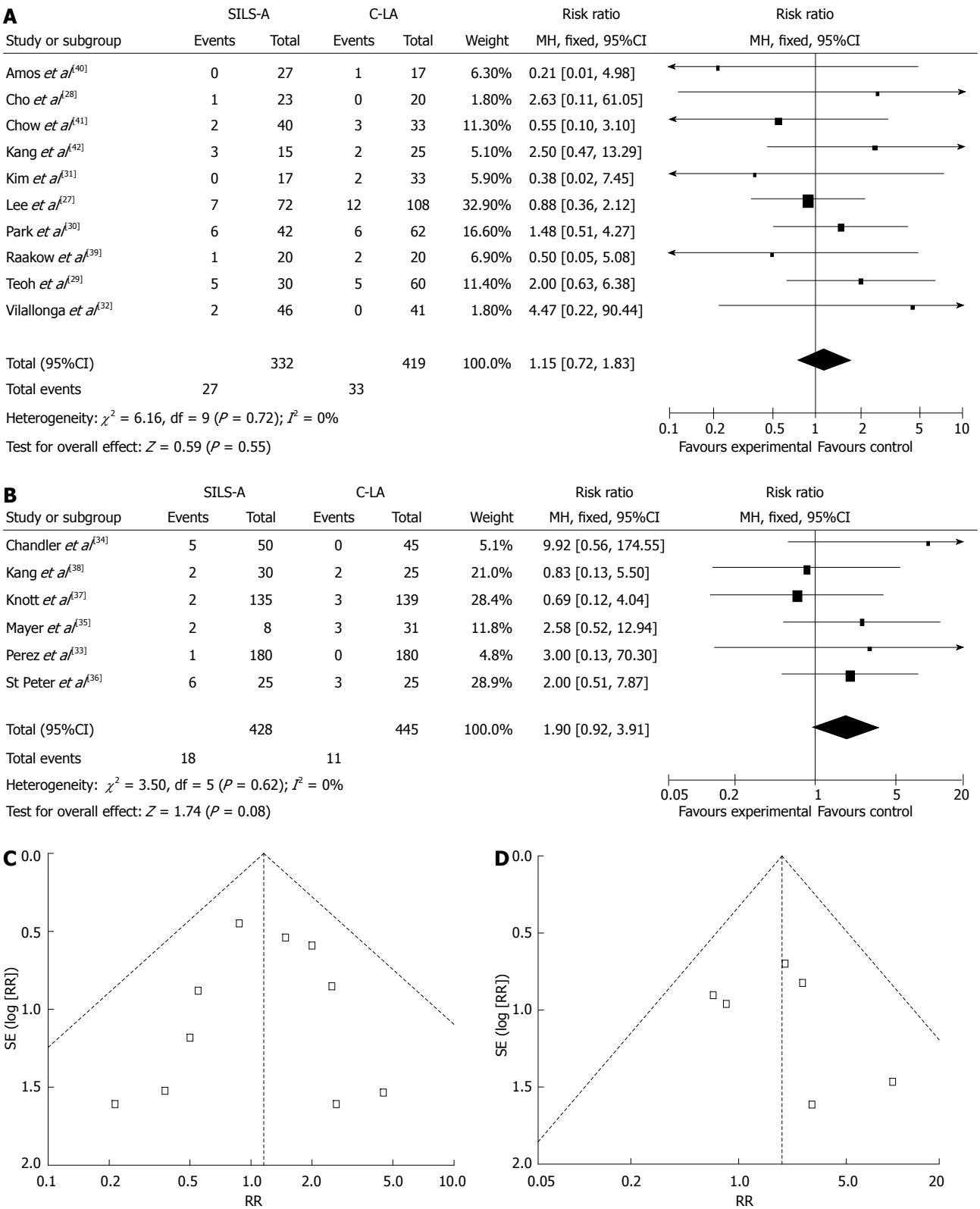


Figure 3 Forest plot of comparison: Single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: Complications. A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for adult; B: SILS-A *vs* C-LA in terms of short-term results for children; C: SILS-A *vs* C-LA in terms of short-term results for adult, risk ratios (RRs); D: SILS-A *vs* C-LA in terms of short-term results for children, RRs. RRs are shown with 95%CI.

have the similar levels of complications [WMD a pooled RR of 1.9 (95%CI: 0.92-3.91), $P > 0.05$]. The χ^2 and I^2 were 3.5 ($P = 0.62$) and 0%, which excluded heterogeneity in the studies (Figure 3B).

Wound infection: Seven studies (577 patients) provided

Table 2 Main characteristics of the six included studies in children

Ref.	Year	SILS-A (*n*)	C-LA (*n*)	Age		M:F	
				SILS-A	C-LA	SILS-A	C-LA
Perez *et al*[33]	2012	25	25	8.7 ± 0.6	8.9 ± 0.6	10:15	15:10
Chandler *et al*[34]	2010	50	45	11.1 ± 3.6	11.7 ± 3.8	26:24	34:11
Mayer *et al*[35]	2011	8	31	12.3 ± 2.4	12.3 ± 2.4		
St Peter *et al*[36]	2011	180	180	11.1 ± 3.5	11.1 ± 3.3	99:81	92:88
Knott *et al*[37]	2012	135	139	11.0 ± 3.5	10.9 ± 3.4	72:63	70:69
Kang *et al*[38]	2011	30	25	9.3 ± 4.0	8.7 ± 3.5	17/13	14/11

SILS-A: Single-incision laparoscopic surgery for appendectomy; C-LA: Conventional laparoscopic appendectomy; M: Male; F: Female.

Table 3 Result of the 10 included studies in adult children

Ref.	Postoperative day (d)		Complications		OR time (min)		Wound infection	
	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA
Lee *et al*[27]	2.0 ± 1.4	2.0 ± 1.3	7	12	41.0 ± 13.6	37.1 ± 18.6	4	7
Cho *et al*[28]			1	0	61.8 ± 23.6	61.1 ± 13.7		
Teoh *et al*[29]			5	5	64.67 ± 26.09	71 ± 21.45	2	4
Park *et al*[30]	2.6 ± 1.0	2.9 ± 1.9	6	6	51.6 ± 16.8	55.8 ± 15.2	3	2
Kim *et al*[31]			0	2			0	2
Vilallonga *et al*[32]			2	0	40.4 ± 17.5	35.0 ± 13.6		
Raakow *et al*[39]	4.12 ± 0.61	4.65 ± 0.98	1	2	48.0 ± 13.2	49.0 ± 19.9	1	1
Amos *et al*[40]	3.70 ± 2.52	3.82 ± 1.24	0	1	41.37 ±10.19	54.41 ± 21.93	5	
Chow *et al*[41]	1.36 ± 0.95	2.36 ± 2.62	2	3	60.0 ± 15.56	70.2 ± 21.23	2	2
Kang *et al*[42]	6.8 ± 1.8	6.4 ± 1.6	3	2	62.5 ± 18.7	53.7 ± 11.5	1	1

OR time: Operation time; SILS-A: Single-incision laparoscopic surgery for appendectomy; C-LA: Conventional laparoscopic appendectomy.

Table 4 Result of the ix included studies in children

Ref.	Postoperative day (d)		Complications		OR time (min)		Wound infection		Doses of narcotics	
	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA	SILS-A	C-LA
Perez *et al*[33]			1	0	46.8 ± 3.7	34.8 ± 2.5				
Chandler *et al*[34]	1.1 ± 0.4	1.2 ± 0.5	5	0	33.8 ± 9	26.3 ± 7.5	4	0	0.9 ± 0.9	1.4 ± 1.3
Mayer *et al*[35]	3.63 ± 1.2	3.68 ± 1.3	2	3	68.5 ± 19.9	66.2 ± 19.5			4.75 ± 3.3	7.33 ± 3.0
St Peter *et al*[36]	0.95 ± 0.3	0.93 ± 0.3	6	3	35.2 ± 14.5	29.8 ± 11.6	6	3	9.6 ± 4.9	8.5 ± 4.3
Knott *et al*[37]	0.92 ± 0.2	0.94 ± 0.3	2	3	34.0 ± 13.6	29.6 ± 13.6	2	3	5.7 ± 3.5	5.3 ± 3.2
Kang *et al*[38]	4.0 ± 1.5	3.8 ± 2.0	2	2	46.2 ± 18.5	40.5 ± 15.2	2	1		

OR time: Operation time; SILS-A: Single-incision laparoscopic surgery for appendectomy; C-LA: Conventional laparoscopic appendectomy.

data on wound infections in adults. Wound infections occurred in 13 of 236 (5.5%) patients after SILS-A and in 19 of 341 (5.6%) patients after C-LA. Pooling the results indicated that SILS-A and C-LA have the similar levels of wound infection [WMD 1.01 (95%CI: 0.51-2.0), $P > 0.05$]. The χ^2 and I^2 were 1.44 ($P = 0.96$) and 0%, which excludes heterogeneity in the studies (Figure 4A). Five studies (784 patients) provided data on wound infections in children. Wound infections occurred in 14 of 395 (3.5%) patients after SILS-A and in 7 of 398 (1.7%) patients after C-LA. The results indicated that SILS-A has more wound infections, but at an acceptable level. Pooling the results of wound infection [WMD 1.86 (95%CI: 0.77-4.48), $P > 0.05$]. The χ^2 and I^2 were 2.23 ($P = 0.53$) and 0%, which excluded heterogeneity in the studies (Figure 4B).

Postoperative days (d): Six studies (481 patients) provided data on postoperative days for adult. Pooling the results indicated that SILS-A has the slightly better results than C-LA [WMD -0.25 (95%CI: -0.50-0), $P > 0.05$]. The χ^2 and I^2 were 6.48 ($P = 0.26$) and 23%, respectively, indicating heterogeneity among the studies (Figure 5A). Five studies (822 patients) provided data on postoperative days for children. Pooling the results indicated that SILS-A has the same results as C-LA [WMD, -0.01 (95%CI: -0.05-0.04), $P > 0.05$]. The χ^2 and I^2 were 2.08 ($P = 0.72$) and 23%, respectively, which excluded heterogeneity in the studies (Figure 5B).

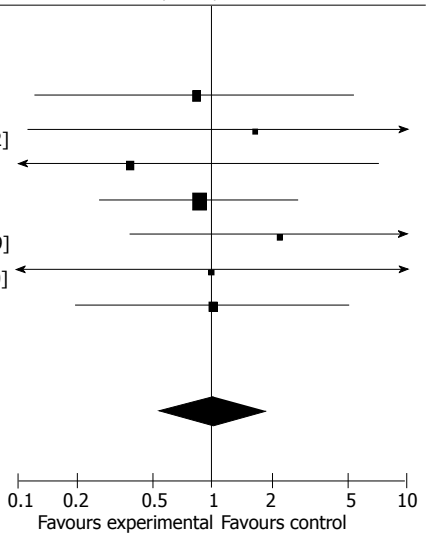
Doses of narcotics: Four studies (768 patients) provided data on doses of narcotics for children. Pooling the results indicated that SILS-A had similar results to C-LA, [WMD -0.25 (95%CI: -0.50-0), $P > 0.05$]. The χ^2 and I^2 were 14.25 ($P = 0.0003$) and 79%, respectively, indicating

A

Study or subgroup	SILSA Events	SILSA Total	C-LA Events	C-LA Total	Weight	Risk ratio MH, fixed, 95%CI
Amos *et al*[40]	0	0	0	0		Not estimable
Cho *et al*[28]	0	0	0	0		Not estimable
Chow *et al*[41]	2	40	2	33	14.1%	0.82 [0.12, 5.54]
Kang *et al*[42]	1	15	1	25	4.8%	1.67 [0.11, 24.72]
Kim *et al*[31]	0	17	2	33	11.1%	0.38 [0.02, 7.45]
Lee *et al*[27]	4	72	7	108	36.0%	0.86 [0.26, 2.82]
Park *et al*[30]	3	42	2	62	10.4%	2.21 [0.39, 12.69]
Raakow *et al*[39]	1	20	1	20	6.4%	1.00 [0.07, 14.90]
Teoh *et al*[29]	2	30	4	60	17.1%	1.00 [0.19, 5.15]
Vilallonga *et al*[32]	0	0	0	0		Not estimable
Total (95%CI)		236		341	100.0%	1.01 [0.51, 2.00]
Total events	13		19			

Heterogeneity: χ^2 = 1.44, df = 6 (P = 0.96); I^2 = 0%

Test for overall effect: Z = 0.04 (P = 0.97)

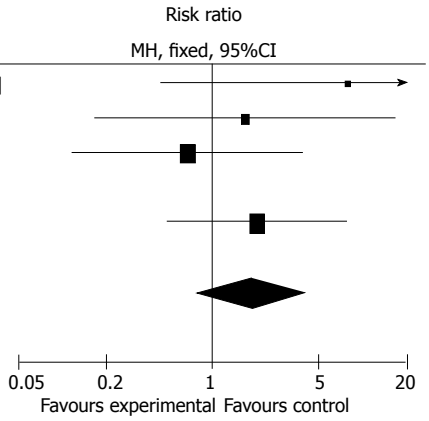


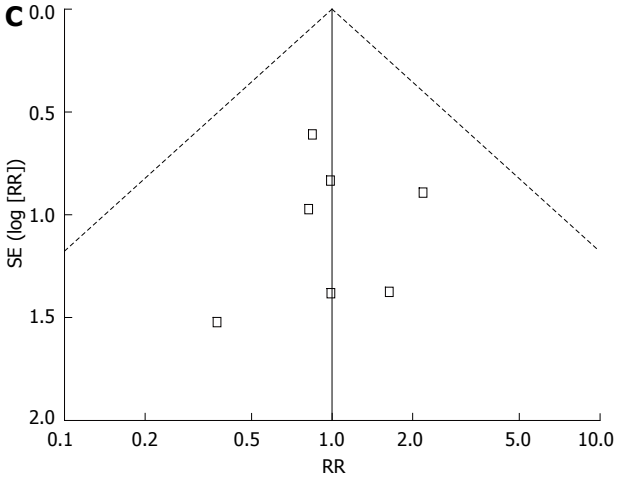
B

Study or subgroup	SILS-A Events	SILS-A Total	C-LA Events	C-LA Total	Weight	Risk ratio MH, fixed, 95%CI
Chandler *et al*[34]	4	50	0	45	6.9%	8.12 [0.45, 146.71]
Kang *et al*[38]	2	30	1	25	14.4%	1.67 [0.16, 17.32]
Knott *et al*[37]	2	135	3	139	39.0%	0.69 [0.12, 4.04]
Mayer *et al*[35]	0	0	0	0		Not estimable
St Peter *et al*[36]	6	180	3	180	39.6%	2.00 [0.51, 7.87]
Total (95%CI)		395		389	100.0%	1.86 [0.77, 4.48]
Total events	14		7			

Heterogeneity: χ^2 = 2.23, df = 3 (P = 0.53); I^2 = 0%

Test for overall effect: Z = 1.39 (P = 0.16)





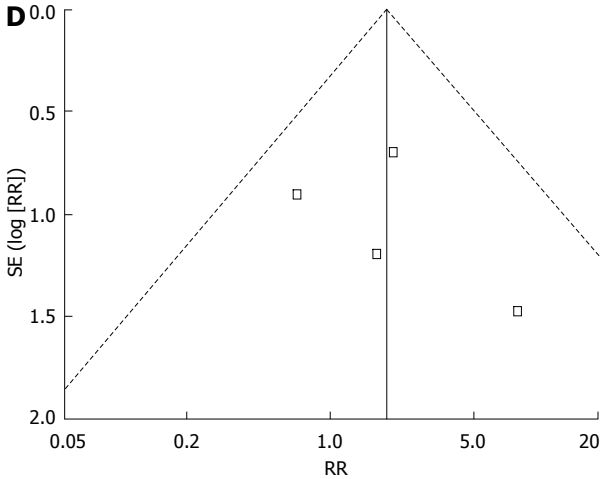


Figure 4 Forest plot of comparison: Single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: wound infection. A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for adult; B: SILS-A *vs* C-LA in terms of short-term results for children; C: SILS-A *vs* C-LA in terms of short-term results for adult, risk ratios (RRs); D: SILS-A *vs* C-LA in terms of short-term results for children, RRs. RRs are shown with 95%CI.

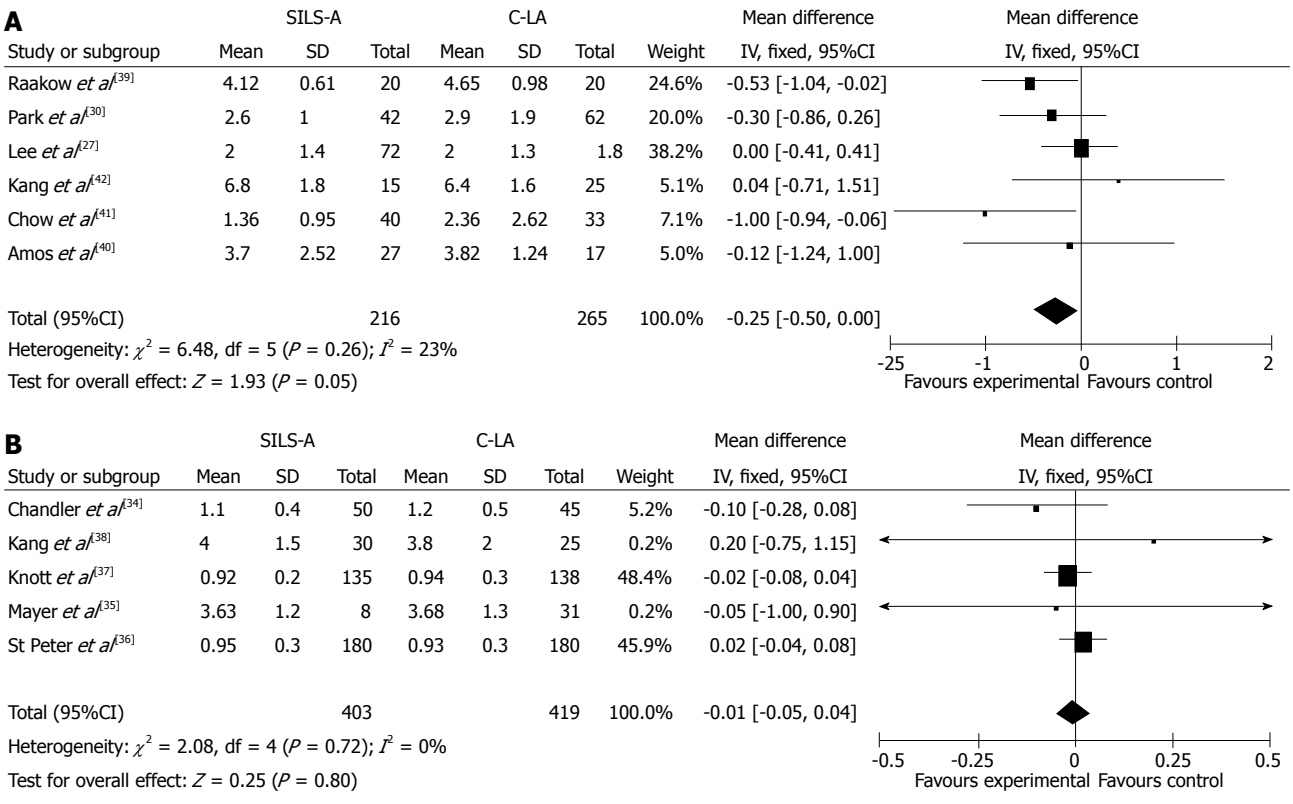


Figure 5 Forest plot of comparison: Single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: postoperative day (d). A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for adult; B: SILS-A *vs* C-LA in terms of short-term results for children; C: SILS-A *vs* C-LA in terms of short-term results for adult, mean differences (MDs); D: SILS-A *vs* C-LA in terms of short-term results for children, MDs. MDs are shown with 95%CI.

heterogeneity among the studies (Figure 6).

Publication bias

A funnel plot was created to access the publication bias of the literature. The shapes of the funnel plots did not reveal any evidence of obvious asymmetry (Figures 2C, 2D, 3C, 3D, 4C, 4D, 5C, 5D and 6B).

DISCUSSION

The straightforward conclusion from the 16 included studies is that compared with C-LA, SILS-A has acceptable complications, similar recovery, and the same OR times for patients.

Arguments against the use SILS-A cite the lack of evidence regarding patient benefit over open surgery or CL-A. The potential requirement for advanced instrumentation may also translate into increased costs. In addition, the lack of pneumoperitoneum leaks, triangulation, and instrument "clashing" are perceived as real disadvantages of this procedure, thereby increasing its difficulty. From our study, the umbilical incision permitted only

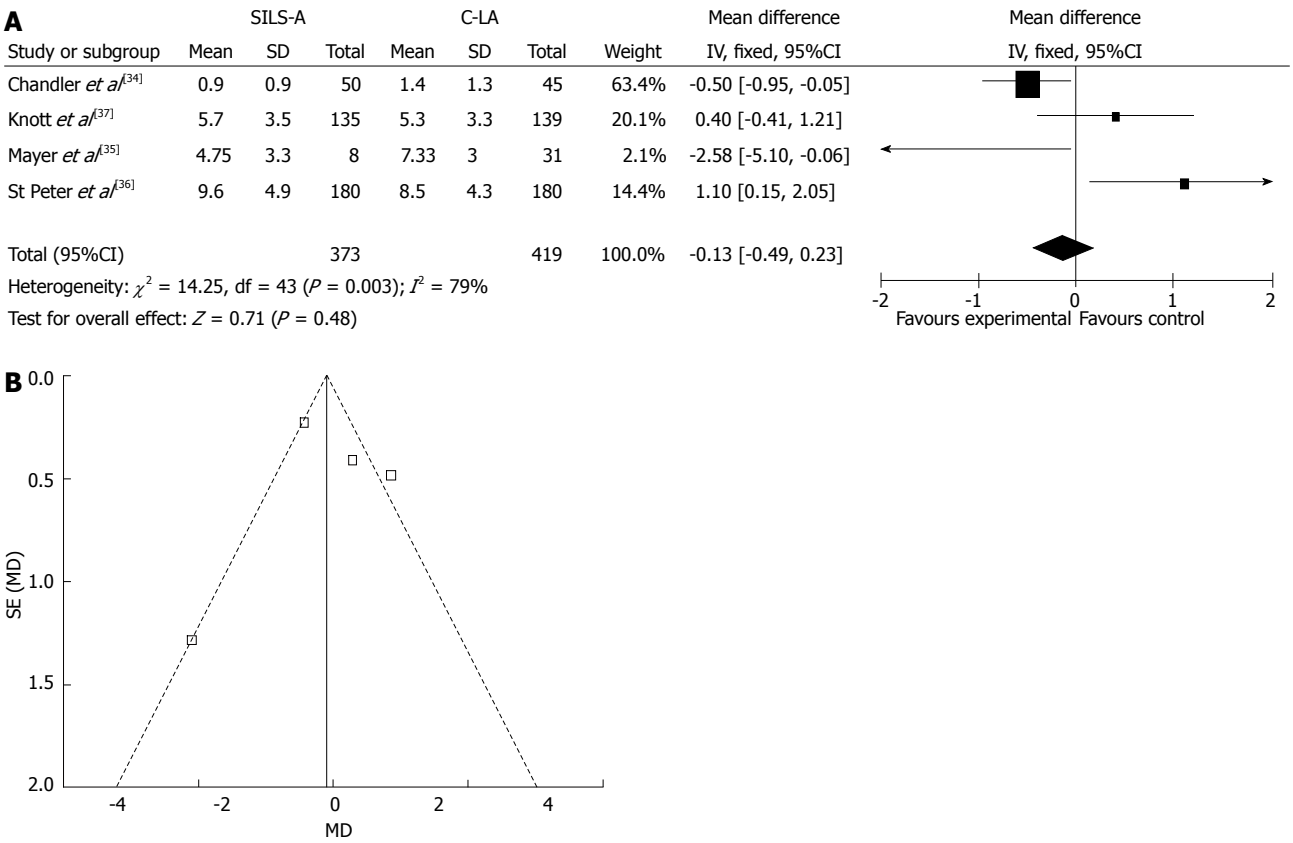


Figure 6 Forest plot of comparison: Single-incision laparoscopic surgery for appendectomies *vs* conventional laparoscopic appendectomy in terms of short-term results, outcome: doses of narcotics. A: Single-incision laparoscopic surgery for appendectomies (SILS-A) *vs* conventional laparoscopic appendectomy (C-LA) in terms of short-term results for children; B: SILS-A *vs* C-LA in terms of short-term results for children, mean differences (MDs). MDs are shown with 95%CI.

one laparoscope and one instrument into the abdominal cavity concomitantly, which ensured less trauma than the C-LA. Coaxiality was not a significant problem, except for a few of patients in whom we adopted flexible and rotating instruments. Moreover, tilting the operating table enabled us to achieve adequate exposure and dissection for the majority of patients. However, ligation of the appendix was a restricted phase of the procedure. In children, the surgery space is smaller and the lack of ancillary equipment increasing the difficulty. This is why SILS-A requires a longer time than C-LA in children at present. However, future research could be oriented toward the development of a 5-mm-diameter clip applier or sealing of the appendiceal base using energy sources, which would resolve this difficulty.

With the emergence of natural orifice translumenal endoscopic surgery, the new transumbilical approach seems to reduce the trauma of surgical access, improving postoperative pain and patient cosmesis compared to the conventional laparoscopic approach. The cosmetic outcomes of SILS-A are expected to be better if the operation is performed through the umbilicus. This is because the surgical wound is hidden within the umbilicus, leaving no visible abdominal scars. From our study, SILS-A has the same of OR times, recovery and complications as C-LA; however, SILS-A has more advantages than C-LA.

The total complication rate of 8% after SILS-A in our series was close to the 9%-14% published in the current literature for C-LA[43,44]. Extraction of the appendix through the abdominal wall is generally performed with a protected method. In our series, the risk of surgical-site infection was similar to C-LA. Although more wound infections occurred in the SILS-A group, this difference did not reach statistical significance. To answer the question of whether the wound infection rate is indeed higher for single-incision compared to C-LA, a larger number of patients are needed.

There are conflicting results regarding doses of narcotics required comparing SILS-A with C-LA, with some studies reporting higher doses of narcotics required after SILS-A[34-37] and others showing no difference. Some scholars reported that in SILS-A, early pain was more severe than in a C-LA. This might be caused by the skin incision. Although the skin incision in the umbilical area is small, the actual length of the fascia incision is much longer, and through the small incision region, all the laparoscopic equipment is used together, which stimulates the incision. From our study, there is no different between SILS-A and C-LA in children.

Postsurgical complications in patients who underwent SILS-A were treated without special side effects or complications, except for wound problems. Thus, SILS-A appears to be safe. Implementation in the identified RCT's showed a fairly low rate of complications in the SILS-A group.

However, major complications were not reduced. More large studies, with more stringent quality criteria, may improve the statistical power and provide proof of reduced morbidities. There is a common perception that although patients are released earlier after SILS-A, there are more readmissions. With a a 90 d follow up period, this is unlikely, especially in children. Although not statistically significant, it seems that SILS-A does decrease morbidities. However, the available data does not provide proof that SILS-A is superior to the conventional technique and more evidence should be provided. In addition, the quality of future trials should be higher to adequately advocate using SILS-A as the gold standard.

There are limitations to this meta-analysis. First, the sample size of some of the studies was quite low, as was the number of studies included in our meta-analysis; this may have biased the results. Second, not all of the included trials were randomized, which caused a lack of the required details. Third, we did not compare improvements in other comorbidities following SILS-A and C-LA, and these factors may be important in assessing and recommending the procedure. SILS-A is a comparatively new procedure that has become popular in recent years; therefore, there is also concern about the long-term results. The follow-up periods in most reports were 3 or 6 mo, and the studies analyzed here provided relatively short-term findings. However, we believe that, with greater awareness and the increasing popularity of SILS-A, studies comparing the two approaches in large volumes with long-term follow-up will be published.

In conclusion, this meta-analysis demonstrated that the SILS-A procedure is associated with significantly less bleeding, while providing an improved cosmetic outcome despite a modest increase the ratio of conversion. SILS-A is a technically feasible and reliable approach with short-term results similar to those obtained with C-LA. Prospective randomized studies comparing the two approaches in large patient cohorts with long-term follow-up will be needed to confirm the results reported.

COMMENTS

Background

Single incision laparoscopic surgery for an appendectomy (SILS-A) is widely accepted and has become the best option for treatment of appendicitis. Compared with conventional laparoscopic appendectomy (C-LA), the safety and efficacy of SILS-A is not known.

Research frontiers

Over the past three decades, many studies have assessed the performance of SILS-A. However, comparisons of SILS-A and C-LA for adults and children have not been published.

Innovations and breakthroughs

Based on this meta-analysis, single incision laparoscopic surgery does not increase the risk for an appendectomy. Similar associations were indicated in subgroup analyses of East Asian, Western, cohort, and high-quality studies. These findings were not presented clearly in previous systematic reviews.

Applications

Single incision laparoscopic surgery appears to be neither directly nor indirectly associated with the risk and pain of appendectomy. Further studies should seek to clarify this conclusion.

Peer review

SILS-A is rapidly becoming the focal point of attraction for specialists worldwide. This article shows the advantages of the procedure for adults and children. This analysis has great practical value for clinicians.

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CASE REPORT

Azathioprine-induced fever in autoimmune hepatitis

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Abstract

Underdiagnosis of drug-induced fever leads to extensive investigation and prolongation of hospitalization, and may lead to multiple unnecessary invasive procedures and a wrong diagnosis. Azathioprine is a widely used immunosuppressive drug. We report a case of a 53-year-old female patient diagnosed with autoimmune hepatitis treated with azathioprine, who presented to the emergency room with a 6-wk history of fever and chills without other associated symptoms. Since the patient's fever was of unknown origin, she was hospitalized. All treatment was stopped and an extensive workup to explore the source of fever and chills was performed. Results of chest X-ray, viral, urine, and blood cultures, autoimmune serology, transthoracic and transesophageal echocardiography, and abdominal ultrasound revealed no source of infection. A rechallenge test of azathioprine was performed and the fever and chills returned within a few hours. Azathioprine was established as the definite cause following rechallenge. Fever as an adverse drug reaction is often unrecognized. Azathioprine has been reported to cause drug-induced fever in patients with inflammatory bowel disease, rheumatoid arthritis, and sarcoidosis. To the best of our knowledge there have been no previous reports documenting azathioprine-induced fever in patients with autoimmune hepatitis. The occurrence of fever following the readministration of azathioprine suggests the diagnosis of drug-induced fever, particularly after the exclusion of other causes. A careful rechallenge is recommended to confirm the diagnosis.

Key words: Autoimmune hepatitis; Adverse drug reactions; Azathioprine; Drug fever

Core tip: Azathioprine is widely used in inflammatory disease such as rheumatoid arthritis, inflammatory bowel disease and post solid organ transplant such as kidney transplantation. Azathioprine is an immune modulator drug that can expose patients to various infections and clinical fever. Azathioprine needs to be remembered as a potential fever provoker in the differential diagnosis of fever origin.

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INTRODUCTION

Drug reaction is an underestimated cause of fever. Drug-induced fever may be defined as a disorder characterized by the appearance of elevated body temperature 7-10 d following the administration of a specific medication, with resolution of the fever upon discontinuation of the suspected agent. Less often, the fever can appear after a long period of treatment. Drug-induced fever is estimated to account for about 10% of elevated body temperature evaluations[1,2] and fever as the sole symptom of a drug's side effect has been reported in about 3%-5%[3].

The diagnosis of drug-induced fever is usually challenging, especially in medically complicated patients with multiple treatment regimens. There are no clear guidelines for the diagnosis, which is usually made by exclusion of the responsible drug. Underdiagnosis of drug-induced fever may lead to extensive investigations, unnecessary antibiotic treatment, and prolonged hospitalization[4].

Azathioprine-induced fever is a relatively rare disorder. It has been reported in only a few patients with inflammatory bowel disease, rheumatoid arthritis, and sarcoidosis[5-7]. In several other reported cases, it was associated with accompanying manifestations such as pruritus and cutaneous rash[8,9].

We report the case of a patient with autoimmune hepatitis who developed fever and chills induced by azathioprine. Rechallenge confirmed the diagnosis. To the best of our knowledge, this is the first described case of azathioprine-induced fever in autoimmune hepatitis.

CASE REPORT

A 53-year-old female of Arab descent was diagnosed in 2009 with autoimmune hepatitis by liver biopsy, which showed moderate inflammatory activity with infiltrating plasma cells suggestive of autoimmune hepatitis. Serological tests for anti-nuclear antibody (ANA), anti-double-stranded antibodies, and anti-parietal antibodies were positive, and serum immunoglobulins IgM 329 mg/dL (normal range 65-280 mg/dL), IgA 786 mg/dL (90-450 mg/dL), IgG 3120 mg/dL (800-1700 mg/dL) were elevated (Table 1). The patient had been treated with budesonide until 6 wk before admission, when azathioprine 100 mg/d was added to the treatment regimen.

Her medical history was notable for valvular heart disease, paroxysmal atrial fibrillation, hypertension, hypothyroidism, diabetes mellitus, and microcytic anemia. Her medications included warfarin 22.5 mg once daily, metformin 850 mg 3 times daily, omeprazole 20 mg once daily, losartan 80 mg twice daily, insulin detemir 20 units once daily, insulin aspart 10 units 3 times daily, metoprolol 25 mg twice daily, spironolactone 100 mg once daily, aspirin 100 mg daily, furosemide 40 mg once daily, azathioprine 50 mg once daily, and thyroxine 50 mg once daily. She presented to the emergency room due to fever and chills of several weeks duration, without other associated symptoms. On admission, the patient was stable; blood pressure was 155/69 mmHg, pulse rate 62 beats/min, and temperature 38.4 ℃. Her physical examination was unremarkable except for small skin erosion with surrounding erythema in her anterior abdominal wall. Blood tests showed a white blood cell count of 7.700 per cubic millimeter (4-10 thousand per cubic millimeter) with 90% neutrophils (40%-70%), 5.5% lymphocytes (15%-41%) and 3.5% monocytes (1%-7%). Hemoglobin was 9.3 g (12-16 g). Results of blood chemistry, including electrolytes and kidney function, were normal except for mild elevation of liver function tests. Urinalysis showed a few leukocytes but otherwise was normal. Nasal viral cultures were negative. C-reactive protein and erythrocyte sedimentation rate were normal. Computed tomography was performed to exclude an intra-abdominal cause of fever and revealed a small area of subcutaneous inflammation adjacent to the skin erosion. Chest X-ray showed no changes suggestive of infection.

Table 1 Revised international autoimmune hepatitis group scoring system for the diagnosis of autoimmune hepatitis

Clinical feature	Score	Patient
Female gender	+2	+2
ALP:AST ratio		+2
< 1.5	+2	
1.5-3.0	0	
> 3.0	-2	
Serum globulin or IgG above normal		+2
> 2.0	+3	
1.5-2.0	+2	
1.0-1.5	+1	
< 1.0	0	
ANA, SMA, LKM1		+3
> 1:80	+3	
1:80	+2	
1:40	+1	
< 1:40	0	
Illicit drug use history		+1
Positive	-4	
Negative	+1	
Average alcohol intake daily		+2
< 25 g/d	+2	
> 60 g/d	-2	
Histologic findings		Lymphoplasmacytic infiltrate
Interface hepatitis	+3	+1
Lymphoplasmacytic infiltrate	+1	
Rosette formation	+1	
None of the above	-5	
Biliary changes	-3	
Other changes	+2	
Other autoimmune disease	+2	No other autoimmune disease
AMA positivity	-4	AMA negative
Hepatitis viral markers		+3
Positive	-3	
Negative	+3	
Aggregate score without treatment		Overall 16 points
Definite AIH	> 15	
Probable AIH	10-15	

ALP: Alkaline phosphatase; AST: Aspertate aminotransferase; AIH: Autoimmune hepatitis; ANA: Anti-nuclear antibody; SMA: Soluble ribonucleic acid; LKM: Liver-kidney microsomal; IgG: Immunoglobulin G.

The patient was admitted to the Department of Internal Medicine with an initial diagnosis of fever, most likely secondary to the localized skin infection in the abdominal wall. Azathioprine was withheld and the patient was treated with antibiotics. Within several hours the fever subsided and the patient remained afebrile throughout 5 d of hospitalization.

Following recovery and resolution of the cellulitis, the patient was discharged and azathioprine was re-administered. Within a few hours, fever and chills recurred and the patient was re-admitted to the hospital. An extensive work-up for fever of unknown origin was done including blood and urine cultures and nasal viral cultures, which were all negative. Serological tests for human immuno-

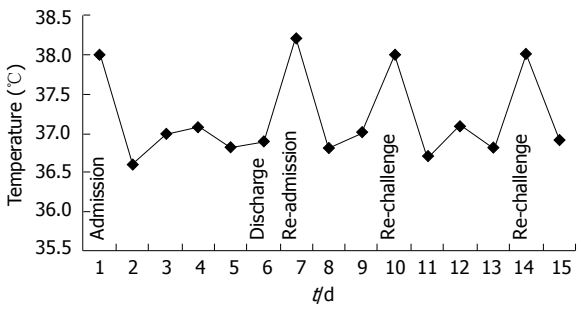

Figure 1 Patient fever scale during hospitalization and rechallenge test.

deficiency virus, hepatitis viruses, brucella, Q fever and toxoplasma were negative. Autoimmune serology showed ANA 2 of 4, anti-parietal cell antibodies 4 of 4; anti-smooth muscle, anti-mitochondrial and immunoglobulins were negative. In addition, ultrasonography of the abdomen revealed no evidence of ascites or infection. Transthoracic and transesophageal echocardiography did not show vegetation or abscess. Purified protein derivative test was negative. Again azathioprine was withheld and the fever resolved within a few hours.

With the patient's informed consent, a rechallenge with one tablet of 50 mg azathioprine was performed twice under observation. Chills appeared within 3 h and there was a gradual increase in temperature up to 38 ℃ within 7 h. Fever and chills resolved completely within 6 h after discontinuation of azathioprine.

DISCUSSION

Drug-induced fever is an underdiagnosed condition, particularly in patients treated with a large number of medications. It is described as a febrile response that coincides temporally with the administration of a drug and disappears after discontinuation of the offending agent. It is usually suspected when no other cause for fever can be explained, leaving drug fever as the diagnosis of exclusion.

Several mechanisms have been implied in the pathogenesis of drug-induced fever, most importantly hypersensitivity reaction involving the formation of antigen-antibody complexes. Common agents associated with hypersensitivity type reaction include penicillins, cephalosporins, phenytoin, methyldopa, procainamide, and antitubercular agents[2,3,10]. Other mechanisms include idiosyncratic reactions, drug effects on the thermoregulatory center, reactions to drug administration, and the extension of drug pharmacological effects as seen in patients who developed cell lysis and release of variable pyrogenic substances following chemotherapy treatment[1,11-13].

Azathioprine suppresses the immune system by inhibiting the activity of T cell lymphocytes. It is a prodrug which, following oral ingestion, is metabolized into active mercaptopurine, a purine synthesis inhibitor that impedes DNA synthesis and inhibits cell proliferation[14]. Azathioprine is known to cause multiple adverse effects, including fever, gastrointestinal symptoms, nervous system symptoms, bone marrow suppression, hepatic symptoms, myalgia, and arthralgia. Discontinuation of azathioprine is seen in about 10%-15% of patients due to side effects[5].

Several cases of azathioprine-induced fever in association with other symptoms have been reported; fever and chills were the only reported symptoms when azathioprine was administered to patients with sarcoidosis and inflammatory bowel disease, and after kidney transplantation[5,7,15,16].

In an observational study, three of 25 patients with RA developed symptoms of fever, chills, skin rash, hepatotoxicity, nausea, and diarrhea 2 wk after starting treatment with azathioprine. One patient developed only fever and chills. Rechallenge was performed in two patients with the appearance of more severe reactions[6]. In these patients, the febrile reaction appeared several days up to several weeks after beginning treatment with azathioprine. In our case the fever appeared within a few hours after restarting the offending agents. Thus the lag period between the initiation of the offending agent and the appearance of fever is highly variable.

In our patient, the sequence of events and the lack of objective evidence of infection are both highly suggestive of azathioprine-induced fever. In addition, previous reports of azathioprine causing drug fever in other disease states support the likelihood of a similar event in this patient[5-7]. With the patient's permission, two rechallenge tests were done in which 50 mg azathioprine was administered. Fever of 38 ℃ was measured about 7 h after administration, without other associated symptoms or objective findings on blood analysis, and without recurrence of the fever upon discontinuation of azathioprine (Figure 1). Thus, fever and chills in our case are the only manifestations of azathioprine and were likely caused by the administration of azathioprine.

In conclusion, drug reaction is an underestimated cause of fever. Clinicians should be aware of the fact that an immunomodulatory drug such as azathioprine, commonly used to treat various autoimmune conditions and to suppress the inflammatory response, can cause fever and chills. It is thus essential to withdraw a suspected medication and perform a rechallenge when fever fails to regress in patients already treated with antibiotics.

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CASE REPORT

Rectal arterio-portal fistula: An unusual cause of persistent bleeding per rectum following a proximal spleno-renal shunt

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Abstract

Gastrointestinal arterio-venous malformations are a known cause of gastrointestinal bleeding. We present a rare case of persistent rectal bleeding due to a rectal arterio-portal venous fistula in the setting of portal hypertension secondary to portal vein thrombosis. The portal hypertension was initially surgically treated with splenectomy and a proximal splenorenal shunt. However, rectal bleeding persisted even after surgery, presenting us with a diagnostic dilemma. The patient was re-evaluated with a computed tomography mesenteric angiogram which revealed a rectal arterio-portal fistula. Arterio-portal fistulas are a known but rare cause of portal hypertension, and possibly the underlying cause of continued rectal bleeding in this case. This was successfully treated using angiographic localization and super-selective embolization of the rectal arterio-portal venous fistula *via* the right internal iliac artery. The patient subsequently went on to have a full term pregnancy. Through this case report, we hope to highlight awareness of this unusual condition, discuss the diagnostic workup and our management approach.

Key words: Portal hypertension; Esophageal varices; Splenorenal shunt; Arteriovenous malformations; Portal vein thrombosis

Core tip: We present a rare case of persistent rectal bleeding due to a rectal arterio-portal venous fistula, in the setting of portal hypertension. Through this case report, we hope to highlight awareness of this unusual condition, and discuss the diagnostic workup and our subsequent management. We believe that this is the first of such cases reported in the literature.

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INTRODUCTION

Arterio-venous malformations occurring in the gastrointestinal tract are a known cause of gastro-intestinal bleeding and can often be missed especially in the presence of other pathology. They can lead to persistent symptoms if left undiagnosed and untreated. We present a patient who had portal vein thrombosis, portal hypertension and continued to have rectal bleeding in spite of a patent spleno renal

shunt to highlight awareness of this unusual condition.

CASE REPORT

A 33-year-old Chinese lady presented with multiple episodes of haematemesis when she was nine years old.She was seen in a secondary hospital and diagnosed to have portal hypertension secondary to portal vein thrombosis (PVT), a possible sequelae of a neonatal umbilical infection. The portal hypertension resulted in her having episodes of bleeding esophageal varices that were treated endoscopically during her childhood years. On clinical examination, she had splenomegaly but no stigmata of chronic liver disease. Investigations revealed that she did not have any liver cirrhosis or prothrombotic disorders. Liver function tests were normal. Radiological investigations confirmed the PVT and splenomegaly. More than twenty years after her initial diagnosis, she started to develop intermittent episodes of rectal bleeding which was attributed to rectal varices. She was anaemic (haemoglobin level 7.0 g/dL) and required intermittent blood transfusions for her symptoms. Her splenomegaly led to consumption thrombocytopenia (platelet levels ranged from 74-103 × 10^9 g/L) and she subsequently sought a second opinion at a tertiary centre, after much investigation including a contrast enhanced computed tomography scan, upper gastrointestinal endoscopy and a colonoscopy.

Pre-operative investigations were reviewed and showed PVT, splenomegaly and a dilated inferior mesenteric vein all the way down to the pelvis. Colonoscopy reported that there were large rectal varices. Based on these investigations, we presumed that the rectal varices were a result of left sided portal hypertension with the pressure transmitted down the inferior mesenteric vein, and thus performed a splenectomy and an end-to-side proximal splenorenal shunt for portal decompression.

After surgery, the patient continued to have rectal bleeding during her follow-up. A flexible sigmoidoscopy was performed and this revealed almost circumferentially dilated pulsatile submucosal vessels at the lower rectum. These were not reported in the colonoscopy that the patient underwent previously in the secondary hospital. A multiphasic computed tomography mesenteric angiogram confirmed a rectal arterio-venous fistula. On the arterial phase of the scan, there was evidence of arterio-venous shunting as evidenced by the dilated superior rectal vein draining into the superior mesenteric vein, with arterial feeders from the internal iliac arteries (Figure 1). A diagnostic catheter angiogram was performed to confirm the complexity of the feeder vessels and assess the potential collateral damage to rectal mucosa that could occur with angioembolisation. This revealed that there were two major arterial feeders arising from the anterior division of the internal iliac arteries on both sides which were supplying the arterio-portal fistula. There was a single tortuous drainage vein into the inferior mesenteric vein (Figure 2).

A super selective angioembolisation of the arterio-portal fistula was performed *via* a left common femoral artery access. The EV3 Apollo microcatheter (ev3 Endovascular Inc., Plymouth, MA, United States) was introduced and advanced into the arterio-portal fistula nidus and embolisation performed using 2cc of Onyx 18 embolic agent (ev3 Endovascular Inc., Plymouth, MA, United States). Completion angiograms *via* both internal iliac arteries showed successful obliteration of the arterio-portal fistula with cessation of arterio-portal shunting (Figure 3).

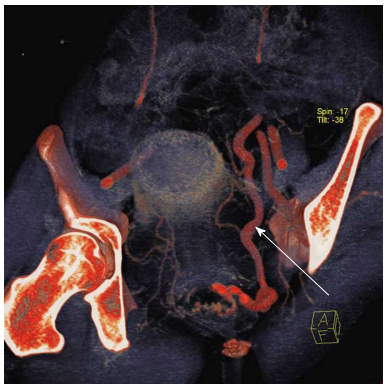

Figure 1 Computed tomography angiography image (post processed with volume rendering technique) showing rectal arterio-portal fistula draining into dilated superior rectal vein (arrow).

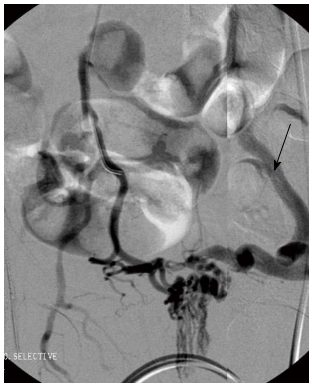

Figure 2 Pelvic angiography image showing the rectal arterio-portal fistula draining into the dilated superior rectal vein (arrow).

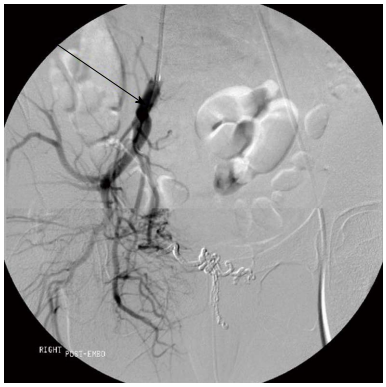

Figure 3 Post embolization angiogram from the anterior branch of the right internal iliac artery (arrow) showing obliteration of blood flow in the arterioportal fistula.

Post procedure, clinical examination and a repeat flexible sigmoidoscopy confirmed that there was no rectal ischemia. However, the patient complained of anal pain and further rectal bleeding, although the quantity of bleeding was much reduced. On clinical examination, she was found to have an anal fissure at 3 o'clock position, possibly as a result of the embolisation. This was initially treated with topical methods but did not resolve her symptoms and thus she underwent a lateral anal sphincterotomy. Since surgery, she has successfully delivered a child after a full-term pregnancy and her haemoglobin (12.0 g/dL) and platelet levels have returned to normal.

DISCUSSION

Arteriovenous malformations (AVMs) in the gastrointestinal tract are a known cause of gastrointestinal bleeding, and are usually difficult to diagnose. In the literature, it has been reported that the highest frequency of gastrointestinal AVMs occur in the right sided colon[1]. Arteriovenous malformations in the rectum are rare and it is often difficult to distinguish between bleeding from the AVM and bleeding from haemorrhoids, leading to diagnostic delays and even unnecessary procedures being performed for haemorroids[2].

Arterio-portal fistulas are a type of arteriovenous malformations defined as abnormal communications between the systemic arteries and the portal circulation[3]. They can be congenital or acquired. Congenital causes include arteriovenous malformations, ruptured aneurysms and hereditary telangiectatic diseases where there might be multiple arterio-portal fistulas present[4-7]. The majority of them are a result of penetrating or blunt trauma to the vessels which can be iatrogenic[8]. Most commonly, arterio-portal fistulas originate from the celiac or splanchnic circulation in particular the hepatic artery or splenic artery due to their close proximity to the portal and splenic veins. Rarely, they can be found to arise from the superior mesenteric or inferior mesenteric arteries[3]. In our patient, the arterio-portal fistula arose from the internal iliac arteries and this is believed to be the first such case reported in the literature.

Portal vein thrombosis is a known cause of extrahepatic presinusoidal portal hypertension. In neonates, this can be caused by umbilical infection, often as a result of umbilical vein catheterisation. The infection spreads along the left portal vein to the main portal vein causing thrombosis[8]. Due to the increase in portal resistance, collaterals arise from the high pressure veins in the portal system to the low pressure veins in the systemic circulation. The reversal of blood flow towards the systemic venous circulation leads to formation of varices at the oesophago-gastric region, along the falciform ligament at the umbilicus, in the retroperitoneum *via* the veins of Retzius and at the anorectal region where the superior haemorrhoidal veins decompress into the middle and inferior haemorroidal veins of the systemic circulation. Our patient presented with bleeding esophageal varices at an early age and this lead to her diagnosis of PVT. The leading cause of mortality in portal hypertension is bleeding esophageal varices[9]. Anorectal varices rarely bleed, likely due to the rich plexus of veins around the rectum which shunts away most of the blood.

In our patient, we offer two possibilities to account for her condition. The first is that she has a congenital pre-existing arterio-portal fistula between the rectal artery and the haemorrhoidal veins. However, when the patient first presented with rectal bleeding in the setting of portal hypertension, the most likely diagnosis of bleeding ano-rectal varices was made and she was treated with the aim of reducing her portal pressures, which was the presumed cause of the ano-rectal varices. In retrospect, this was a wrong postulation. The rectal bleeding did not resolve despite the splenorenal shunt and only on further investigations did we discover the arterio-portal fistula which had initially masqueraded as bleeding ano-rectal varices. Awareness of this entity as a possible differential diagnosis for bleeding ano-rectal varices is important. Initial investigation with a dynamic computed tomography scan with arterial and portal venous phase would have brought the diagnosis to light.

The second possibility is that the arterio-portal fistula was formed due to unintended or unnoticed trauma to the rectal wall. The left sided portal hypertension led to the formation of dilated portal vasculature in the rectum. These rectal varices are in close proximity to the branches of the arteries supplying the rectum, like the middle rectal artery in this case. An episode of unsuspecting trauma to the rectal wall possibly during defecation, led to the formation of the abnormal vascular communication between the middle rectal artery and the rectal varices, propagating the formation of an arterio-portal fistula

Multiphasic contrast enhanced computed tomography scan of the abdomen and pelvis helped to clinch the diagnosis of the arterio-portal fistula in this case, as contrast was seen on the draining dilated superior rectal vein in the arterial phase of the scan. This distinguishes it from a simple rectal bleeding secondary to rectal varices. In the setting of portal hypertension, awareness of the possible diagnoses is critical.

In the era prior to interventional radiology, arterio-portal fistulas were usually treated by open surgical excision. With the advent of super-selective angiographic catheterisation, embolisation is now the definitive treatment of choice for patients if the expertise is available[3]. Inadequate imaging especially in the setting of presumed portal hypertension has led to the delayed diagnosis of this arterio-portal fistula and local transanal treatment could have been catastrophic. Endoscopic ablation of the nidus of dilated collaterals in the rectum using sclerosant or banding could have led to massive haemorrhage from high pressure arterial bleeding if it was simply treated as for rectal varices following portal decompression.

In conclusion, arterio-portal fistulas are a known cause of portal hypertension and can also cause a diagnostic dilemma in the setting of portal hypertension. An-

giographic embolisation is the treatment of choice and this can be achieved with minimal morbidity.

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CASE REPORT

Endoscopic retrieval of 28 foreign bodies in a 100-year-old female after attempted suicide

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Abstract

Foreign body ingestion is a common emergency situation in children with one or a few objects having been ingested. Here we report our experience using endoscopic retrieval in a female centenarian with dyspnea and foreign bodies in the esophagus. She attempted suicide by swallowing 26 coins and two other foreign bodies. A gastroscope was used to remove all foreign bodies in the lower esophagus. In total, 26 coins, one ferrous ring and one cylindrical plastic object were retrieved. To our knowledge, this is the first clinical report on retrieval of so many foreign bodies in a single case.

Key words: Foreign body; Esophagus; Endoscopy; Coin; Gastroscope; Retrieval basket

Core tip: Foreign body ingestion is typically a childhood phenomenon, and generally involves one or a few objects. Here, we report our experience using emergency endoscopy in a centenarian with dyspnea who had swallowed 26 coins and other foreign bodies. Rescue measures were applied to maintain stable vital signs and airway patency, while an alligator forceps or basket was inserted through a flexible gastroscope to remove all foreign bodies. The objects removed from the patient included 26 coins, a ferrous ring, and a cylindrical plastic object.

Li QP, Ge XX, Ji GZ, Fan ZN, Zhang FM, Wang Y, Miao L. Endoscopic retrieval of 28 foreign bodies in a 100-year-old female after attempted suicide. *World J Gastroenterol* 2013; 19(25): 4091-4093 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4091.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4091

INTRODUCTION

Ingestion of foreign bodies that lodge in the upper gastrointestinal (GI) tract is common. Most objects pass through the GI tract spontaneously, but some need endoscopic or surgical removal. Here, we report the first case of a centenarian who had swallowed several foreign bodies, which were safely removed by endoscopy.

CASE REPORT

Foreign body ingestion is a commonly encountered clinical problem in pediatric emergency cases, but generally it involves only one or a few objects. Here, we report a case of ingestion of foreign bodies in a centenarian.

A 100-year-old woman complaining of retrosternal pain and tachypnea visited the emergency room. She had swallowed several foreign bodies in a suicide attempt due to intolerable pain induced by a fracture she suffered 3 mo previously. She was bedridden and had a depressed mood, which caused her to attempt suicide. She was admitted to hospital and a routine chest roentgenogram showed the incidental finding of suspicious foreign bodies, which were located in the lower esophagus (Figure 1). Blood

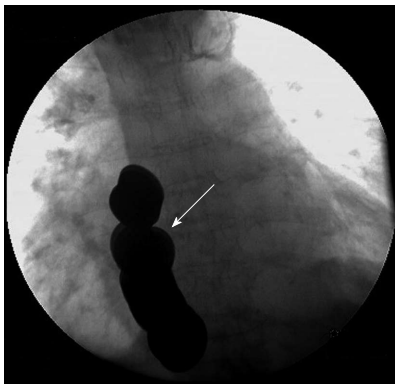

Figure 1 A roentgenogram showing foreign bodies in the lower esophagus (arrow).

Figure 3 Foreign bodies after removal. These included 26 coins, a ferrous ring, and a cylindrical plastic object.

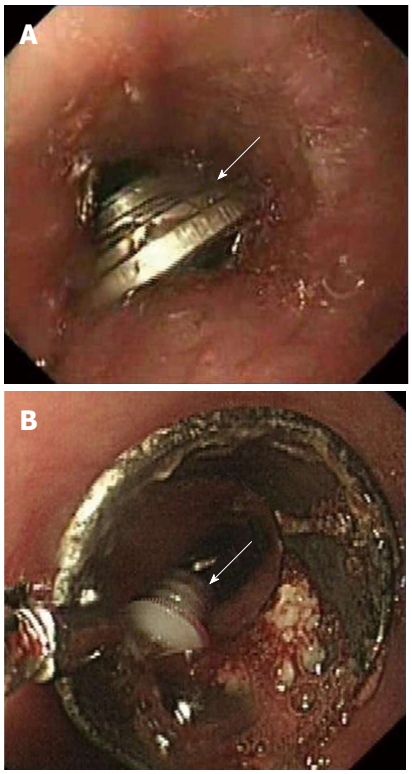

Figure 2 Gastroscope examination. A: Gastroscopy displayed several coins (arrow) in the lower esophagus; B: A cylindrical plastic foreign body impacted in the esophageal wall (arrow).

tests were normal, and an electrocardiogram showed premature ventricular extrasystole. Further gastroscopy examination using a flexible endoscope under a conscious state showed that several coins were located in the lower esophagus (Figure 2A). Endoscopic removal of these coins was performed, but failed because of their smooth edges. Therefore, an alligator clamp was employed to secure the coins, and more than 10 coins were successfully removed. Additionally, a cylindrical plastic foreign body was difficult to extract using the alligator clamp (Figure 2B). After several attempts, this foreign body was taken out via a stone retrieval net. Subsequently, additional foreign bodies were found and taken out using the alligator forceps. The foreign bodies removed from the patients included 26 coins, a ferrous ring, and a cylindrical plastic object (Figure 3). The procedure took about half an hour. When we reviewed the gastroscopy procedure, there was no block and no active bleeding in the esophagus.

DISCUSSION

Ingestion of foreign bodies is common in clinical practice. Most foreign body ingestion is accidental, and often occurs in the pediatric population, with a peak incidence between 6 mo and 6 years of age[1]. In contrast to the high frequency of foreign body ingestion in children, the occurrence rate of foreign body ingestion is relatively low in adults. The situation in adults occurs more commonly in patients with psychiatric disorders, mental retardation, or impairment caused by alcohol[2]. This is the first report of a centenarian ingesting foreign bodies in a suicide attempt due to intolerable pain. Additionally, the location of the foreign bodies in this case was in the lower esophagus, which was different from previous reports in which the majority of foreign bodies were located in the upper esophagus[3,4]. Foreign bodies in the upper esophagus may cause mechanical compression of the airway while in the lower esophagus may cause functional narrowing of the cardia. In this case, the patient's dyspnea was caused by both mechanical compression of the airway and functional narrowing of the cardia because of the irritation of so many foreign bodies.

The type of foreign body may influence the risk of complications. The types of foreign objects are different for different ages and cultures. In children, coins are a common type of foreign body[5]. In Asian countries, bone foreign bodies have been regarded as the most common type due to traditional habits of swallowing fish bones or eating soup from which fish bones are not removed[6,7]. The current case was rare in that a centenarian swallowed the coins.

Endoscopic removal of foreign bodies and impacted food boluses is a reliable and safe procedure in the hands of a skilled endoscopists, and it has a high success rate and low level of significant complications[8]. However,

the effectiveness of endoscopic removal of the foreign bodies was challenged by the smooth edges of the coins in the current case. Therefore, an alligator clamp was utilized. Additionally, a stone retrieval net was also employed to extract a cylindrical plastic foreign body in the present case. Many foreign bodies were removed, which is another important point in this case.

According to our experience, the key factor in shortening the procedure time was to catch and remove the foreign bodies in a short time using a powerful retrieval device. If the surface of the foreign body is smooth, it may be much more difficult. Each object is a challenge to the endoscopist. This was the reason why it took us 0.5 h to remove all the foreign bodies using a common stone extraction device. Fortunately, a novel retrieval basket is being developed by Detian Medical (Changzhou, China). Based on our animal and *in vitro* experiments, it is much easier for an endoscopist to catch foreign bodies and polyps and hold them stably in this basket device compared with traditional retrieval devices.

In conclusion, we reported our experience with retrieval of 26 coins and another two foreign bodies from the esophagus of a 100-year-old suicidal patient with dyspnea. To our knowledge, this is the first clinical report of retrieval of so many foreign bodies in a single case.

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CASE REPORT

Epstein-Barr virus negative primary hepatic leiomyoma: Case report and literature review

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Author contributions: Luo XZ and Ming CS contributed equally to this work; Gong NQ and Chen XP designed the research; all authors performed the research, analyzed the data, wrote the paper, and approved the final manuscript.

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Abstract

Primary hepatic leiomyoma is a neoplasm of mesenchymal origin and occurs only rarely. Secondary to benign smooth muscle proliferation, it is usually found in adult women and is associated with Epstein-Barr virus (EBV) infection. Here, we report the 29th case of primary hepatic leiomyoma with its unique features related to diagnosis, treatment and developmental biology. A 48-year-old man, with an immunocompromised status, complained of pain in the upper quadrant of the abdomen. Serological analysis indicated no presence of hepatitis virus, no human immunodeficiency virus, and no EBV infection. The levels of α-fetoprotein and carcinoembryonic antigen were normal. A mass was detected in segment Ⅲ of the hepatic lobe by ultrasonography and an abdominal computed tomography scan. Endoscopy had negative findings. Exploratory laparotomy found no existing extrahepatic tumor and left lateral lobectomy was performed. Pathological examination showed the mass to be a typical leiomyoma. The cells were positive for α-smooth muscle actin and desmin, and negative for the makers of gastrointestinal stromal tumor (GIST), including CD117, CD34 and DOG1 (discovered on GIST1). *In situ* hybridization revealed negative status for EBV-encoded small RNA. After left lateral lobectomy, the patient was not given chemotherapy or radiotherapy. During a 2-year follow-up, no sign of local recurrence or distant metastasis was observed. In conclusion, we report a rare case of primary hepatic leiomyoma in a male patient without EBV infection. Hepatic resection was curative. This case presents data to expand our knowledge concerning the complex and heterogeneous nature of primary liver leiomyoma, indicating that EBV infection is important but neither necessary nor sufficient for the development of primary liver leiomyoma.

Key words: Epstein-Barr virus; Primary hepatic leiomyoma; Cancer diagnosis; Tumor resection; Developmental biology

Core tip: Primary hepatic leiomyoma is usually found in adult women and is associated with Epstein-Barr virus (EBV) infection. We report the 29th case worldwide in a 48-year-old kidney allograft recipient without EBV infection and extrahepatic tumor. He achieved clinical cure by mass resection. The leiomyoma was positive for α-smooth muscle actin and desmin, and negative for gastrointestinal stromal tumor markers, including CD117, CD34 and DOG1 (discovered on gastrointestinal stromal tumor 1). The tumor was negative for EBV-encoded small RNA. The data indicate that EBV infec-

tion is important but neither necessary nor sufficient for development of primary liver leiomyoma.

Luo XZ, Ming CS, Chen XP, Gong NQ. Epstein-Barr virus negative primary hepatic leiomyoma: Case report and literature review. *World J Gastroenterol* 2013; 19(25): 4094-4098 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i25/4094.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i25.4094

INTRODUCTION

Primary hepatic leiomyoma occurs rarely. The first case was described by Demel[1] in a 42-year-old woman. To date, only 28 cases have been reported worldwide in the literature (Table 1). Secondary to benign smooth muscle proliferation, primary hepatic leiomyoma is usually found in adult women, and is associated with Epstein-Barr virus (EBV) infection. Due to its low prevalence, the diagnosis, treatment and biological behavior remain elusive and require further investigation[2-5]. More data to provide essential information concerning this disease are keenly awaited.

CASE REPORT

We report a 48-year-old man who complained of pain in the upper quadrant of the abdomen for 1 year and was admitted 2 years ago. He had received a renal graft 9 years before with the immunosuppressive regimens of cyclosporine A, mycophenolate mofetil and prednisone. Due to the calcineurin inhibitor nephrotoxicity found by fine-needle aspiration biopsy 3 years ago, cyclosporine was changed to tacrolimus. Two years ago, mycophenolate mofetil was replaced by azathioprine due to persistent diarrhea. On the day of admission, he was receiving tacrolimus (4.1 ng/dL), azathioprine (50 mg/d), and prednisone (5 mg/d). Routine blood analysis showed a white blood cell count of 7.2×10^9/L and lymphocyte count of 2.4×10^9/L. His liver function was normal and graded as A (score: 6) by Child-Turcotte-Pugh classification. There was no evidence of hepatitis B or hepatitis C virus infection. Human immunodeficiency virus (HIV) testing was negative. Serological testing for EBV was also negative. α-fetoprotein was 5.27 ng/mL (range: 1.09-8.04 ng/mL), and carcinoembryonic antigen was 2.55 ng/mL (normal range: 0-5 ng/mL). Ultrasonography revealed a mass in the left region of the liver, and an abdominal computed tomography (CT) scan showed a tumor of 3.7 cm × 4.9 cm in segment Ⅲ of the hepatic lobe (Figure 1). No tumor was found by esophagogastroduodenscopy and colonoscopy.

After diagnosis with a liver tumor, the patient underwent exploratory laparotomy. A solitary tumor was found in segment Ⅲ of the liver (Figure 2A). No tumors were present at extrahepatic sites; particularly in the pelvis. Left lateral hepatectomy was performed. The patient recovered with an uneventful postoperative course and abdominal pain disappeared.

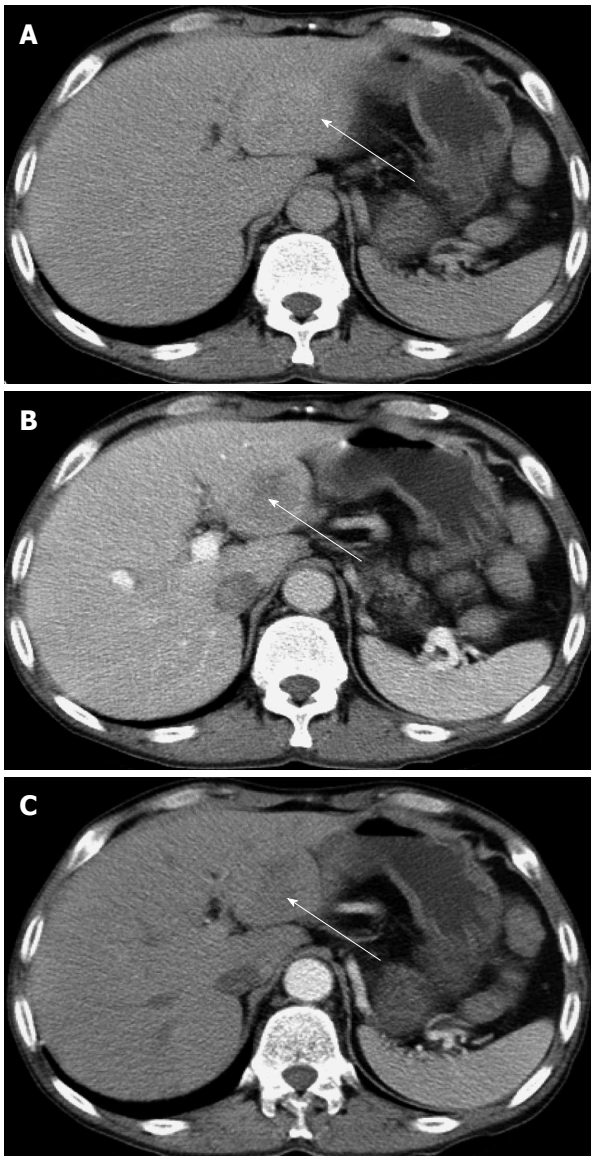

Figure 1 Abdominal computed tomography scan shows a mass in segment Ⅲ of the liver. A: Hepatic equilibrium phase; B: Portal venous phase; C: Hepatic arterial phase. The arrows indicate the tumor in the liver.

Histopathological examination of the resected specimen showed that the tumor consisted of spindle cells with scarce mitotic figures. The cells had elongated nuclei and eosinophilic cytoplasm forming a fabric-like structure, and neither giant cells nor anaplasia were present (Figure 2B). Immunohistochemical staining showed that the cells were positive for α-smooth muscle actin and desmin (Figure 2C and D), and negative for the gastrointestinal stromal tumor (GIST) markers, including CD117, CD34 and DOG1 (discovered on GIST1). *In situ* hybridization revealed that the nuclei of the tumor cells were negative for EBV-encoded small RNA (EBER) (Figure 3).

Diagnosis of primary hepatic leiomyoma was then made. As a benign tumor, neither chemotherapy nor radiotherapy was administered to the patient. During a

Table 1 Summary of the published cases of primary liver leiomyoma

Author	Age/sex	EBV infection	Symptoms	Location/size (cm)	Immunosuppression	Treatment
Demel[1]	42/F	Unknown	RUQ pain	RL/12	NS	Laparotomy
Rios-Dalenz *et al*	87/F	Unknown	RUQ pain/bleeding	LL/-	NS	Autopsy
Ishak *et al*	64/M	Unknown	Abdominal mass	RL/-	NS	Laparotomy
Hawkins *et al*[2]	66/M	Unknown	Abdominal mass	LH/13	NS	Left hepatectomy
Rummeny *et al*	46/F	Unknown	RUQ pain	NS	NS	NS
Hollands *et al*[6]	17/M	Unknown	Abdominal pain	LH/9	NS	Left hepatectomy
Herzberg *et al*	30/F	Unknown	RUQ fullness	RL/19	NS	Partial right hepatectomy
Doyle *et al*[11]	1.5/F	Positive	Incidental	LL/3	Yes	LL segmentectomy
Reinertson *et al*	32/F	Unknown	RUQ pain	LH/10	NS	Left hepatectomy
Hailer *et al*	9/M	Unknown	Incidental	LH/5.6	Yes	Partial hepatectomy
Davidoff *et al*[12]	5/M	Positive	Incidental	RR/15	Yes	Right trisegmentectomy
Yoon *et al*	41/F	Unknown	RUQ discomfort	RL/19	No	Right hepatectomy
Yanase *et al*	59/F	Unknown	Liver dysfunction	RL/13	NS	Right hepatectomy
Mesenas *et al*	59/M	Unknown	NS	RL/3.6	NS	Segmentectomy (S5)
Belli *et al*[7]	67/F	Unknown	Abdominal mass	RL/30	NO	Right extended resection
Sclabas *et al*[13]	30/F	Positive	Epigastric pain	LL/4.4, 0.6	Yes	LL sectionectomy
Cheuk *et al*[14]	37/M	Positive	Abdominal discomfort	LH/3.5, 1	Yes	Conservative management
Kanazawa *et al*	31/M	Unknown	None	LL/3.5	No	LL sectionectomy
Beuzen *et al*	36/F	Unknown	RUQ pain	LL/5	No	LL sectionectomy
Imasato *et al*[3]	61/F	Unknown	None	S1/4.5	No	Right hepatectomy
Urizono *et al*	71/M	Unknown	NS	S1/3	No	Partial hepatectomy
Marin *et al*	64/F	Unknown	None	RL	No	Right hepatectomy
Sousa *et al*	61/F	Unknown	Dyspepsia	LL/9.5	No	Left hepatectomy
Kalil *et al*	44/F	Unknown	Abdominal mass	RL/7	No	Atypical resection
Santos *et al*	28/F	Unknown	Incidental	RL (S6)/5.5	No	Segmentectomy
Raber *et al*	46/F	Unknown	Incidental	RL/2.8	Yes	Conservative management
Perini *et al*[5]	45/M	Positive	Epigastric pain	LL/4.3	Yes	LL sectionectomy
Perini *et al*[5]	45/F	Unknown	RUQ pain	RL (S6)/16.5	No	Segmentectomy

LL: Left lateral; NS: Not stated; RL: Right lobe; RUQ: Right upper quadrant; S: Segment; LH: Left hepatic lobe; EBV: Epstein-Barr virus.

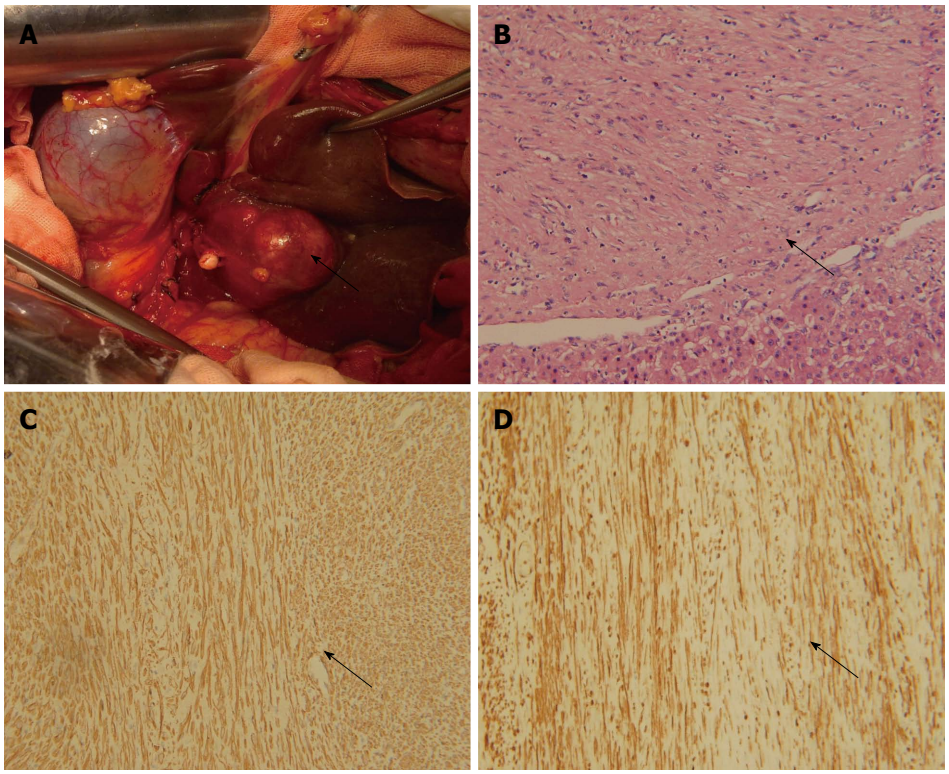

Figure 2 Pathological characteristics of the primary liver leiomyoma. A: Tumor (arrow) located in segment Ⅲ of the liver; B: Tumor (arrow) and normal liver tissue, hematoxylin and eosin staining, × 200; C: α-smooth muscle actin staining (arrow) of tumor tissues, immunohistochemical staining, × 200; D: Desmin staining (arrow) of tumor tissues, immunohistochemical staining, × 200.

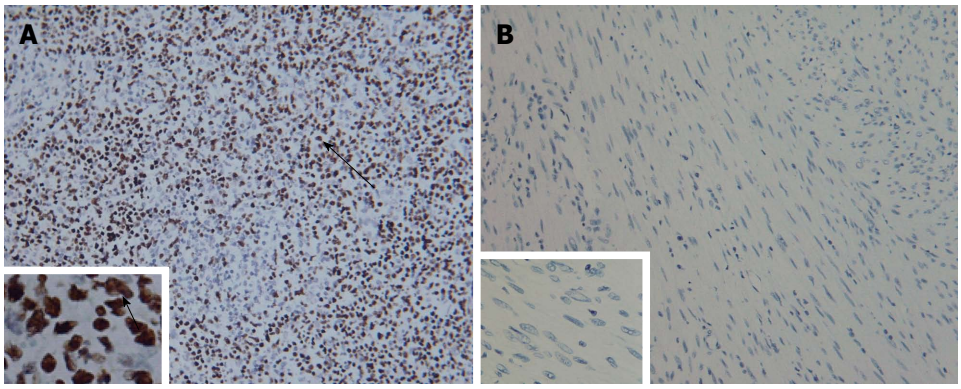

Figure 3 Tumor cells stained negative by *in situ* hybridization with Epstein-Barr virus-encoded small RNA. A: Positive control staining × 200, × 1000; B: Tumor cell staining × 200, × 1000. Arrows indicate positive staining of the nuclei.

24-mo postoperative follow-up, no sign of local recurrence or distant metastasis was observed, indicating a clinical cure in this case.

DISCUSSION

Primary hepatic leiomyoma occurs rarely. The first case was described by Demel[1] in a 42-year-old woman. To date, only 28 cases have been reported worldwide (Table 1). Secondary to benign smooth muscle proliferation, primary hepatic leiomyoma is usually found in adult women, and is associated with EBV infection. Due to its low prevalence, diagnosis, treatment and biological behavior remain elusive and require further investigation.

Leiomyoma is relatively common and tends to originate from the muscularis of the gut or the media of the blood vessels, and usually develops in the urogenital and gastrointestinal tracts. Primary hepatic leiomyoma is rare and has its own particular clinical and biological features.

To diagnose primary hepatic leiomyoma, Hawkins *et al*[2] has proposed the following criteria: (1) the tumor is composed of leiomyocytes; and (2) the presence of a leiomyomatous tumor at other sites can be excluded. Moreover, this liver tumor must be distinguished from GIST[3,4]. In the present case, we excluded the presence of hepatocellular carcinoma, and laboratory tests and histopathological examination were the first step in this process. Then, the diagnosis of leiomyoma was established on the basis of its pathological features. GIST makers (CD117, CD34 and DOG1) were also negative. Combining the findings of ultrasonography, abdominal CT scan, esophagogastroduodenscopy, colonoscopy and exploratory laparotomy, the final diagnosis of primary hepatic leiomyoma was made.

Although no standard therapy is available at present, consistent with the existing reports (Table 1), the tumor was successfully excised and neither chemotherapy nor radiotherapy was applied. Our experience supports that hepatic resection is both diagnostic and curative for primary hepatic leiomyoma.

Some unique characteristics should be noted in this case. First, the patient was male, and primary hepatic leiomyoma is more likely to be found in adult women (18 out of the total 28 cases were female) (Table 1). The relevance of sex may partly be due to the activity of the smooth muscle cells in female urogenital tissue in tumorigenesis and progenesis. The cellular origin of primary hepatic leiomyoma remains unclear and may arise from vessels or the biliary tree[6-8]. In this report, the patient had negative findings in the pelvis and for detection of GIST markers. More observations are required to explore the cellular source of primary hepatic leiomyoma. Second, this case was an adult patient. To date, a total of four pediatric cases (< 18 years) have been identified with primary hepatic leiomyoma (Table 1). Whether or not the developmental mechanisms are different between children and adults requires further investigation. Third, it could be deduced that EBV infection plays a critical role in development of primary hepatic leiomyoma[9,10]. Based on the reported literature, five patients were examined for EBV infection and all of them were positive[11-14]. The relationship between development of primary hepatic and EBV infection and immunocompromised status is also interesting. Seven out of the 28 patients (25%) were immunocompromised (6 transplanted and 1 HIV infection), and five of the seven cases (71.4%) were EBV-positive (4 transplanted and 1 HIV infection). However, in the present case, EBER *in situ* hybridization, which is the gold standard for detection and localization of latent EBV in tissues, showed that the patient did not have EBV infection, which was different from the status of other patients currently being studied.

Our data indicate that EBV infection is important but neither necessary nor sufficient for the development of primary liver leiomyoma. This observation highlights the complex and heterogeneous nature of the disease and raises the question whether EBV is a passenger rather than a causative agent for this tumor. Due to the rare occurrence of the tumor, an international primary hepatic leiomyoma sample bank, which needs worldwide cooperation of the involved institutions, will contribute to untangling the complex pathogenesis using omics- and system-based methodologies, and therefore to clarify the underlying mechanism behind this interesting tumor.

In conclusion, this report of the 29th case of primary hepatic leiomyoma with its unique features related to diagnosis, treatment and developmental biology contributes to our knowledge of the tumor.

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Acknowledgments

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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